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CORNEAL EPITHELIAL AND RETINAL PIGMENT EPITHELIAL CELL CULTURE ASSAYS AS POTENTIAL ALTERNATIVES TO ANIMAL EXPERIMENTATION FOR THE EVALUATION OF OCULAR TOXICITY

Anne Huhtala

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ABSTRACT

The widely criticized Draize rabbit test is the only eye toxicity test officially accepted worldwide for regulatory purposes in the classification of slightly and moderately irritating chemicals. Today, there are no *in vitro* alternatives that could be used as a complete replacement for the Draize eye test. Moreover, in the current OECD guidelines, there are no tests for retinal toxicity, not even *in vivo*.

This study was undertaken to develop corneal epithelial and retinal pigment epithelial cell culture assays as pre-screens and potential alternatives in a more comprehensive battery test for *in vitro* eye toxicity testing. Two basal cytotoxicity tests with effective 96-well techniques and plate readers were established to evaluate the adverse ocular effects of selected test compounds: WST-1 test as an index of mitochondrial function and cell viability/proliferation, and lactate dehydrogenase (LDH) leakage test as an index of cell membrane integrity.

The confluent and post-confluent human corneal epithelial (HCE) cell line in culture medium was found to express cornea-specific cytokeratin 3, but it also expressed simple epithelium-specific cytokeratins 7, 8, 18 and 19. In most cases in the WST-1 and LDH testing, the pre-confluent HCE cell line was as sensitive to the test compounds as the pre-confluent primary cultures of rabbit corneal epithelial epithelium. The WST-1 test appeared to be an earlier indicator of toxicity than the LDH test, which also showed great variations in the cytotoxicity results. In the HCE-WST-1 interlaboratory study, the most reproducible results were obtained when the cells were exposed to the test substances for one hour in the absence of serum. The use of the human retinal pigment epithelial cell line D407 and of pig primary retinal pigment epithelial cell cultures in the WST-1 testing also yielded comparable results.

In all cases, the use of serum in culture medium resulted in lower toxicity, and thus its use is not recommended. The assays studied, especially those based on cell lines, are reliable, transferable, easy-to-perform tests for ocular toxicity screening.

PREFACE

This thesis was completed as a continuation for a larger European research consortium aimed to improve the scientific and technical evaluation of oculotoxicity *in vitro* (Biomed project, BMH4-97-2324). The thesis was carried out during the years 1998-2003 at the University of Tampere, Medical School, in collaboration with the University of Bremen, Institute of Experimental Physics (Germany); the University of Pisa, Department of Bioorganic Chemistry and Biopharmaceutics (Italy); the University of Ioannina, Medical School, Department of Pharmacology (Greece); Santen Oy (Tampere, Finland); and Orion Pharma (Turku, Finland). However, despite all the intensive collaboration I remained faithful to my old seat of learning, Helsinki University of Technology.

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LIST OF PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals I-V.

I Huhtala, A., Nurmi, S.K., Tähti, H., Salminen, L., Alajuuma, P., Rantala, I., Helin, H., and Uusitalo, H. (2003). The Immunohistochemical Characterization of an SV40-Immortalized Human Corneal Epithelial Cell Line. Altern Lab Anim *31*, Issue 4, in press.

II Huhtala, A., Mannerström, M., Alajuuma, P., Nurmi, S., Toimela, T., Tähti, H., Salminen, L., and Uusitalo, H. (2002). Comparison of an Immortalized Human Corneal Epithelial Cell Line and Rabbit Corneal Epithelial Cell Culture in Cytotoxicity Testing. J Ocul Pharm Ther *18*, 163-175. Reprinted with Permission from Mary Ann Liebert, Inc.

III Huhtala, A., Alajuuma P., Burgalassi, S., Chetoni, P., Diehl, H., Engelke, M., Marselos, M., Monti, D., Pappas, P., Saettone, M.F., Salminen, L., Sotiropoulou, M., Tähti, H., Uusitalo, H., and Zorn-Kruppa, M. (2003). A Collaborative Evaluation of the Cytotoxicity of Two Surfactants by Using the Human Corneal Epithelial Cell Line and the WST-1 Test. J Ocul Pharm Ther *19*, 11-21. Reprinted with Permission from Mary Ann Liebert, Inc.

IV Huhtala, A., Tähti H., Salminen, L., and Uusitalo, H. (2002). Evaluation of Adverse Ocular Effects of 5-Fluorouracil by Using Human Corneal Epithelial Cell Cultures. J Toxicol Cutaneous Ocul Toxicol *21*, 283-292. Reprinted with Permission from Marcel Dekker, Inc.

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The author's contribution to the publications

I The author defined the research plan for the cell culture studies, carried out the cell culture experiments, and was the major contributor of the manuscript.

II The author defined the research plan with the coauthors, carried out the experiments, interpreted the results, and wrote the manuscript.

III The author defined the research plan for all the participating laboratories and carried out the Finnish experiments. The author was the major contributor in the interpretation of the results and wrote the manuscript.

IV The author defined the research plan, carried out the experiments, interpreted the results, and wrote the manuscript.

V The author defined the research plan, interpreted the results and wrote the manuscript together with the coauthors.

ABBREVIATIONS

ADR	Adverse drug reaction
APAAP	Alkaline phosphatase anti-alkaline phosphatase
BAC	Benzalkonium chloride
BCOP	Bovine corneal opacity and permeability test
BGA/BMBF	Bundesgesundheitsamt/Bundesministerium für Bildung und Forschung (German Federal Health Office/German Federal Ministry of Education and Research)
BgVV	Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin (German Federal Institute for Health Protection of Consumers and Veterinary Medicine), since November 2002, BfR (Bundesinstitut für Risikobewertung, Federal Institute for Risk Assessment)
BSA	Bovine serum albumin
CAM	Chorioallantoic membrane
CAMVA	Chorioallantoic membrane vascular assay
CE	Corneal epithelial
CEET	Chicken enucleated eye test
CHL	Chinese hamster lung cells
CI	Confidence interval
CK	Cytokeratin
COLIPA	European Cosmetic, Toiletry, and Perfumery Industry Association
CPSC	Consumer Product Safety Commission, USA
CTFA	Cosmetic, Toiletry and Fragrance Association, USA
CV	Coefficient of variation
CVS	Crystal violet staining
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EC_{50}	Concentration that induces 50% response, in this study the effective concentration that reduces viability to 50% of controls
EC	European Commission, Electron coupling reagent
ECETOC	European Centre for Toxicology and Ecotoxicology of Chemicals
ECM	Extracellular matrix
EEC	European Economic Community
ECVAM	European Centre for the Validation of Alternative Methods
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
EINECS	European Inventory of Existing Chemical Substances
EPA	Environmental Protection Agency, USA
ex vivo	"From the living", studies done in the isolated tissues
EU	European Union
EYTEX™	Eye irritancy test kit
FBS	Fetal bovine serum
FCS	Fetal calf serum
FDA	Food and Drug Administration, USA
FL	Fluorescein leakage

5-FU	5-Fluorouracil
GLP	Good laboratory practice
HBSS	Hank's balanced salt solution
HCE	Human corneal epithelial
HD	Hemoglobin denaturation
HeLa	Human cervical carsinoma cells
HESI	Health and Environmental Sciences Institute
HET-CAM	Hen's egg test on the 2-chorioallantoic membrane
HO	British Home Office
ICE	Isolated chicken eye
ILSI	International Life Sciences Institute
in ovo	"In the egg", studies made on animals of the embryonated stage
in vitro	"In glass", non-animal experiments, studies made with cultured cells or isolated tissues
in vivo	"In the living (thing)", in animals or man, studies made on the living animal
IRAG	Interagency Regulatory Alternatives Group, USA
IRE	Isolated rabbit eye
kDa	Kilodalton (1 dalton = approximately the mass of a hydrogen atom, $1.66 \cdot 10^{-24}$ g)
LDH	Lactate dehydrogenase
LVET	Low-volume eye test
mAB	Monoclonal antibody
MAS	Maximum average score in the Draize test
MHW/JCIA	Japanese Ministry of Health and Welfare/Japanese Cosmetics Industry Association
MMAS	Modified maximum average score in the Draize test
MTA	Microculture tetrazolium assay
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulpholphenyl)-2H-tetrazolium, inner salt
MTT	Mitochondrial tetrazolium test, methyl-thiazolyl tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
NRR	Neutral red release
NRU	Neutral red uptake
OECD	Organization for Economic Cooperation and Development
PBS	Phosphate buffered saline
PCA	Principal components analysis
PI	Prediction interval
PLS	Partial least squares
PM	Prediction model
PSE	Polyoxyethylene-20-stearyl ether, Brij®78
PTG	Pollen tube growth test
QSAR	Quantitative structure-activity relationship
R41	Risk of severe damage to the eye (Risk rating by the EU regulations)
R36	Irritating to the eye (Risk rating by the EU regulations)
RBC	Red blood cell

RCE	Rabbit corneal epithelial
REC	Human reconstituted epithelial culture
RPE	Retinal pigment epithelial
RS	Reference standard, Mitochondrial succinate-tetrazolium-reductase system
SD	Standard deviation
SEM	Standard error of mean
SIRC	Staatens Seruminstitute rabbit corneal cells
SM	Silicon microphysiometer
SV40	Simian virus 40
TEA	Tissue equivalent assay
TER	Transcutaneous electrical resistance
TB	Tryban blue
TBS	Tris-buffered saline
Vol	Volume
WST-1	4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate
XTT	Sodium (2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide, inner salt
ZEBET	Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergänzungsmethoden zum Tierversuch (National Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments), Germany

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1. INTRODUCTION

In today's chemical world the need for toxicity evaluations is continuously increasing. More than 100,000 chemicals are in commercial use, and it has been estimated that approximately 2,000 new chemicals are introduced to the market each year (Köeter, 1994). According to current EU regulations, all the new chemicals on the market have to be tested for various kinds of toxicity, including testing for eye irritation. In the European Inventory of Existing Chemical Substances (EINECS), a total of 100,195 existing chemicals are listed. These are chemicals that have been declared to be on the market before September 1981. About 3,200 substances have been noted since 1981 (European Chemicals Bureau, 2003). Although the current regulation provides sufficient data on the new chemicals, the data on the existing chemicals is far from complete. The recent EC White Paper on a *Strategy for a Future Chemicals Policy* aims to close the knowledge gap on the existing chemicals by the end of 2012 (European Commission, 2003).

The main goal of toxicological scientific endeavors is to safeguard human beings against the possible adverse effects of diverse types of chemicals, including pharmaceuticals, cosmetics, household products, industrial chemicals, and agrochemicals. The exposure can be incidental, accidental, or intentional, as with cosmetics and certain drugs. Even today, the final preclinical safety assessment of chemicals is largely based on animal experiments. The search for *in vitro* methods that could be used as alternatives or complete replacements for animal experiments has been the emphasis of toxicological research for several decades now, not only for ethical reasons, but also for scientific and economic reasons. The Draize rabbit eye test used for the assessment of ocular irritation is the most widely criticized single toxicity test (Worth and Balls, 2002a). It seems that more effort has been focused on finding alternatives to the Draize rabbit eye test than on all the other acute *in vivo* toxicity tests combined (Balls et al., 1999). Furthermore and despite everything, there are no specific tests for retinal toxicity in the generally accepted guidelines, although systemic and intravitreally administrated drugs can induce adverse drug reactions (ADR).

The present study was undertaken to develop cytotoxicity tests based on novel cell culturing techniques to be used as pre-screens in pharmaceutical and other chemical industry and as possible alternative methods in a more comprehensive test battery aimed to replace the Draize eye test completely. In this study, animal primary cultures and human cell lines from both corneal epithelium and retinal pigment epithelium were employed. Two basal cytotoxicity tests based on effective multititer techniques and plate readers were established to evaluate the adverse ocular effects of selected model drugs: the WST-1 test as an index of mitochondrial function and cell proliferation/viability, and the lactate dehydrogenase (LDH) leakage test as an index of cell membrane integrity.

2. REVIEW OF THE LITERATURE

2.1. Anatomical View of the Eye

The various parts of the eye can be damaged by substances coming into contact with it: cornea, conjunctiva, iris, and even lens and retina. A schematic representation of the structure of the eye is shown in Figure 1. The current eye irritation test based on animals evaluates only the changes observed in three tissues of the eye: cornea, conjunctiva, and iris. The cornea forms the outermost part of the eye globe exposed to the outside environment. The main function of the cornea is to protect the eye and to maintain its shape. The cornea is also a powerful refracting surface; it provides about 2/3 of the eye's focusing power. The transparent, avascular human cornea is approximately 0.5 mm thick and consists of five layers (Figure 1): the surface epithelium, Bowman's membrane, the stroma, which forms the major part of the cornea, Descemet's membrane, and the endothelium (Beuerman and Pedroza, 1996; Newell, 1996). The corneal epithelium has a rich nerve supply and consists of 4-6 layers of stratified epithelial cells. The outside of the corneal epithelium is moistened by tear film. Beneath the corneal epithelium lies the Bowman's membrane, a resistant acellular collagen structure. The corneal stroma forms 85-90% of the thickness of the entire cornea. It consists of regularly arranged collagen fibrils, which are responsible for corneal transparency. The collagen matrix contains keratocytes, fibroblast-like cells, which produce substances essential for the maintenance of the hydration of the cornea. The non-cellular Descement's membrane, secreted by the cells of the corneal endothelium, is located between the stroma and the internal endothelium. The corneal endothelium consists of a single layer of polygonal, flattened cells. Their main role is to extract water from the stroma so that the arrangement of the collagen matrix remains regular. Corneal transparency has been found to depend on many factors: rapid renewal of the epithelium, maintenance of the integrity of its structure, the state of relative dehydration of the stroma, absence of blood vessels, and the normal metabolic activity of keratocytes and the cells of corneal endothelium, which have a vital role in the maintenance of the transparency and the normal function of the cornea (Hubert, 1992).

The internal surface of the eyelid is covered by conjunctival mucosa, which extends into the conjunctival cul-de-sac and passes to the anterior surface of the eyeball (Newell, 1996). The conjunctiva has a rich blood supply. The iris forms the anterior part of the uvea. It is bathed by the aqueous humour. The stroma of the iris, rich with blood and nerve supply, consists of a network of collagen. The iris contains large chromatophores responsible for the color of the eye and fibroblasts (Saari, 1972). Its smooth muscle forms the pupillary dilator. Just behind the iris is located the lens, focusing light onto the retina. The cornea is responsible for most of the refraction and the crystalline lens fine-tunes the focus. In a healthy eye, the lens can accommodate, *i.e.* change its shape to provide a clear vision at various distances.

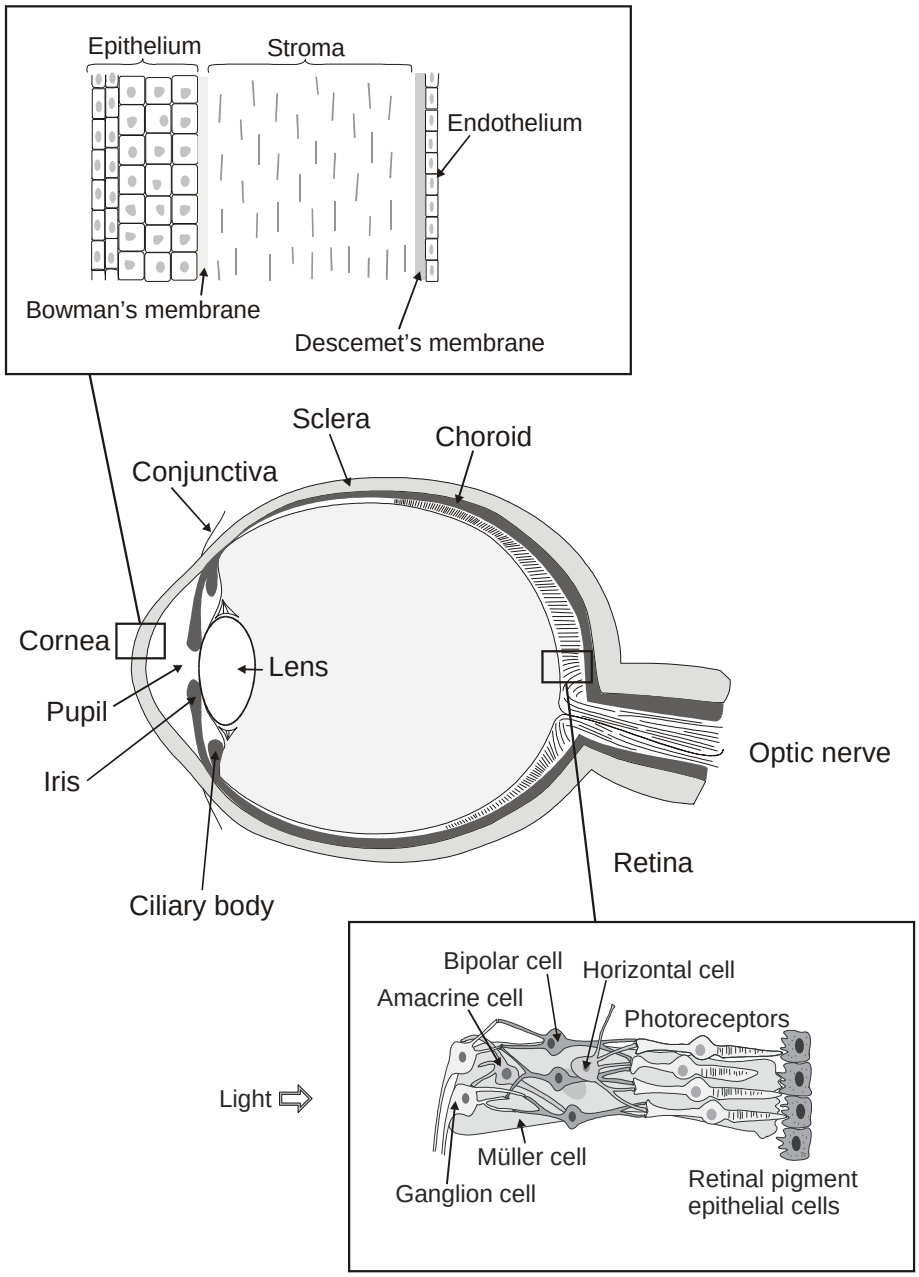

Figure 1. Anatomy of the eye.

The retina is approximately 0.5 mm thick and it lines the back of the eye. The retina consists of an outer pigment epithelium layer and an inner neurosensory retinal layer (Figure 1). The output neurons of the retina, the ganglion cells, lie innermost in the retina, closest to the front of the eye, while the photoreceptors (the rods and cones) lie outermost in the retina against the pigment epithelium and the choroid (Newell, 1996). The pigment epithelium consists of a single layer of pigmented cuboidal cells, and it extends to the optic nerve margin posteriorly and to the ora serrata anteriorly. Retinal pigment epithelial (RPE) cells have an important role in the maintenance of homeostasis, the metabolic activity of the photoreceptors and the blood-retinal barrier (Marmor, 1998).

2.2. Draize Eye Test

The currently used rabbit eye test is based on the method that was originally developed in the early 1940's in the United States. The classic paper by Draize and coauthors (Draize et al., 1944) is a refinement of another paper published by Friedenwald and coworkers (Friedenwald et al., 1944). Draize and his colleagues developed standardized testing protocols for the evaluation of dermal and ocular toxicity by assessing the effects of acute, intermediate, and chronic exposures of chemical compounds applied to the skin, penis, and eyes of rabbits (Draize et al., 1944; Wilhelmus, 2001). Other investigators soon adopted the method to screen many different compounds. From the early 1960's scientists began to refer to this technique as the Draize test. To distinguish the target organ, the ocular test was called the Draize eye irritation test or, for short, the Draize eye test. The Draize eye test has since been modified in Europe, North America, and Asia (Wilhelmus, 2001). Albino rabbit (*e.g.* New Zealand white rabbit) is the usual test species. Groups of 3-6 animals are normally used. In the original Draize test, the lower eyelid is pulled away from the eyeball, and depending on the test material (liquid, ointment, paste, or solid), 0.1 ml or 0.1 g of the test compound is installed in the resulting conjunctival cul-de-sac (Draize et al., 1944). The materials can also be placed directly onto the cornea. The other eye is left untreated or treated with the vehicle or excipient. A topical anesthetic drug is sometimes instilled before the test agent to avoid unnecessary discomfort. A washing procedure may also be included. The Draize test was designed to mimic the human eye in the clinical situation, *i.e.* exposure when chemical substances come into contact with the eye, the time-scale, and the normal wash-out-procedure of the substances from the eye. Therefore, the test has been found to be most useful in pharmaceutical industry. However, the original test is based on the subjective scoring of the responses from only three tissues of the eye: cornea, conjunctiva, and iris.

2.2.1. Draize Test Scoring System

In the Draize test, the evaluations of ocular lesions are generally made at 1, 4, 24, 48 and 72 hours after exposure, and if needed at 4, 7 and 21 days (Wilhelmus, 2001). There is no "standard" Draize test. Several grading systems have been proposed, but the original Draize scoring method remains widely used (Table 1). The scoring method involves weighting and summing six components of directly observable changes on the anterior segment of the eye, including the density and area of corneal opacification, the severity of

iritis, conjunctival redness, edema, and discharge. An illustrated standard guide is used to score irritancy. The eye irritation potential is often summarized as the "Maximum Average Score" (MAS), which is obtained by averaging the weighted scores for individual animals at the each time of observation (*e.g*. as 4, 24 and 48 hours) and selecting the highest of these averages. The scores in the Draize test can range from 0 to 110 points. From the maximum score of 110 points, 80 points (73% of the total score) can result from the severity and size of the corneal opacity, 20 points from the conjunctival irritation, and 10 points from the severity of iritis. Although Draize did not translate the severity score into the interpretive assessment, since the early 1960's others have attempted to classify severity levels (Kay and Calandra, 1962). Historically, test chemicals have been categorized into several irritation levels, *i.e.*, non-irritating, slightly irritating, mildly irritating, moderately irritating, or severely irritating. Minor eye irritation, such as allergic reactions, is difficult to categorize by the Draize test. And because there are a number of modifications of the original Draize scoring method, diverse irritation categories can be found for some chemical substances (Gupta et al., 1993). Moreover, the Draize test based on only the subjective scoring without histological characterization cannot provide comprehensive test results.

There are several structural, physiological, and biochemical differences between the human and the rabbit eye. Rabbits have a relatively low tear production, blink frequency, and ocular surface sensitivity (Swanston, 1985; Wilhelmus, 2001). The anatomy of the rabbit eye is also different from that of the human eye. Rabbits have a nictitating membrane, a relatively larger corneal surface area, and a thinner cornea. The Draize test has been criticized for many reasons, such as dosing of test materials, methods of exposure, subjectivity of observations and scoring, lack of discrimination of fine response differences, and overestimation of the human response (Swanston, 1985; Schlatter and Reinhardt, 1985; Sharpe, 1985; York and Steiling, 1998). Also, the reproducibility of the Draize test has been found to be poor within and among laboratories (Weil and Scala, 1971; Williams et al., 1982; McCulley and Stephens, 1994; Earl et al., 1997). The test volume used in the original Draize eye test (0.1 ml) exceeds about ten times the normal volume of fluid residing in the human eye. The low-volume eye test (LVET) test, sometimes also called the Griffith's test, is a refinement of the original Draize test and uses only one-tenth of the volume of a test agent placed in the eye (Griffith et al., 1980; Bruner et al., 1992; Lambert et al., 1993; Cormier et al., 1996). The level and duration of eye irritation in the LVET test is less than in the original Draize test, which makes it a less stressful procedure. The LVET test has also been reported to lead to a closer correlation with the human eye response.

Table 1. Draize test scoring for grading the severity of ocular effects (Draize et al., 1944).

Ocular Effects	**Grade**
1. CORNEA	
A. Opacity - Degree of density (Area most dense taken for reading)	
No opacity	0
Scattered of diffuse area, details of iris clearly visible	1
Easily discernible translucent areas, details of iris slightly obscured	2
Opalescent areas, no details of iris visible, size of pupil barely discernible	3
Opaque, iris invisible	4
B. Area of cornea involved	
One-quarter of less, but not zero	1
Greater than one-quarter, but less than one-half	2
Greater than one-half, but less than three-quarters	3
Greater than three-quarters up to whole area	4
SCORE (A x B) x 5 TOTAL MAXIMUM = 80	
2. IRIS	
A. Values	
Normal	0
Folds above normal, congestion, swelling, circumcorneal injections (Any one or all of these or combinations of any thereof), iris still reacting to light (Sluggish reaction is positive)	1
No reaction to light, haemorrhage, gross destruction (Any or all of these)	2
SCORE A x 5 TOTAL MAXIMUM = 10	
3. CONJUNCTIVAE	
A. Redness (Refers to palpebral conjunctivae only)	
Vessels normal	0
Vessels definitely injected above normal	1
More diffuse, deeper crimson red, individual vessels not easily discernible	2
Diffuse beefy red	3
B. Chemosis (Oedema)	
No swelling	0
Any swelling above normal (Includes nictitating membrane)	1
Obvious swelling with partial eversion of the eyelids	2
Swelling with lids about half closed	3
Swelling with lids about half closed to completely closed	4
C. Discharge	
No discharge	0
Any amount different from normal (Does not include small amounts observed in inner canthus of normal animals)	1
Discharge with moistening of the lids and hairs just adjacent of the lids	2
Discharge with moistening of the lids and hairs, and considerable area around the eye	3
SCORE (A + B + C) x 2 TOTAL MAXIMUM = 20	
MAXIMUM POSSIBLE SCORE = 80 + 10 + 20 = 110	

2.2.2. Reference Chemicals Data Bank

In 1992, a list of 55 chemicals with comprehensive rabbit eye irritation data was published by ECETOC, European Centre for Toxicology and Ecotoxicology of Chemicals (Bagley et al., 1992a). Similar data for 77 chemicals was published in 1999 (Bagley et al., 1999b). These 132 chemicals have been assessed in 149 *in vivo* studies in rabbits, and the data is usually obtained from at least three animals at the same time. In the Draize test, 0.1 ml or equivalent weight was instilled into the conjunctival sac, and evaluations were made at least 1, 2 and 3 days after installation. The chemicals were ranked for eye irritation potential on the basis of a "Modified Maximum Average Score" (MMAS), which represents the maximal score calculated after 24 h or longer exposure. This reference chemicals data bank was generated for the use in the validation studies of promising *in vitro* alternatives. The reference chemical data bank represents the whole range of the Draize scale, and the chemicals are classified into 23 categories: acetates, acids, acrylates/metacrylates, acyl halides, alcohols, aldehydes, alkalis, aromatics, brominated derivatives, esters, ethers, fatty acids, heterocyclis, hydrocarbons, inorganic chemicals, ketones, nitriles, organophosphates, pesticides, soaps/surfactants (anionics, cationics, nonionics, zwitterionics), sulfur-containing compounds, triglycerides, and miscellaneous compounds.

Instead of the MMAS, most of today's regulatory systems, including the European Union, US Environmental Protection Agency (EPA), US Food and Drug Administration (FDA), US Consumer Product Safety Commission (CPSC), and Canadian workplace systems classify chemicals according to their effects in the individual tissues of the eye, *i.e.* conjunctiva, cornea, and iris (Green et al., 1993; Balls et al., 1999). Despite the criticism against its scientific validity and its ethical acceptability, the Draize eye test remains the officially accepted government-recognized procedure for predicting the potential irritant effect of chemicals in the eye, at least for moderately and slightly irritating chemicals. With the development of alternative non-animal methods to replace the Draize eye test, the data generated by the Draize test has also been used as a "gold standard", to which the performance of *in vitro* methods has been compared.

2.3. Methods Proposed as Alternatives for Ocular Irritation

The idea of using alternative methods for animal testing emerged at the turn of the 1960's, and many alternatives have since been proposed. Alternatives are methods that are used for reducing the number of animals in research, and, if possible, for replacing the use of animals by other techniques. The principle of the Three Rs approach - *reduction*, *refinement*, and *replacement* - was introduced by Russell and Burch in 1959 (Russel and Burch, 1959). According to this approach, three types of alternatives can be recognized: *reduction* alternatives, which decrease the number of animals needed to perform a particular test or a group of tests; *refinement* alternatives, in which the pain, stress, and discomfort experienced by laboratory animals are minimized by improving the design and/or efficiency of the test; and *replacement* alternatives, which entirely eliminate the need for animal testing. In 1986, these principles were also embedded in EU Directive

86/609/EEC to control the use of animals in scientific experiments (EEC, 1986; Louhimies, 2002).

A large number of *in vitro* tests for the Draize eye test have been developed during the last two decades. Many of them have been initially developed as basic cytotoxicity tests. Since chemical substances that are severe irritants or corrosive to the skin are mostly also irritating to the eye (Williams, 1984; Gad et al., 1986), many methods proposed as alternatives for ocular safety assessment have been developed together with the proposed alternative methods for the assessment of skin irritation. In 1987, in the most frequently cited review of potential alternatives for the Draize eye test by Frazier and co-authors, 34 *in vitro* tests were outlined (Frazier et al., 1987). In 1992, Atkinson *et al*. claimed that there were over 60 alternative methods proposed for ocular irritation (Atkinson et al., 1992). In 1998, it was estimated by ECVAM (European Centre for the Validation of Alternative Methods) that there are approximately 70 different alternative methods for the assessment of eye irritation potential (ECVAM, 2003). These methods can be divided into several categories, such as computer models based on structure-activity relationships and physicochemical parameters, tests with plants and microorganisms, cell culture methods, chorioallantoic membrane (CAM)-based assays in fertilized hen´s eggs, organotypic models, and three-dimensional tissue culture models (Table 2, pp. 24-25). In the following, the most frequently used and the most promising methods are reviewed. (For more comprehensive reviews see: Wilcox and Bruner, 1990; Atkinson et al., 1992; Goldberg and Silber, 1992; Chu and Toft, 1993; McCulley and Stephens, 1994; Herzinger et al., 1995; Curren and Harbell, 1998; Balls et al., 1999; Wilhelmus, 2001).

2.3.1. Physicochemical Detection Systems

The studies of the relationships between a chemical's structure and/or physicochemical properties and its biological activity have been found helpful at the early stage of safety assessment. Several physicochemical parameters, such as physical state, octanol-water partition coefficient, pH value, buffering capacity, and osmolality have been found to be useful parameters in ocular safety assessment (Atkinson et al., 1992; Regnier and Imbert, 1992; Cronin et al., 1994; Abraham et al., 1998). Materials at the pH extremes of 2 or below or of 11.5 or above may be considered to be ocular irritants (Murphy et al., 1982). According to the OECD guideline, a test substance that is suspected to be corrosive to the eye on the basis of measurements of pH does not need to be tested in animals (OECD, 1987; Hurley et al., 1993).

Information on the structural analogues of specific chemicals may also give insights on eye irritation potential. Consequently, computer programs based on quantitative structure-activity relationships (QSARs) have been developed for predicting ocular irritancy (Kulkarni and Hopfinger, 1999; Patlewicz et al., 2000; Gerner et al., 2000). Several computer programs based on QRAS have been developed, such as TOPKAT, Multi-CASE, HAZARD-EXPERT, and DEREK (Atkinson et al., 1992). QSAR analysis on structural analogues can also be used at the early stage in the development of safe drugs and cosmetics (Cronin, 2002). The interaction with lipids and proteins has been shown to contribute to a chemical's irritant effect (Helenius and Simons, 1972; Miyazawa et al., 1984; Imokawa et al., 1989). As a result of these findings, the preparations of liposomes

preloaded with a fluorescent dye have been proposed for predicting the eye irritation of surfactants (Taniguchi et al., 1988; Kato et al., 1988; Bean et al., 1991). The Irritection system, formerly known under the trade name of EYTEX, is a commercially available test kit by InVitro International (Irvine, CA, USA), and one of the simplest and most widely used *in vitro* tests developed for testing ocular irritation potential (Gordon and Bergman, 1987; Bruner et al., 1991a; Courtellemont et al., 1992; Gordon, 1992a; Gordon, 1992b; Sina et al., 1995; Gettings et al., 1996a; Curren et al., 1997; Courtellemont et al., 1999b). The non-living system is based on modeling corneal opacity by measuring the ability of test substances to interact with the conformation and hydration of a three-dimensional synthetic protein matrix.

2.3.2. Tests with Plants and Microorganisms

The tobacco (*Nicotiana sylvestris*) pollen tube growth test (PTG test) is one of the best-validated non-animal systems developed for toxicity screening (Kappler and Kristen, 1987). The growth of pollen tubes is closely related to the integrity of their plasma membranes and the test is based on the photometric quantification of pollen tube mass production. It uses suspensions of tobacco pollen, which provide a sensitive indication of bioactive materials on a cellular level, since the growth of pollen tubes is enhanced or inhibited in the presence of substances interfering with a broad range of intracellular processes (Kristen and Kappler, 1995; Kristen, 1997; Kristen et al., 1999; Kristen et al., 2002).

The effect of chemicals on the swimming patterns of the protozoans *Tetrahymena termophila* and *Tetrahymena pyriformis* has been evaluated microscopically and related to the prediction of eye irritancy (Silverman, 1983; Silverman and Pennisi, 1987; Bruner et al., 1991a). The luminescent bacteria (*Vibri fischeri*, formerly *Photobacterium phosphoreum*) toxicity test is provided under the trade name of Microtox by AZUR Environmental, formerly Microbics Corporation and now owned by Strategic Diagnostics Inc. (Newark, DE, USA). The bacteria generate light by a biochemical process coupled to bacterial respiration, and the bacterial luminescence is measured as an indicator of toxicity (Bulich et al., 1990; Sina et al., 1995; Curren et al., 1997). A decrease in light emission indicates altered respiratory metabolism.

Table 2. *In vitro* methods proposed as alternatives for ocular irritation.

Test System	Examples of Assays	Classified as/ Type of Chemical (Regulatory Acceptance)
Physicochemical properties	pH testing	
	-Corrosive when 2<pH<11.5	R41 chemicals, Risk of severe damage to the eye (OECD)
	Octanol-water partition coefficient	-
	Buffering capacity	-
	Osmolality	-
Quantitative structure-activity relationships	Computer programs	-
Biological activity	Interactions with lipids	-
	Interactions with proteins (Irritection)	-
Plants and microorganisms	Tobacco pollen tube growth test	-
	Protozoan motility	-
	Luminescent bacteria toxicity test	-
Cell culture methods	Cell count, cell detachment, colony-forming efficiency	-
	Morphological changes	-
	Nutrient transport	-
	Cellular protein	-
	Energy metabolism disturbances	-
	Membrane changes	
	-Neutral red uptake assay	Phototoxic (EU)
	-Neutral red release assay	Cosmetics (France)
	Agar diffusion assay	Cosmetics (France)
	Evaluation of pH changes	-

Test System	Examples of Assays	Classified as/ Type of Chemical (Regulatory Acceptance)
Chorioallantoic membrane (CAM) assays	Hen's egg test on the chorioallantoic membrane (HET-CAM) assay	R41 (Germany, France, UK, Netherlands)
	Chorioallantoic membrane vascular assay (CAMVA)	-
	Chorioallantoic membrane-trypan blue (CAM-TB) test	-
Organotypic test models	Isolated chicken eye (ICE)	R41 (Germany, France, Netherlands)
	Isolated rabbit eye (IRE)	R41 (Germany, France, UK)
	Bovine corneal opacity and permeability test (BCOP)	R41 (Germany, France, UK, Netherlands), Drug formulations (Belgium)
	Isolated lens	-
	Skin explant	
	-Rat skin transcutaneous electrical resistance test	Corrosive chemicals (OECD)
	Isolated rabbit vagina, ileum	-
Tissue equivalents	Three-dimensional skin models	
	-Epiderm	Corrosive chemicals (OECD)
	Three-dimensional corneal models	-

2.3.3. Methods Based on Cell Cultures

Most of the proposed alternative methods are based on the use of cultured mammalian cells and the variations of basal cytotoxicity assays that are simple and reproducible and that yield a defined endpoint. One major advantage of cell culture systems is the possibility of obtaining cells from a particular target organ and, in some cases, also of human origin. A variety of cellular systems, both presumed target cells, such as corneal epithelial and conjunctival cells, and non-target cells have been proposed for the assessment of ocular irritation.

2.3.3.1. Cell Types Used in Ocular Toxicology

Since corneal epithelial (CE) cells form the outermost layer of the eye and are thus readily exposed to injury, they have been found to be a promising tool for *in vitro* ocular toxicity testing. The use of rabbit primary CE cells in cytotoxicity testing is well known (Lazarus et al., 1988; Grant and Acosta, 1990; Grant et al., 1992; Tripathi et al., 1992; Grant and Acosta, 1994; Yang and Acosta, 1994; Yang and Acosta, 1995; Grant and Acosta, 1996a; Grant and Acosta, 1996b; Grant and Acosta, 1997). However, the use of human primary CE cells has been limited, due to the limited availability of donor corneas and the difficulties encountered with cell culture methods that work well with rabbit cells (Ebato et al., 1987). Other primary cell cultures include rabbit conjunctival cells, human skin fibroblasts, human skin keratinocytes, human buccal mucosa cells, human gingival fibroblasts, rat peritoneal cells, and isolated red blood cells from bovine, rat, rabbit, dog, and human. Today, the use of cell lines is favored, as they are more easily manageable than primary cell cultures (Guillot, 1992). Rabbit fibroblastic corneal cells (SIRC) and mouse fibroblasts (Balb/c3T3) are among the most widely used cell lines. Several other cell lines, both target and non-target ones, have also been introduced, such as human dermal fibroblasts, human epidermal keratinocytes, mouse fibroblast cells (L929), Chinese hamster lung fibroblasts (V79), human erythroleukemia cells (K562), hamster kidney cells (BHK-21), Madin-Darby canine kidney (MDCK) cells, Chinese hamster ovary (CHO) cells, human hepatoma cells (Hep 2, Hep G2), and human cervical carsinoma cells (HeLa). The development of toxicity tests based on human corneal cell lines has been held out against the fact that to date only a few human cell lines have been reported to have been developed (Kahn et al., 1993; Araki-Sasaki et al., 1995; Griffith et al., 1999), and that of these cell lines only CE cells are also commercially available.

2.3.3.2. Simple Cytotoxicity Tests

A large number of cytotoxicity assays have been proposed as alternative methods for ocular irritation. However, the classification of these assays is difficult, since many endpoints may reflect the results of multiple biological events (Herzinger et al., 1995). The surviving cells after an exposure to a test substance can be counted by using a hemocytometer (Benoit et al., 1987) or an electronic cell counter (Bracher et al., 1987;

Kennah et al., 1989). The cells detached from the substratum can also be determined (Reinhardt et al., 1985; Bracher et al., 1987). Colony-forming efficiency assays are used to assess the capacity of cells to continue cell division after toxic exposure (North-Root et al., 1982; Borenfreund and Borrero, 1984; North-Root et al., 1985; Sasaki et al., 1991). Morphological changes can be observed by simple microscopic observation allowing a rapid evaluation of the minimal dose of a test material inducing a change in cellular morphology (Borenfreund and Borrero, 1984; Borenfreund and Puerner, 1984; Borenfreund and Puerner, 1985).

2.3.3.3. Detection of Nutrient Transport, Cellular Protein, and Energy Metabolism Disturbances

The integrity of cellular nutrient transport systems can be determined by measuring the uptake of radiolabeled precursors, such as thymidine (Lazarus et al., 1988), leucine (Sina et al., 1992), and uridine (Shopsis and Sathe, 1984; Borenfreund and Borrero, 1984; Borenfreund and Shopsis, 1985; Jacobs et al., 1988). Today, however, the use of radioactive labels is no longer favored. The total cellular protein can be determined by various dyes, such as Coomassie brilliant blue (Shopsis and Eng, 1985), kenacid blue (Balls and Horner, 1985; Knox et al., 1986; Clothier et al., 1988; Pape and Hoppe, 1991), crystal violet (Itagaki et al., 1991), and the Lowry reagent (De Angelis et al., 1986). The amount of protein-bound dye is usually proportional to the cell number. The impairment of mitochondrial energy metabolism can be measured by using tetrazolium salt dyes, which are readily reduced to their respective formazans by metabolically active cells. The most widely utilized tetrazolium salt dye is the methyl-thiazolyl tetrazolium salt, MTT, used as a convenient microtiter format (Mosmann, 1983; Grant et al., 1992; Sina et al., 1992; Yao and Acosta, 1992; Grant and Acosta, 1994; Yang and Acosta, 1994; Saarinen-Savolainen et al., 1998). For spectrophotometric measurements, MTT-formazan crystals need to be solubilized, and a wide range of solubilization protocols can be used, such as acidified isopropanol, propanol/ethanol solution or dimethyl sulfoxide, DMSO (Marshall et al., 1995).

2.3.3.4. Detection of Membrane Changes

Certain classes of chemicals may cause damage to plasma membranes and the denaturation of membrane proteins and other cellular proteins. These events can be correlated with the initial inflammatory response in tissue irritation and with the changes in protein conformation, such as those changes observed in the opacification of the cornea. The red blood cell (RBC) hemolysis assay is widely used in the soap and detergent industry to assess membrane injury (Pape et al., 1987; Pape and Hoppe, 1990; Pape and Hoppe, 1991; Sugai et al., 1991; Sugai et al., 1993; Pape et al., 1999). Red blood cells are readily available and their handling does not require cell culture facilities. The RBC assay measures two endpoints, cellular lysis and changes in protein conformation, which are detected by spectrophotometric changes in hemoglobin absorption.

One of the simplest methods for viable cell counting is the trypan blue dye exclusion method (Wong et al., 1991; Chang et al., 1995; Uliasz and Hewett, 2000). Trypan blue staining method is being used widely in cell culture systems to detect damage and denaturation of the cell membrane. It is based on the principle that viable cells do not take up certain dyes, while non-viable cells do. Changes in the cell membrane integrity can also be measured by simultaneous monitoring of changes in the relative fluorescence intensity of the two dyes fluorescein diacetate and ethidium bromide, which stain viable and non-viable cells, respectively (Scaife, 1985; Altman et al., 1993). In the propidium iodide fluorescence staining method, DNA-binding dye propidium iodide binds to the nuclei of cells whose plasma membranes have become permeable due to cell death (Nieminen et al., 1992; Grant and Acosta, 1994; Dengler et al., 1995; Saarinen-Savolainen et al., 1998). Membrane injury can also be investigated by measuring the leakage of intracellular enzymes into culture medium, such as LDH (Korzeniewski and Callewaert, 1983; Decker and Lohmann, 1988; Grant et al., 1992; Yao and Acosta, 1992; Grant and Acosta, 1994; Yang and Acosta, 1994) or alkaline phosphatase (Scaife, 1985).

The most widely studied cytotoxicity test is the neutral red uptake (NRU) test, which is based on the uptake and accumulation of the vital dye neutral red in the lysosomes of viable cells (Borenfreund and Puerner, 1984; Borenfreund and Shopsis, 1985; Borenfreund and Puerner, 1985). Neutral red is selectively retained by the lysosomes of living cells, because of the differential pH between the inside of the lysosome and the surrounding cytoplasm. Alterations in the lysosomal membranes result in reduced uptake and binding of neutral red. After the incubation period, excess dye is removed. Then intracellular neutral red is extracted and measured spectrophotometrically. The NRU test can be easily automated, and thus many samples can be studied at the same time. For eye irritation testing, the NRU test has been performed on normal primary human keratinocytes, normal rabbit CE cells (Torishima et al., 1995), and the rabbit SIRC cell line (Roguet et al., 1992). Some other fibroblast cell lines have also been used (Rasmussen, 1995; Jones et al., 1999). CornePack® (Kurabo Industries Ltd., Osaka, Japan) is based on the use of normal epithelial cells from rabbit cornea and the NRU test (Uchiyama et al., 1999). The NRU test has also been evaluated in several validation studies. Membrane damage can also be measured by the neutral red release (NRR) test. In the NRR test, cells are first preloaded with neutral red and then exposed to a toxic substance. The amount of dye released from the cells will reveal the degree of membrane damage (Reader et al., 1989; Reader et al., 1990; Zuang, 2001). A commercially available test kit under the trade name of PrediSafe (Biopredic, Rennes, France) is based on the use of SIRC cells and the NRR test (Guyomard et al., 1994; Courtellemont et al., 1999a).

Fluorescein leakage (FL) tests model the corneal epithelium barrier, which is normally impermeable due to cellular junctional formation, especially to tight junctions (Cottin and Zanvit, 1997; Zanvit et al., 1999). Cells are grown to confluence on microporous filters and exposed to fixed concentrations of a test material. The degree of junction disruption results in proportional increase in permeability, measured as a passage of the relatively non-toxic dye sodium fluorescein across the barrier (Tchao, 1988; Shaw et al., 1990; Shaw et al., 1991). This leakage can be measured spectrophotometrically. The system also has the capability to assess recovery from exposure.

2.3.3.5. Evaluation of Solid Materials and pH Changes

In cell culture, solid products which are non-soluble in culture media can be studied by the agar diffusion assay, in which a diffusion matrix (agar or agarose) is used for the delivery of a test material to a monolayer of cultured cells (Guess et al., 1965; Wallin et al., 1987; Jackson et al., 1988) or, alternatively, cells can be suspended in agarose medium (Wallin et al., 1987; Jackson et al., 1988; Cottin et al., 1993). After the incubation time, cell viability can be examined microscopically or by using the NRR and MTT tests (O'Brien et al., 1990). The silicon microphysiometer (SM), also known under the trade name of Cytosensor (Molecular Devices Corp., Sunnyvale, CA, USA) is a biosensor based on silicon chip technology, which detects changes in the physiological rate of cultured cells by monitoring the rate at which cells excrete their acidic products of metabolism (Parce et al., 1989; Bruner et al., 1991b; Catroux et al., 1993; Harbell et al., 1999). The SM makes it possible to examine the changes of the cellular acidification rate by measuring continuously the pH value in the culture medium.

2.3.4. Chorioallantoic Membrane (CAM) Assays

In the CAM-based assays, test material is applied to the chorioallantoic membrane of embryonated chicken eggs. Originally, CAM-based assays were considered to provide a model for the conjunctival tissue of the eye. They are the only *in vitro* models that evaluate the effects of chemicals to blood vessels, which plays an important role in the inflammatory process *in vivo*. A significant benefit of the CAM assays is that they can be used to test any water-soluble or insoluble material or product. Since CAM assays as *in ovo* assays use a living non-mammalian organism at a low developmental stage, they are not *in vitro* systems in the strict sense. In principle, two types of CAM-based assays are currently being used. The hen's egg test on the chorioallantoic membrane (HET-CAM) assay was developed by Luepke in Germany (Luepke, 1985), and the chorioallantoic membrane vascular assay (CAMVA) by Bagley in the United States (Bagley et al., 1991). In the HET-CAM assay, the chorioallantoic membrane of the fertilized hen's egg is exposed to a test substance, and the blood vessels, capillaries, and albumin are examined and scored for irritant effects (hemorrhage, coagulation and lysis). There are a number of modifications of the original HET-CAM (Gilleron et al., 1996; Gilleron et al., 1997; Steiling et al., 1999). As endpoints, the CAMVA measures hemodynamic effects, injury, and anti-angiogenic effects to the membrane microvasculature correlated to eye irritation potential (Bagley et al., 1992b; Bagley et al., 1994; Bagley et al., 1999a). The chorioallantoic membrane-trypan blue staining (CAM-TB) method was developed to make the HET-CAM assay a more objective and quantitative test (Hagino et al., 1991; Hagino et al., 1993). The injury induced by test substances is determined by measuring the amount of trypan blue dye absorbed by the CAM. The CAM-TB method can be performed in combination with the HET-CAM assay by using the same eggs.

2.3.5. Organotypic Test Models

The most obvious approach to eliminate the use of animals in toxicology studies is the employment of isolated target organs (*i.e. ex vivo* experiments). The organotypic systems include enucleated rabbit and chicken eyes, isolated corneas, and cultured bovine and porcine lenses (Sivak et al., 1994; Sivak et al., 1995; Chamberlain et al., 1997; Oriowo et al., 2002). Non-ocular tissues such as skin explants from various species, isolated rabbit vagina, and rabbit ileum have also been used (Muir, 1983). The enucleated eye test with isolated rabbit eyes was first introduced in the early 1980's (Burton et al., 1981; Whittle et al., 1992). Corneal thickness, corneal opacity, and fluorescein retention were measured to assess the possible adverse eye effects of test materials. Next, slaughterhouse waste tissue was investigated as a source of eyes in order to limit the use of laboratory animals. The chicken enucleated eye test (CEET) was proposed as a practical pre-screen for the assessment of eye irritation potential (Prinsen and Koeter, 1993; Prinsen, 1996). The test is based on the scoring of corneal swelling, corneal opacity, and the fluorescein retention of damaged epithelial cells. Today, the isolated chicken eye (ICE) test and the isolated rabbit eye (IRE) test use the normally discarded eyes from food production and from laboratory animals that have been used for other scientific purposes (Chamberlain et al., 1997; Cooper et al., 2001).

The cornea is a very important tissue in the original Draize eye test, since damage to the cornea contributes more to the Draize score than does damage to any other ocular tissue (Draize et al., 1944; Wilhelmus, 2001). 73% of the maximum Draize score can derive from the severity and size of the corneal opacity. The bovine corneal opacity and permeability (BCOP) assay is based on the method of Muir (Muir, 1984; Muir, 1985) and focuses primarily on corneal damage. The BCOP assay employs the freshly collected corneas from the cattle used for meat production, and two endpoints are investigated, corneal opacity and the disruption of the corneal barrier by the passage of a fluorescent dye (Gautheron et al., 1992; Vanparys et al., 1993; Taniguchi et al., 1994; Gautheron et al., 1994; Cooper et al., 2001).

2.3.6. Tissue Equivalents

A more recent and very promising approach for *in vitro* ocular testing is the use of tissue equivalent assays (TEA), which have also been designed to mimic the corneal response (Whalen et al., 1994; Decker and Harber, 1994; Donnelly et al., 1994; Osborne et al., 1995). There are several commercial three-dimensional dermal tissue models, *e.g.* EPISKIN™, SKIN^{2TM}, Epiderm™, and MATREX™. At least the SKIN^{2TM} models (Pirovano et al., 1993; Decker and Harber, 1994; Osborne et al., 1995; Espersen et al., 1997; Southee et al., 1999; Kurishita et al., 1999) and the MATREX™ model (Taniguchi et al., 1994; Decker and Harber, 1994; Rasmussen, 1995; Kasai et al., 1995; Espersen et al., 1997; Southee et al., 1999; Ohuchi et al., 1999) have also been used for ocular irritation studies. The EPISKIN™ model is a three-dimensional human skin model consisting of human keratinocytes grown on a collagen to support reconstituted epidermis and a functional stratum corneum. It was first marketed in the form of a kit by the Imedex

Company (Chaponost, France) and bought by L'Oréal in April 1997. The SKIN^{2TM} ZK1100 model consists of human dermal fibroblasts grown in a three-dimensional nylon mesh matrix, while the SKIN^{2TM} ZK1200 model consists of cocultured stratified squamous epithelium and stromal fibroblasts derived from human skin, grown on a nylon mesh (Advanced Tissue Sciences, La Jolla, CA, USA). The Epiderm™ model by MatTek Corp. (Ashland, MA, USA) consists of human-derived epidermal keratinocytes cultured on the cellulose acetate filter to form a multilayered model of the human epidermis. The MATREX™ consists of human dermal fibroblasts cultured in a contracted collagen lattice, which maintains a three-dimensional structure (originally developed by Organogenesis, Inc. in the 1980's, produced by Toyobo Co., Ltd., Japan). In dermal models, viability has been measured by using the MTT, NRU and NRR tests. Membrane damage has been determined by the LDH and sodium fluorescein permeability assays.

The three-dimensional human reconstituted epithelial culture (REC) model by SkinEthic Laboratories (Nice, France) has been constructed with transformed human keratinocytes cultured on polycarbonate filter inserts at the air-liquid interphase to form a stratified epithelium similar to that found in the human cornea (Doucet et al., 1998; Doucet et al., 1999). Cell viability has been determined by using the MTT test. Another commercially available corneal tissue model, EpiOcular™ (MatTek) consists of primary human-derived epidermal keratinocytes and has also been used with the MTT test (Stern et al., 1998). The HCE-T model was developed by using a transfected HCE cell line cultured on a collagen membrane at the air-liquid interphase (Kruszewski et al., 1997; Clothier et al., 2000). In this model, barrier function has been determined by measuring transepithelial permeability to sodium fluorescein and transepithelial electrical resistance (Ward et al., 1997), and cell viability by using the MTT, Alamar Blue™, and lactate assays (Clothier et al., 2000). In another HCE model, immortalized HCE cells were grown on filters with various filter materials and coating procedures (Toropainen et al., 2001). In their study, transepithelial electrical resistance and transmission electron microscopy were used. Permeabilities of H-3-mannitol and 6-carboxyfluorescein were determined to evaluate the intercellular spaces of the epithelium. Rhodamine B was used as a lipophilic marker of transcellular permeability. A reconstituted corneal epithelium model consisting of immortalized HCE cells is also being marketed by SkinEthic Laboratories.

Three-dimensional corneal models mimicking the entire cornea are currently being developed for ocular toxicity studies. These corneal models consist of a stromal analogue composed of keratocytes in a collagen matrix cocultured with epithelial cells (Parnigotto et al., 1998; Germain et al., 1999; Orwin and Hubel, 2000). A more complex corneal model that mimics the normal cornea even more closely is composed of corneal stromal keratocytes embedded in a collagen matrix with an underlying layer of endothelial cells, and covered with multilayered CE cells (Germain et al., 2000). These kinds of corneal models have been constructed with primary bovine cells (Minami et al., 1993), primary fetal pig cells (Schneider et al., 1997; Schneider et al., 1999), and human cell lines (Griffith et al., 1999). The interest for the development of three-dimensional human corneal models is continuously increasing, since an *in vitro* model based on human cells is expected to approximate better the range of species-specific cellular targets and responses to toxic injury that occur in the human eye *in vivo*. Especially corneal wound healing and recovery can be studied more accurately in three-dimensional corneal models, as the cell-matrix interactions and the cell-to-cell contacts are closely modeled.

2.4. Evaluation of Alternative Methods

In the context of *in vitro* toxicology, the term validation is the process by which alternative methods are independently assessed for their relevance, reliability, reproducibility, and interlaboratory transferability before being evaluated for their acceptability for integration with or as replacements for animal toxicity testing (Balls and Clothier, 1987; Balls et al., 1990; Bruner et al., 1996; Bruner et al., 1998a; Balls and Fentem, 1999). The prediction model (PM), which defines how to use the results from an alternative method to predict an *in vivo* toxicity endpoint, has an important role in the validation process. As a matter of fact, an alternative method for the replacement or the partial replacement of an animal test can be thought to be a combination of a test system and a PM (Bruner et al., 1996; Archer et al., 1997; Worth and Balls, 2001a). The test system provides a means of producing physicochemical or other *in vitro* data for chemicals of interest, whereas the PM is an explicit algorithm for converting these data into predictions of *in vivo* toxicity in animals or human. In order to validate an alternative method, it is necessary to show that for its intended purpose the test system has a reliable scientific basis (relevance), the predictions made by the PM are sufficiently accurate (reliability), and the results are sufficiently reproducible over time, within and between laboratories (reproducibility).

Before its formal validation, an *in vitro* test must be properly developed. In test development, the purpose of the test and the need for its comparison with other tests are defined (Balls and Fentem, 1999). The scientific basis of the test is studied and a protocol and standard operation procedures are produced. A prediction model is developed and its use with a set of reference chemicals is defined. In prevalidation, *i.e.* in a step between test development and formal validation, an experienced laboratory, other than the developer of the test, ensures that an optimized test protocol is available, which can be transferred to other laboratories (Curren et al., 1995). The feasibility and reproducibility of the test are then examined in an interlaboratory study using a set of coded test agents. If the method performs well, a database is established by testing an extended set of chemicals with known *in vivo* irritancy data. Finally, appropriate statistical methods are used to compare the *in vitro* data to the *in vivo* data. The Draize eye irritation test is the most widely criticized toxicity test and, consequently, several national and international validation studies of alternatives for ocular toxicology have been organized. Six major evaluation and validation studies were carried out in 1988-1997 with the most promising ocular *in vitro* alternative methods.

2.4.1. BGA/BMBF Validation Study

The BGA/BMBF study by the Bundesgesundheitsamt/Bundesministerium für Bildung und Forschung (German Federal Health Office/Federal Ministry of Education and Research) was a national validation study carried out in Germany between 1988 and 1995 (Kalweit et al., 1990; Spielmann et al., 1991; Spielmann et al., 1993; Spielmann et al., 1996). The study was coordinated by ZEBET (German Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments) at the Bundesgesundheitsamt

(BGA), and supported by the German Ministry of Research and Technology. Two *in vitro* assays, the hen's egg chorioallantoic membrane (HET-CAM) assay and the NRU test using the 3T3 mouse fibroblast cells were selected for validation as promising tests for the identification of severe eye irritants. The validation study was conducted in two phases, and a total of 200 chemicals (147 new chemicals and 53 existing chemicals) was tested.

During the first phase (1988-1990), standardized protocols were developed for the HET-CAM and 3T3-NRU tests and the test methods were established in 13 laboratories. 34 independently coded chemicals, supported by high quality *in vivo* data, were tested under blind conditions. Both *in vitro* tests were found to have satisfactory intralaboratory and interlaboratory reproducibilities. The 3T3-NRU test was found to be more reproducible than the HET-CAM test, while the HET-CAM test was better at identifying severe eye irritants. Both tests were able to classify the test chemicals in a similar order to that arrived at the Draize test. In the second phase (1990-1994), both methods were evaluated in two laboratories by testing under blind conditions 166 industrial chemicals which were different from the 34 chemicals tested in the first phase. The authors of the study concluded that chemicals can be classified as severe irritants (as R41 chemicals according to the EU classification) with sufficient reliability by the combined use of the HET-CAM test and the 3T3-NRU test. Since 1992, the German regulatory authorities have accepted the use of HET-CAM data for the classification of R41 chemicals in the safety assessment of new industrial chemicals. According to EU regulations, the risk rating R41 (risk of severe damage to eyes) is used to classify substances that can cause irreversible corneal opacity and/or serious damage to the eye. The risk rating R36 (irritating to eyes) is used for substances that induce reversible changes in the cornea, iris, and/or conjunctiva.

2.4.2. CTFA Validation Study

The CTFA (Cosmetics, Toiletries and Fragrance Association) conducted a six-year validation program in the United States in three phases in 1990-1996. In each phase approximately 24 *in vitro* assays, not counting the variations of the tests, were tested with specific groups of products: 10 hydroalcoholic formulations were tested in Phase I (Gettings et al., 1990; Gettings et al., 1991); 18 oil-water based emulsions in Phase II (Gettings et al., 1994); and 25 surfactant-based formulations in Phase III (Gettings et al., 1996b). An independent laboratory coded all test materials, and both *in vitro* and *in vivo* tests were conducted under blind conditions.

After the experimental stages of each phase, the chemical identities were revealed, and the relationship between the *in vivo* and *in vitro* data was analysed by statistical methods. First, a concordance analysis was carried out in which a comparison was made between the *in vivo* and *in vitro* rankings of the test materials. The *in vitro* tests which performed to a certain level in the concordance analysis were then analyzed by non-linear regression to approximate the relationship between the *in vivo* and the *in vitro* data. In this analysis, a 95% prediction interval (PI) was used to reflect the variability both in the *in vivo* and the *in vitro* test. By this approach the observer can visualize the range of *in vivo* scores predicted by a given *in vitro* result. The variability of the Draize test in each phase of the

study was high, even though all tests had been made in the same laboratory. The performance of the *in vitro* tests also varied between the phases. For Phase I and Phase III, hydroalcoholic and surfactant-based formulations, the concordance of the *in vitro* tests was higher and the prediction intervals were narrower than for Phase II, oil-water based materials. It is noteworthy in this validation study that the variability of the Draize test scores was taken into account when determining the performance of the *in vitro* tests (Balls et al., 1999). The predictivity of *in vitro* tests was also shown to vary according to the type of the chemical tested.

2.4.3. IRAG Validation Study

The IRAG (Interagency Regulatory Alternatives Group) study was organized by three US regulatory agencies (FDA, EPA, and CPSC) between the years 1991 and 1994 (Bradlaw et al., 1997; Bradlaw and Wilcox, 1997). The evaluation study was based on the existing Draize test and the *in vitro* data submitted in parallel from laboratories all over the world. Over 60 data sets from 41 laboratories were received for 29 different test methods. The *in vitro* data were not only compared with the MMAS, but also with tissue scores representing damage to the cornea, conjunctiva, and iris. A set of guidelines was developed to standardize the data submissions and to advance their review (Scala and Springer, 1997). Methods were grouped into five categories: organotypic models (Chamberlain et al., 1997), CAM-based assays (Spielmann et al., 1997), cell function-based assays (Botham et al., 1997), cytotoxicity assays (Harbell et al., 1997), and other systems (Curren et al., 1997). Also a statistical committee was established to facilitate the planning and analysis of the study (Feder et al., 1997).

In the study, the variabilities of both *in vitro* and *in vivo* tests were taken into account. There were differences in the predictivity between test methods for the same type of chemicals and between chemical types for the same test method. None of the test methods showed a satisfactory performance across all chemical groups. In general, *in vitro-in vivo* correlations were influenced by the variability of the Draize test scores. The IRAG study group concluded that none of the tested *in vitro* tests, and no combination of them, could be used as a replacement for the Draize test. However, some of the alternative methods were reported to have the potential to reduce the use of animals, if the tests are used in validated and well-defined conditions.

2.4.4. MHW/JCIA Validation Study

The MHW/JCIA study was a national validation study carried out in Japan by the Japanese Ministry of Health and Welfare and the Japanese Cosmetics Industry Association in 1991-1995 (Ohno et al., 1999). A total of 27 laboratories participated in this study, and twelve alternative methods were assessed: the HET-CAM method (Hagino et al., 1999); the HET-CAM-trypan blue staining (CAM-TB) method (Hagino et al., 1999); the RBC haemolysis method (Okamoto et al., 1999); the haemoglobin denaturation (HD) method (Hatao et al., 1999); the artificial skin models SKIN™ (Kurishita et al.,

1999) and MATREX™ (Ohuchi et al., 1999); the CornePack™, the NRU test based on normal rabbit CE cells (Uchiyama et al., 1999); the crystal violet staining (CVS) method using SIRC cells (SIRC-CVS)(Tani et al., 1999); the CVS method using Chinese hamster lung cells (CHL-CVS)(Okumura et al., 1999); the NRU method using SIRC cells (SIRC-NRU)(Tani et al., 1999); the MTT test with HeLa cells (MTT-HeLa)(Chiba et al., 1999); and EYTEX™ (Matsukawa et al., 1999). Each test (except for MATREX™ and CHL-CVS) was tested in at least five laboratories. Most of the participating laboratories assessed more than one method. The test chemicals, 38 cosmetic ingredients, were tested in three phases. The test materials were coded and supplied to the participating laboratories, and the tests were conducted under the principles of good laboratory practice (GLP). *In vitro* data was compared to the Draize data obtained from a single laboratory.

Interlaboratory variability assessed by the mean CV (the coefficient of variation averaged by all chemicals) was less than 50% for all the *in vitro* tests except for the HET-CAM and HD tests, for which CVs were higher than 50%. By excluding the non-irritating ingredients from the analysis, the mean CVs for these tests were also reduced to below 50%. The variability in the *in vivo* data was higher, especially for the MMAS in the range of 15 to 50, which is important for the evaluation of cosmetic ingredients. The correlation between the *in vitro* data and the MMAS data was high (Pearson's coefficient higher than 0.8) for CAM-TB, HD, SIRC-CVS, SIRC-NRU, HeLa-MTT, and CHL-CVS, but low for EYTEX (0.3). For cytotoxicity tests the Pearson's correlation coefficients were more than 0.8 when acids, alkalis, and alcohols were excluded. When MMAS scores were grouped into five categories according to Kay and Calandra (Kay and Calandra, 1962), a high rank correction (Spearman's coefficient greater than 0.8) was found between these categories and *in vitro* data for the HET-CAM and the CAM-TB tests. As a result of these findings, it was concluded that none of these alternative methods could be used to test all types of test substances, and that a battery of tests would be needed to optimize the prediction of eye irritancy.

2.4.5. EC/HO Validation Study

The EC/HO study, set up by the European Commission and the British Home Office, was conducted in 1992-1995 (Balls et al., 1995). The validation study was organized by the UK and funded by the EU. Nine of the tested *in vitro* methods included four cell culture methods (RBC, NRU, FL, and SM), three *ex vivo* tests (IRE, ICE, and BCOP), the HET-CAM test, and the EYTEX™. 60 chemicals were tested under blind conditions in 37 laboratories.

The data generated by the laboratories were analyzed independently. The interlaboratory Pearson's correlation coefficients of the *in vitro* scores for each endpoint were determined to assess the reliability of each test. There was good reproducibility between different laboratories conducting the same test. The predictivity of each test was assessed by preparing scatter plots showing the relationship between the *in vitro* data and *in vivo* scores, by calculating the Pearson's and Spearman's correlation coefficients for the relationships between each alternative test endpoint and the MMAS, and by deriving a linear regression equation to predict the MMAS from each alternative test endpoint and to

determine a 95% confidence interval (CI) for this prediction. For the full set of tested chemicals, the *in vitro-in vivo* correlations were generally low (less than 0.6) and 95% CIs were generally wide (greater than ± 40 MMAS units). A multivariate analysis was also undertaken to determine if the combined use of more than one non-animal test could be useful, to determine which combinations of tests may provide improved predictions, and to assess the utility of the rabbit eye test for the evaluation of non-animal alternative methods (Balls et al., 1999). This multivariate analysis involved an examination of 20 non-animal test measures and *in vivo* scores for 59 test substances. Principal components analysis (PCA) was used to identify the endpoints, which explained the greatest variation in the data, and partial least squares (PLS) analysis was used to develop models for predicting eye irritation potential from a combination of *in vitro* test results. The analyses showed that FL, IRE, ICE, and NRU tests explained more of the variability in the data than any other single test used alone. Several factors were reported to be possible reasons for the low precision of the predictions obtained in this study, including the choice of test materials, the evaluated test methods, the variability of the *in vivo* data, the use of MMAS as the *in vivo* endpoint, and the choice of statistical methods. None of the nine tests alone was found to be sufficiently predictive of *in vivo* eye irritation for the full set of the test chemicals, even though some of the tests were sufficiently reproducible. However, the EC/HO study made valuable contributions to the validation processes by highlighting the importance of optimizing the test protocols and refining the PMs before entering them into a large-scale validation study (Bruner et al., 1996).

2.4.6. COLIPA Validation Study

The European Cosmetic, Toiletry and Perfumery Industry Association carried out the COLIPA international validation study in 1994-1997 (Brantom et al., 1997). Ten proposed alternative methods were assessed: the CAMVA (Bagley et al., 1999a), EYTEX™ (Courtellemont et al., 1999b), the FL test (Zanvit et al., 1999), the HET-CAM test (Steiling et al., 1999), the NRU test (Jones et al., 1999), the PTG test (Kristen et al., 1999), the NRR test as the PrediSafe™ test kit (Courtellemont et al., 1999a), the RBC assay (Pape et al., 1999), the SM assay (Harbell et al., 1999), and the Skin^{2TM} ZK1200 assay (Southee et al., 1999). The COLIPA validation study was designed to take into account the lessons already learned in the EC/HO study. Five of the tests (EYTEX™, HET-CAM, NRU, RBC, and SM) had similar protocols with the EC/HO study. 55 substances (23 cosmetic ingredients and 32 formulations, such as make-up products, hair dyes, and shampoos) were tested under blind conditions in 32 laboratories. The study was carried out at two stages: a dry run on ten test substances, and a main run on all test substances. Sample coding, randomization, and supply to the participating laboratories were done under the GLP principles. The raw data had a quality check before they were independently analyzed. According to the predefined criteria for reliability, none of the methods could be confirmed as a valid replacement for the Draize test across the full range of eye irritancy. The most promising tests were the FL test, the RBC test, and the Skin^{2TM} ZK1200 tissue equivalent assay, but their validity could not be confirmed, which indicates a need for follow-up studies. A multivariate analysis of the data was also conducted by the PCA and PLS techniques similar to those used in the EC/HO study. As

with the EC/HO study, improved PMs could be developed by the combinations of *in vitro* endpoints.

2.4.7. Summary of the Validation Studies

Historically, most of the proposed *in vitro* tests have been designed to model a small fraction of the complex process of eye irritation. As in the Draize test, other ocular toxicity, such as retinal toxicity, has been largely ignored. Many of the proposed *in vitro* alternative methods have been designed to measure the corneal damage only. Most of the proposed alternatives are good at classifying a certain types of chemicals, but not all types of chemicals across the full range of eye irritancy. Moreover, a number of them appear to be capable of distinguishing between non-irritants and severe irritants, but they are not especially good at classifying between materials of mild and moderate toxicity. The outcome of the most recent validation studies has been that no test, combination of tests, or testing strategy has been found capable of replacing the Draize eye test completely, but some of the assays have shown a considerable promise as screens for ocular irritancy (Balls et al., 1999; Worth and Balls, 2002a). The most developed and the most widely used alternatives are BCOP, HET-CAM, CAM-TB, IRE, ICE, RBC, agarose diffusion method, and EpiOcular™ tissue model (Worth and Balls, 2002a). The Irritection system, formerly known as EYTEX™, is however not recommended, because there are no standardized protocols, and because no good *in vitro-in vivo* correlations have been found.

The use of *in vitro* methods as screening tests is widespread in industry, since a number of alternative methods has been found to work well in-house (LeClaire and de Silva, 1998). It has been estimated that each year thousands of new products and materials are successfully tested worldwide in *in vitro* alternative studies, but only a small fraction of the results are being published (Curren and Harbell, 2002). Nevertheless, validation studies have not been able to establish this satisfactorily when *in vitro* test results have been compared to the historical Draize test data (Balls et al., 1999). The main reason for this is the subjectivity of the Draize test, which provides variability in the estimation of eye irritation. Furthermore, the *in vitro* alternatives tested in the recent validation studies only partially model the complex *in vivo* eye irritation response. Moreover, the test protocols and prediction models may have been insufficiently developed. The choice of statistical methods used for analyzing the data may also have been inappropriate.

Today, no single alternative method is expected to take the place of the multipurpose Draize eye test. In fact, it is now generally considered that a set of *in vitro* tests, which model the many mechanisms of eye irritation, is needed for the complete replacement of the Draize test (Worth and Balls, 2002a). Moreover, these methods should reflect various ocular target sites and the recovery from injury.

2.5. Current Approaches in Eye Irritation Testing

In 1991, ECVAM (European Centre for the Validation of Alternative Methods, located at the EC Joint Research Centre at Ispra, Italy) was established (Balls, 2002) to coordinate the validation of alternative methods and to promote the regulatory acceptance of alternative methods in the EU (Marafante et al., 1994; Worth and Balls, 2001b; Worth and Balls, 2002b). In 1995-1996, the EU member countries, the US regulatory agencies, and the OECD officially accepted the ECVAM validation procedure (Spielmann and Liebsch, 2001). In 1999, ECVAM organized a workshop on *Eye Irritation Testing: The Way Forward* (Balls et al., 1999). Four parallel approaches were then suggested as means for reducing the use of animals and ultimately for replacing the Draize eye test completely; (1) a reference standards approach, (2) a review of tiered testing strategies, (3) further analysis of data obtained in the previous validation studies, and (4) further research on the mechanisms of eye irritation. These approaches remain of importance even today.

2.5.1. Reference Standards (RS) Approach

It is widely considered that the reference standards (RS) approach would improve the predictivity of *in vitro* tests, which do not have relevant and reliable *in vivo* benchmark data (Worth and Balls, 2002a). An RS is a substance with a known degree of toxicity *in vivo*. ECETOC has published a comprehensive reference data bank including 132 chemicals (Bagley et al., 1999b). RSs can be used *in vitro* to determine the degree of toxicity of test substances, whose effects are scaled relative to the RSs (Balls et al., 1999). In industry, RSs are widely used for making safety decisions regarding the acceptability of new formulations of existing ingredients. In 1999-2000, ECVAM organized a reference standard study with five *in vitro* methods for ocular irritation: the ICE test, the BCOP test, the combined use of the HET-CAM and the NRU test, the EpiOcular corneal model, and the RBC test (Zuang, 2002). Four groups of chemicals were tested; neutral organics with BCOP, alcohols and esters with HET-CAM/NRU and EpiOcular, surfactants with ICE, HET-CAM/NRU and RBC, and siloxanes with BCOP and ICE. The management team of the ECVAM reference standards study recommended that the *in vivo* data should be analyzed by discriminating between different mechanisms of chemical action, by focusing on the effects of chemicals on the various parts of the eye, and on the different time-scales of effect (Worth and Balls, 2002a). The limitations of each *in vitro* assay should also be defined in terms of chemical classes. The existing *in vitro* data need to be re-evaluated to identify how to define better the groups of chemicals suitable for testing in each *in vitro* assay. The application fields of specific methods should therefore be narrowed. A decision tree for method selection and a database for reference standards should also be developed.

2.5.2. Tiered Testing Strategies

The OECD proposed a stepwise approach to hazard identification in 1996, which underwent revisions in 1998 and 2001 (OECD, 2001). Governmental commissions for the United States have also proposed a tiered approach to hazard identification (Wilhelmus, 2001). In this sequential approach (Figure 2), new chemicals are first evaluated by a literature review and physicochemical tests (such as the pH test). The data from skin irritation tests are also used as a pre-screen to detect the most severely irritating materials, assuming that materials that are severely irritating to the skin are most likely significantly irritating to the eye (Williams, 1984; Gad et al., 1986). In the following step, the compound is tested with structure-activity relationship studies and by a battery of *in vitro* tests. Only then negative results are confirmed with animal testing. The stepwise strategy therefore reduces and refines the use of animals, but the *in vivo* test is not completely replaced. The tiered testing strategy is particularly useful for eye irritation testing, since it is very unlikely that any single *in vitro* test will be capable of reproducing the complexity of the *in vivo* response.

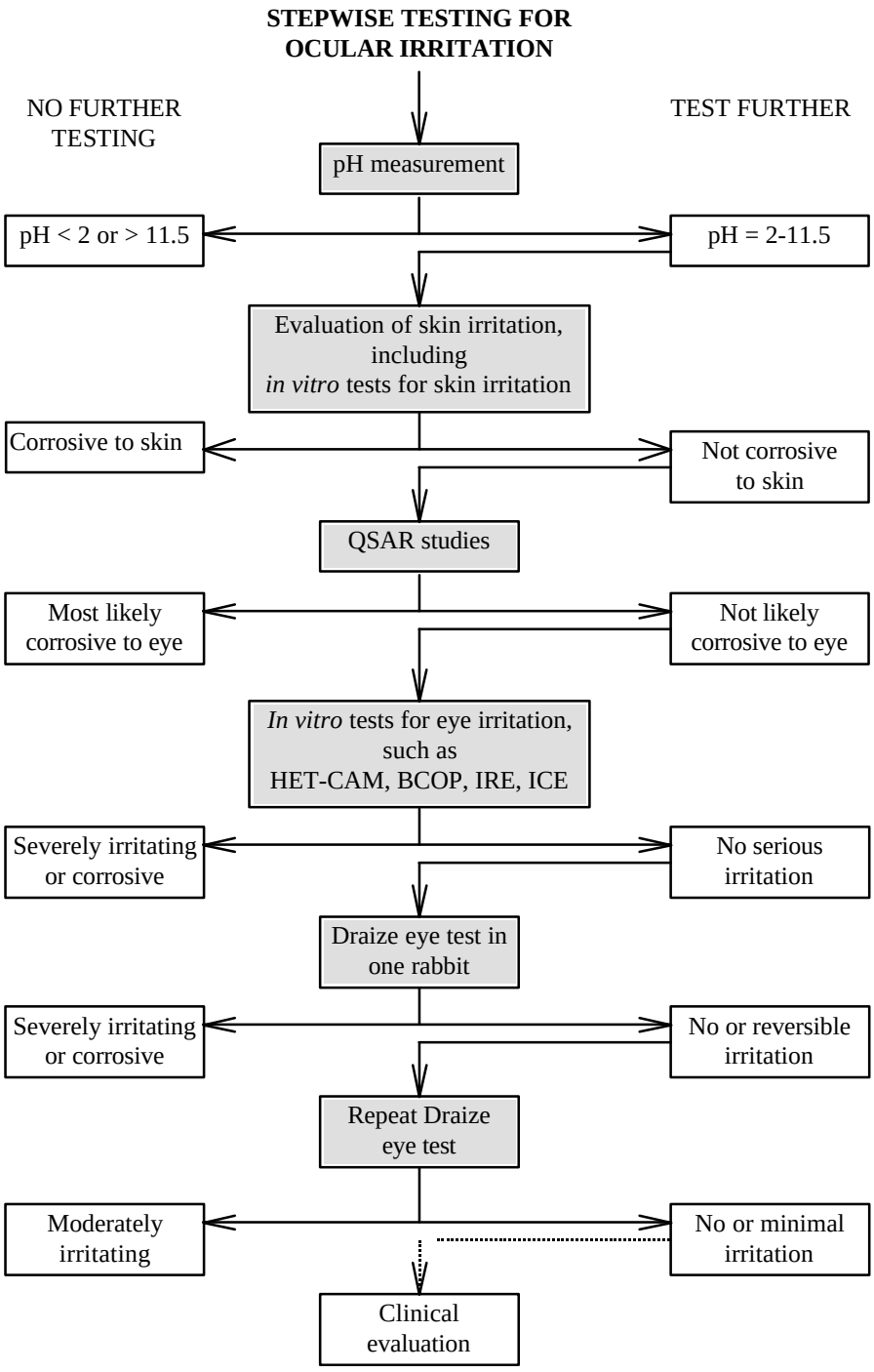

Figure 2. Stepwise testing strategy for ocular irritation used in the OECD (modified from (Wilhelmus, 2001)).

2.5.3. Mechanisms of Eye Irritation

More mechanistic approaches, which use *in vitro* systems rather than conventional animal analogy models, are now being proposed for toxicity testing (Balls, 1998; Bruner et al., 1998b). There is a need to develop early markers of eye injury, to evaluate the area and depth of corneal injury as markers of eye injury, and to develop methods for assessing wound healing, pain and the kinetics of the eye response (Balls et al., 1999). The COLIPA Eye Irritation Task Force has developed a research programme on the mechanisms of eye irritation with academic partners and cosmetics industry (Bruner et al., 1998a). The programme aims to find new *in vitro* endpoints that are more predictive of the human eye response than the traditional Draize test. The study focuses on the kinetics and patterns of changes in physiological function, the signals of injury released from *in vitro* cornea, and recovery after chemical injury as potential markers of eye damage. The circumstances where it is suitable to use different types of test systems, such as simple CE cell cultures or three-dimensional corneal constructs, will be defined. The International Life Sciences Institute (ILSI) and the Health and Environmental Sciences Institute (HESI) are also organizing studies on the development of a mechanistic-based eye irritant classification system (ILSI, 2003).

2.5.4. Current Status of In Vitro Alternatives

In June 2000, the first three experimentally validated *in vitro* tests were accepted by the EU for regulatory purposes: the 3T3-NRU *in vitro* phototoxicity test and two skin corrosivity tests; the human skin model EPISKIN™, and the rat skin transcutaneous electrical resistance (TER) test (Spielmann and Liebsch, 2001; Liebsch and Spielmann, 2002). The skin corrosivity tests and the 3T3-NRU phototoxicity test were adopted as test methods B.40 and B.41 of Annex V of EU Directive 67/548 EEC on the Classification, Packaging and Labeling of Dangerous Substances (European Commission, 2000). Since the EPISKIN™ model was not commercially available after it had been validated, another human skin model, the EpiDerm™ was validated in an ECVAM catch-up-study, in which prevalidation and validation studies were combined to facilitate the validation process and its regulatory acceptance as an alternative corrosivity test. As of May 2002, skin alternative methods were also approved as OECD test guidelines 430 and 431.

Four *in vitro* methods have been accepted for regulatory purposes according to EU Directive 86/906/EEC for the classification of severely eye-irritating materials (Liebsch and Spielmann, 2002). These methods are the HET-CAM assay, BCOP, and isolated chicken and rabbit eye tests. Many national authorities have already accepted these assays for the identification of severe eye irritants, classified as R41 chemicals (Zuang, 2002). Chemicals which provide negative results in any of these four tests still need to be tested in the Draize test in 1-3 rabbits to confirm the absence of eye irritation potential. In Germany, the BgVV has accepted the use of HET-CAM, BCOP, IRE, and ICE tests for the classification of R41 chemicals (Worth and Balls, 2002a). If the chemical is corrosive to the skin or very acidic or basic, it can be classified as severely irritating to the eye without further testing. In France, the HET-CAM, BCOP, IRE, and ICE tests have also

been accepted for the positive classification of R41 chemicals. Additionally, the NRR and the agarose-diffusion methods are accepted for the safety assessment of cosmetics. In the UK, the IRE, BCOP, and HET-CAM assays are accepted for the classification of severe eye irritants. Negative results still need to be confirmed by an *in vivo* test. In Belgium, the Pharmaceutical Commission has accepted the BCOP test for the identification of drug formulations that are ocular irritants. In the Netherlands, the ICE, BCOP, and HET-CAM tests have also been accepted for screening severe eye irritants. No *in vitro* test is accepted as a single test for the classification of irritating (R36) and non-irritating substances. In Finland, only the methods mentioned in the Annex V of Directive 67/548/EEC are officially accepted. Non-validated *in vitro* methods are assessed on a case-by-case basis. A chemical can be classified as irritating to the eye by using such an *in vitro* test, but the classification as non-irritating must be confirmed by the methods mentioned in Annex V. The FDA, EPA, and other US agencies no longer require the Draize test for corrosive products but still recognize the value of animal testing in product safety assessment (FDA, 2003). ECVAM highly recommends national regulatory authorities to consider their positions on the acceptance of the BCOP, HET-CAM, IRE, ICE, and other non-animal tests for eye irritation (Zuang, 2002).

The objective of the recent European Commission White Paper on a *Strategy for a Future Chemicals Policy* is to collect sufficient information about all chemicals, both new and already existing in the EU, by the end of 2012 (Vogelgesang, 2002). The White Paper proposes a stepwise method to all the chemicals that are produced in the amounts of 1 tonne/year/manufacturer. Specifically, it aims to collect information on the existing chemicals, which represent more than 99% of the chemicals on the market. Present toxicity databases, structure-activity analysis, in-use history, and *in vitro* data need to be combined in an intelligent way when making safety decisions in ocular toxicology (Curren and Harbell, 2002). Therefore, the search for a relevant, reliable, and largely available testing strategy is going on, to replace the Draize eye test and to meet the needs of the recent White Paper policy and whatever the future 7th Amendment to the Cosmetics Directive (EEC, 1976; EEC, 1993) will hold. The establishment of a testing strategy for ocular toxicology requires an *in vitro* test battery that is based on the methods giving complementary results of the different mechanisms and target sites involved in ocular irritation.

3. AIM OF THE STUDY

The present study was undertaken to develop ocular toxicity tests based on novel cell culturing techniques to be used as pre-screens for ocular irritancy and retinal toxicity and ultimately as potential alternatives in a more comprehensive test battery aimed to replace the traditional time-honored Draize eye test. The purpose of the study was to evaluate ocular toxicity by testing, as positive controls, a set of model compounds which are known to have adverse ocular effects. To evaluate the ocular toxic effects of the selected test compounds, effective multititer techniques and two basal cytotoxicity tests were established: the WST-1 test as an index of mitochondrial function and cell proliferation and viability, and the lactate dehydrogenase (LDH) leakage test as an index of cell membrane integrity, based on animal primary cultures and human cell lines. The specific aims of the study were:

- To characterize immunohistochemically an SV40-immortalized human corneal cell line (HCE) grown in culture medium, and to compare the cytokeratins (CKs) induced in culture conditions to the CKs of the human cornea *in vivo* (I).
- To compare the HCE cell line to rabbit primary corneal epithelial cells (RCE) in the WST-1 and LDH cytotoxicity testing (II).
- To investigate the reproducibility and test conditions of the HCE-WST-1 test on the interlaboratory level (III).
- To investigate the use of HCE cells and the WST-1 and LDH tests for the evaluation of long-term adverse effects (IV).
- To compare a human RPE cell line D407 to pig primary RPE cells in the WST-1 testing (V).

4. MATERIALS AND METHODS

4.1. Cell Cultures and Tissues

In the present study, human cell lines and animal primary cell cultures from corneal epithelium and retinal pigment epithelium were used for cytotoxicity studies. Human corneas were used when the cytokeratins expressed by the immortalized HCE cell line were compared to the cytokeratins *in vivo*.

4.1.1. Human Corneas (I)

Peripheral corneal rims were obtained from cadaver donors without any known corneal diseases for corneal transplantation at Helsinki University Central Hospital (Finland). The corneas were excised within 12 hours of the death of the donor. The centres of the corneas were used in transplantation, and we used the peripheral area of the cornea. The peripheral corneal specimens were snap-frozen and stored at -80°C until cut into 6 µm thick sections and placed on vectabond slides, 2 sections per slide. These sections were then stored at -20°C until analyzed by immunohistochemistry.

4.1.2. Immortalized Human Corneal Epithelial (HCE) Cell Line (I-IV)

The SV40-immortalized HCE cell line was a generous gift of the developers' of the cell line, Araki-Sasaki and colleagues (Araki-Sasaki et al., 1995). Mycoplasma-free HCE cells were grown at 37ºC in a humidified 5% CO_2/95% air atmosphere in a culture medium containing 1 vol of Dulbecco's modified Eagle's medium (DMEM) and 1 vol of Ham's nutrient mixture F-12 (from Gibco, Paisley, UK) supplemented with 15% (v/v) fetal bovine serum (FBS, from Gibco), 1% (v/v) antibiotic, antimycotic solution (penicillin 10,000 U/ml, streptomycin 10,000 µg/ml and amphotericin B 25 µg/ml, from Gibco), 2 mmol/l L-glutamine (Gibco), 5 µg/ml insulin (Sigma, St. Louis, MO), and 10 ng/ml human epidermal growth factor (EGF; Sigma). The cells were harvested with trypsin-EDTA (Gibco).

For immunohistochemistry (I), HCE cells were seeded on 8-well chamber slides at a density of 30,000 cells/well (38,000 cells/cm^2) in a total volume of 200 µl. The slides were incubated in a humidified atmosphere at 37ºC in 5% CO_2/95% air for 2, 4, and 14 days for pre-confluent, confluent and post-confluent cell cultures, respectively. The culture medium was changed every 2 days. After the incubation, the cells were washed with Hank's balanced salt solution (HBBS) and fixed in methanol-acetone (1:1) for 10 min, then air-dried and stored at -20ºC until analyzed by immunohistochemistry.

For cytotoxicity tests (II-IV), HCE cells were seeded on 96-well flat bottom microwell plates at the density of 15,000-30,000 cells/well in (45,000-90,000 cells/cm^2) in a total volume of 100 µl and incubated in a humidified atmosphere at 37ºC in 5% CO_2/95% air. The cells were exposed to test compound-containing medium 24 hours after plating before the cell cultures started to form multilayers and became confluent.

4.1.3. Rabbit Primary Corneal Epithelial (RCE) Cell Cultures (II)

Primary cultures of RCE cells were established by an endothelium-free explant method, which is a modification of the methods described by Ebato *et al.* (Ebato et al., 1987), Araki *et al*. (Araki et al., 1993), and Kahn *et al.* (Kahn et al., 1993). Most of the corneas were obtained from rabbits that were used for other experimental studies. A detailed description of the method used to establish the RCE cultures is given in the publication II. RCE cells were grown using the same culture medium as with HCE cells.

For cytotoxicity tests, passage three RCE cells were seeded on 96-well plates at a density of 15,000-20,000 cells/well (45,000-60,000 cells/cm^2) in a total volume of 100 µl. After plating, the cells were allowed to grow for 24 hours in a humidified atmosphere at 37ºC in 5% CO_2/95% air. Cytotoxicity tests were conducted before the cells in culture had reached full confluence.

4.1.4. Human Retinal Pigment Epithelial (RPE) Cell Line D407 (V)

The human RPE cell line D407 is a spontaneously growing, apparently transformed cell line that has been cloned from primary cultured human RPE cells (Davis et al., 1995). The D407 cell line was a generous gift of Dr. Hunt from the University of South Carolina, USA.

For cytotoxicity testing, D407 cells were plated into 96-well plates at the density of 3,000-15,000 cells/well (10,000-50,000 cells/cm^2) in 100 µl DMEM supplied with 3% (v/v) FBS and 1% antibiotic/antimycotic solution. The cells were grown in a humidified atmosphere at 37ºC in 5% CO_2/95% air for 24 hours before drug exposure.

4.1.5. Pig Primary RPE Cell Cultures (V)

The pig primary RPE cell cultures were established by the method described by Mäenpää and coworkers (Mäenpää et al., 1997). Pig eyes were obtained from the local slaughter-house. The eyes were kept in ice-cold HBSS with 1% (v/v) antibiotic-antimycotic solution until used within 3-4 hours after death. 20-30 eyes were used to start one RPE culture. The eyes were aseptically opened by a circumferential incision behind the ora serrata, and the vitreous body and neuroretina were discarded. The eyecups were incubated with 0.25% (v/v) trypsin buffer for 2 hours to isolate RPE cells. The cells were then collected

and plated in 75 cm^2 T-flasks at the densities of 40,000-60,000 cells/cm^2 in DMEM supplied with 20% (v/v) FBS and 1% antibiotic-antimycotic solution. The cells were grown in a humidified atmosphere at 37ºC in 5% CO_2/95% air. After 24 hours, half of the medium was changed and then twice a week with DMEM containing 10% (v/v) FBS and 1% (v/v) antibiotic/antimycotic solution. The cells reached confluence within one week and were subcultured.

For cytotoxicity tests, passage two cells were plated into 96-well plates at the densities of 15,000-43,000 cells/well (50,000-130,000 cells/cm^2) in a total volume of 100 µl DMEM supplied with 10% (v/v) FBS and 1% (v/v) antibiotic/antimycotic solution, and grown for 24 hours before exposure to selected drugs.

4.2. Immunohistochemistry

The cultured immortalized HCE cells and the peripheral corneal sections were analyzed by using the Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP) staining method (Mason et al., 1983; Mason, 1985) and by using 13 different monoclonal antibodies (mAB) to CKs (I). At least four different corneas from different cadaver donors were used with each monoclonal antibody specific to a certain cytokeratin. In HCE cell cultures, the stainings with each antibody were also made at least in quadruplicate.

4.2.1. Monoclonal Antibodies (I)

All the 13 monoclonal antibodies to CKs, ranging from CK3 to CK20, were commercially manufactured. They are listed in Table 3. The antibodies were appropriately diluted in Tris-buffered saline (TBS, pH 7.6) containing 1% (w/v) bovine serum albumin (BSA).

Table 3. Mouse monoclonal antibodies used, their descriptions and manufacturers.

Antibody	Antigen	Description	Manufacturer
AE5	CK3	Basic 64 kDa keratin	ICN Biochemicals, Inc., Aurora, Ohio, USA
6B10	CK4	Basic 59 kDa keratin	Neomarkers, Inc., Fremont, CA, USA
LHK6B	CK6	Basic 56 kDa keratin	Neomarkers
K72.7	CK7	Basic 54 kDa keratin	Neomarkers
TS1	CK8	Basic 52.5 kDa keratin	Neomarkers
LHP1	CK10	Acidic 56.5 kDa keratin	Neomarkers
1C7+2D7	CK13	Acidic 54 kDa keratin	Neomarkers
LL002	CK14	Acidic 50 kDa keratin	Neomarkers
LL025	CK16	Acidic 48 kDa keratin	Neomarkers
E3	CK17	Acidic 46 kDa keratin	Neomarkers
DA-7	CK18	Acidic 45 kDa keratin	Neomarkers
A53-B/A2.26	CK19	Acidic 40 kDa keratin	Neomarkers
Ks20.8	CK20	Acidic 46 kDa keratin	Neomarkers

4.2.2. APAAP Staining Method (I)

The APAAP method is a three-step staining method, in which the tertiary antibody is conjugated with APAAP molecules (Mason et al., 1983; Mason, 1985). Prior to the staining, the frozen corneal sections were first fixed in acetone at -20°C for 10 min and then air-dried. The cultured HCE cells were fixed before the storage at -20°C. The slides containing either corneal sections or HCE cells were incubated in 0.5% (v/v) Triton X-100 for 10 min to enhance the penetration of the antibodies. In order to minimize any non-specific binding of the antibodies, the slides were then incubated in 1% (w/v) BSA for 20 min.

In the first step of the APAAP method, HCE cells and corneal sections were incubated with the primary antibody (anti-CK mAB) for 30 min. In the second phase, after a thorough rinsing, the cells were incubated with the secondary antibody (link) for 30 min, followed by the third phase 30-min APAAP incubation. Finally, the cells were incubated in alkaline phosphatase substrate for 20 min when a positive reaction appeared as a reddish color. In controls, the primary antibody was omitted.

4.3. Cytotoxicity Tests

Two *in vitro* cytotoxicity tests were used in the present study: the WST-1, which is a colorimetric assay for the detection of mitochondrial enzyme activity and viability/proliferation, and the LDH test that measures the leakage of LDH into culture medium. The cytotoxicity of selected drugs used in topically applied eye drops was evaluated with the HCE cell line and primary RCE cell cultures. The cytotoxicity of a set of systemic and intravitreally dosed drugs was assessed by using pig primary RPE cell cultures and the RPE cell line D407.

Table 4. Model compounds tested for cytotoxicity in this study.

Drug	Molecular Weight (g/mol)	Manufacturer	Cell Type	Test Conditions
Benzalkonium Chloride	360	FeF Chemicals A/S, Køge, Denmark	HCE RCE	0% FBS, 15% FBS 0% FBS, 15% FBS
EDTA Disodium	372	Merck KGaA, Darmstadt, Germany	HCE RCE	0% FBS, 15% FBS 0% FBS, 15% FBS
PSE, Brij®78	1152	Fluka, Buchs, Switzerland	HCE	0% FBS, 15% FBS
5-Fluorouracil	130	Pharmacia AB, Stockholm, Sweden	HCE D407 pig RPE	0 % FBS, 15% FBS 0% FBS, 3% FBS 0% FBS, 10% FBS
Tamoxifen Citrate	564	Orion Pharma, Espoo, Finland	D407 pig RPE	0% FBS, 3% FBS 0% FBS, 10% FBS
Toremifen Citrate	598	Orion Pharma	D407 pig RPE	0% FBS, 3% FBS 0% FBS, 10% FBS
Chloroquine Diphosphate	516	Orion Pharma	D407 pig RPE	0% FBS, 3% FBS 0% FBS, 10% FBS
Ganciclovir Sodium	277	Roche Pharma AG, Basel, Switzerland	D407 pig RPE	0% FBS, 3% FBS 0% FBS, 10% FBS
Gentamicin Sulphate	556	Hoechst Marion Roussel Ltd., Middlesex, UK	D407 pig RPE	0% FBS, 3% FBS 0% FBS, 10% FBS

4.3.1. Exposure of Cell Cultures to Test Compounds (II-V)

Selected test compounds and cell cultures used to test each drug are listed in Table 4. The test compounds were chosen as positive controls based on previous knowledge about their ocular toxicity. The concentrations of the compounds tested were within the relevant range considering their concentrations in topically applied ocular drugs, or the serum and tissue levels of the systemic and intravitreally administrated drugs. Benzalkonium chloride (BAC), polyoxyethylene-20-stearyl ether (PSE, Brij®78), disodium ethylenediamine tetraacetic acid (EDTA), 5-fluorouracil (5-FU), and gentamicin were dissolved in culture medium. Tamoxifen and toremifene were dissolved in water containing 10% (v/v) DMSO (Sigma). The final concentration of DMSO in the microtiter wells was 0.5% (v/v). Chloroquine and ganciclovir were dissolved in water. With HCE and RCE cells, cytotoxicity was assessed in serum-free medium or in medium containing 15% (v/v) FBS. With D407 cells and pig primary RPE cells, cytotoxicity was evaluated in serum-free medium and in medium containing either 3% or 10% (v/v) FBS, respectively. The exposure times varied from 5 to 60 min for BAC, PSE, and EDTA, and from 1 hour up to 96 hours for 5-FU. The rest of the test compounds were exposed for 24 hours. All cytotoxicity experiments were made at least in triplicate using at least 6 wells per concentration.

4.3.2. WST-1 Cytotoxicity Test (II-V)

WST-1 test is based on the cleavage of the tetrazolium salt WST-1 (slightly red) to formazan (dark red) by various mitochondrial dehydrogenase enzymes (Figure 3). The number of viable cells is proportional to the absorbance detected at the wavelength of 450 nm. The cytotoxicity test was based on the ready-to-use cell proliferation reagent WST-1, containing WST-1 {4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate} and an electronic coupling reagent, diluted in phosphate buffered saline (cat no. 1644807, Roche Diagnostics GmbH, formerly Boehringer Mannheim GmbH, Germany). With all cell types, 24 hours after plating the growth medium was discarded and the cells were exposed to different test agent concentrations in a total volume of 100 µl for selected treatment periods.

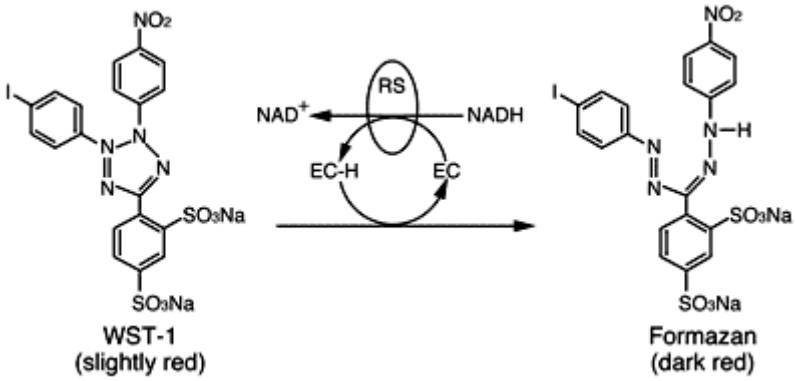

Figure 3. Cleavage of the tetrazolium salt WST-1 {4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate} to formazan (Takara Bio, 2003). Mitochondrial succinate-tetrazolium-reductase system (RS), electron coupling reagent (EC).

In the cytotoxicity tests with CE cells (II-IV), after the test period and the removal of the test compound-containing medium, the cells were rinsed once with serum-free basal medium (Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12, 1:1), and then 100 µl of fresh growth medium containing 15% (v/v) FBS was added. When the cells were exposed for a short period of time (5 to 60 min), they were returned to the incubator for a one-hour recovery period, and then the WST-1 test was performed. In part of the experiments, in certain 5-minute exposures with BAC and in the long-term exposures with 5-FU (from one hour up to 72 hours), the one-hour recovery time was omitted. After the medium change and eventual recovery period, 10 µl of WST-1 reagent was added to the medium in each well. The cells were then incubated with the WST-1 reagent in a humidified atmosphere at 37ºC in 5 % CO_2/95 % air for two hours, the multititer plate was thoroughly shaken for one minute, after which the absorbances were measured using the wavelength of 450 nm. The performance of the WST-1 test using HCE cells was also investigated in a multilaboratory study within the EU-funded Biomed project (BMH4-97-2324). In addition to our laboratory, three other laboratories participated, those from the universities of Bremen (Germany), Pisa (Italy), and Ioannina (Greece).

In the long-exposure (from 24 to 72 hours) cytotoxicity tests with RPE cells (V), the WST-1 reagent was added directly on the cells in test-compound containing medium, and the cells were then incubated in a humidified atmosphere at 37ºC in 5 % CO_2/95 % air for one hour. Then the absorbances were read at 450 nm.

The use of the two-hour incubation period with CE cells (II-IV) and the one-hour incubation period with RPE cells (V) was based on a series of preliminary experiments. In corneal cell cultures, the background absorbance was measured on wells containing only the dye solution and the culture medium. In RPE cell cultures, the possible effect of the solvent was taken into account in the controls. The mean optical density values

corresponding to the non-treated controls were taken as 100 %. The results were expressed as percentages of the optical density of treated *vs.* untreated controls.

4.3.3. Lactate Dehydrogenase (LDH) Leakage Test (II, IV)

LDH assay as an index of plasma membrane integrity measures the leakage of the cytosolic enzyme lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, E.C.1.1.1.27) into culture medium. The activity of LDH is measured by monitoring the rate at which the substrate, puryvate, is reduced to lactate. The procedure is based on the following reaction:

$$\text{Puryvate} + \text{NADH} + H^+ \equiv \text{L-lactate} + NAD^+ \qquad [1]$$

NADH has a high absorbance at 340 nm compared to NAD. The reaction is measured in terms of the rate of decrease in absorbance at 340 nm.

The LDH test used was modified from the "Automated Analysis Boehringer Mannheim LD/LDH assay" (Boehringer Mannheim GmbH, Germany, cat. no. 191353). It is an optimized standard method conforming to the recommendations of the Scandinavian Committee on Enzymes (Stromme and Eldjarn, 1974). 25 µl of test compound-containing sample was pipetted into a 96-well microtiter plate, and 250 µl of coenzyme/buffer containing 56 mmol/l Tris buffer (pH 7.4), 0.17 mmol/l NADH and 5.6 mmol/l EDTA was added. The reaction was started by the addition of 25 µl of 14 mmol/l pyruvate solution. 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. The background absorbance was measured from the wells containing the culture medium with or without serum. Enzyme leakage into the medium was expressed as percentage of controls (untreated cultures).

4.3.4. Statistics (II-V)

Dose-response curves were drawn from the results expressed as mean ± standard error of mean (SEM). The EC_{50} values, the concentrations of test agents that decreased the WST-1 reduction values to 50% of the controls, were determined when possible from the dose-response curves fitted by using a non-linear regression analysis (GraphPad Prism, GraphPad™, San Diego, USA; SigmaPlot, SPSS Science, USA). The statistical significance of the differences between the cultures exposed to test agents without serum or with serum was determined with Student's two-tailed *t*-test (GraphPad Prism). Statistical differences between the treated cells and the controls were determined by using one-way ANOVA with Dunnett's post (GraphPad Prism). Differences were considered significant when $P<0.05$.

5. RESULTS

5.1. Immunohistochemical Characterization of the HCE Cell Line (I)

The SV40-immortalized HCE cell line (Araki-Sasaki et al., 1995) was grown in culture medium and characterized immunohistochemically by using 13 different commercial mABs to CKs, ranging from CK3 to CK20. The results were compared to the corneal epithelium of the human corneal cryostat sections. The human corneal cryostat sections were found to express CK3 very prominently (I, Fig. 1A, Table 1). The suprabasal layer of the cornea also expressed CK4 (I, Fig. 1D), and the limbal part of the cornea expressed CK19 (I, Fig. 2C). The cells reached confluence in the 8-well chamber slides after 3 days of culture and grew as an evenly spread monolayer. After four days of culture, the cells started to pile up and form stratified piles. The immunohistochemical stainings were made with cells grown for 2, 4 and 14 days for pre-confluent, confluent, and post-confluent cell cultures, respectively. The partly stratified confluent and post-confluent HCE cells were found to express cornea-specific CK3 (I, Fig. 1C, Table 1), and thus the HCE cell line resembled the HCE cells *in vivo*, but it also expressed CKs 7, 8, 18 and 19 (I, Fig. 1F, 2A, 2B, 2D), which are characteristic of simple epithelium.

5.2. Corneal Cell Cultures in the WST-1 and LDH Cytotoxicity Testing (II)

The HCE cell line as a cell culture model was compared to RCE cell cultures by using the WST-1 test and the LDH leakage test. Cell cultures were exposed to test compounds for 5 and 60 minutes in serum-free medium and in normal growth medium containing 15% (v/v) FBS. BAC, a cationic surfactant, and disodium EDTA were used as model compounds when evaluating the performance of the corneal cell models and cytotoxicity tests. At the time of exposures, both cell cultures were pre-confluent and at the same stage of confluence.

The comparison of the EC_{50} values obtained by the WST-1 test for the HCE and RCE cells, exposed to BAC is presented in Figure 4. In the WST-1 test, the EC_{50} value after one-hour exposure to BAC in serum-free medium was about the same for both corneal cultures (0.0011 % (w/v), 0.03 mol/l). When serum was used, the EC_{50} values increased significantly. Therefore, it is obvious that serum protects the cells from the toxic effect to some extent. Only 5-minute BAC treatment caused an equally severe effect on the HCE cells as the one-hour treatment. When the WST-1 test was performed immediately after 5-minute BAC exposure, the EC_{50} value was higher than in the test performed after one-hour recovery time. However, this was not statistically significant.

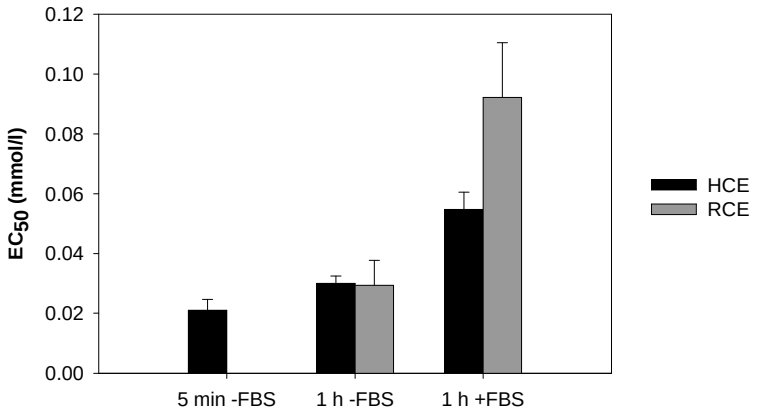

Figure 4. Comparison of HCE and RCE cell cultures exposed to BAC for 5 min or one hour. Cytotoxicity was evaluated by the WST-1 test in the absence of fetal bovine serum (-FBS) and in the presence of serum (+FBS).

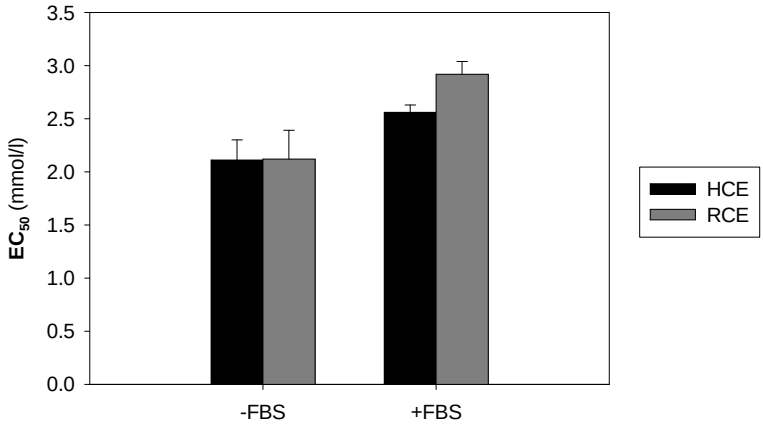

Figure 5. Comparison of HCE and RCE cell cultures exposed EDTA for one hour. Cytotoxicity was evaluated by the WST-1 test in the absence or presence of serum (FBS).

Both corneal cell cultures were equivalently sensitive to disodium EDTA, which was found to be far less cytotoxic than BAC (Figure 5). Serum protected RCE cell cultures slightly from EDTA. 5-Minute EDTA treatment for up to 0.5% (w/v), 13.43 mmol/l caused only a small (about 20%) cytotoxic effect (II, Fig. 3B). Also, the one-hour recovery period did not affect the 5-minute EDTA exposure, unlike in the BAC exposure.

In the LDH test, after one-hour exposure considerable LDH leakage was seen in both corneal cell cultures in serum-free medium starting from BAC concentration 0.005% (w/v), 0.14 mmol/l (II, Fig.4). More leakage was found to occur in RCE cell cultures than in HCE cells. 5-Minute exposure to 0.01% (w/v), 0.28 mmol/l BAC caused a similar LDH leakage in HCE cells as one-hour exposure (II, Fig. 6A). Higher BAC concentrations (0.05%-0.1 (w/v), 1.39-2.78 mmol/l) reduced the LDH activity after 5-minute treatment. After one-hour EDTA treatment in serum-free medium, LDH leakage started at 0.1% (w/v), 2.69 mmol/l EDTA (II, Fig 5). The leakage was more prominent in RCE cell cultures than in HCE cells. In serum-containing medium, serum protected HCE cells across the whole concentration range tested (0.1-1.0% (w/v), 2.69-26.87 mmol/l EDTA). In RCE cell cultures, LDH leakage started at 0.1% EDTA whether or not serum was used (II, Fig. 5B). 5-Minute EDTA treatment had only a small effect on HCE cells across the full concentration range tested (II, Fig. 6B). In both corneal cell types, with both test substances there were great variations in the LDH leakage results when serum was not added to the exposure medium.

5.3. Interlaboratory Evaluation of the HCE-WST-1 Cytotoxicity Test (III)

To investigate the reproducibility and the test conditions of the WST-1 test using the HCE cell line, the cytotoxicity of two surfactants, BAC and PSE (Brij®78), a non-ionic surfactant, was evaluated in four laboratories in the EU (University of Tampere in Finland, University of Bremen in Germany, University of Ioannina in Greece, and University of Pisa in Italy). Cytotoxicity was assessed after 5 min, 15 min, and one-hour exposures in serum-free and serum-containing medium using a mutually agreed HCE-WST-1 test protocol. The cells were plated at the density of 30,000 cells/well (90,000 cells/cm^2) in microtiter plates, and they were exposed to the test compounds 24 hours after plating at 70% confluence. The results were collected and compared to find the best test conditions for the use of the HCE-WST-1 test (Figures 6 and 7).

The cytotoxicity of BAC and PSE was dose-dependent, and it was influenced by the time of exposure and the presence of serum in the culture medium. The two substances gave similar responses. In BAC-treated cells, after one-hour exposure, the EC_{50} value in the presence of serum was 0.0650 mmol/l (SD 0.0284)(0.00234±0.00102% (w/v)) and in the absence of serum 0.0296 mmol/l (SD 0.0081)(0.00107±0.00029% (w/v)). The corresponding values for PSE were 0.0581 mmol/l (SD 0.0300)(0.00669±0.00346% (w/v)) and 0.0228 mmol/l (SD 0.0063)(0.00263±0.00726% (w/v)). The cytotoxicity data was found not to be influenced by the laboratory cell culture experience.

When HCE cells were exposed to test agents for 15 min or one hour, the EC_{50} values were in serum-containing exposures about twice as high as in serum-free exposures. In the 5-minute exposure the effect of serum was less notable. Although serum protected the cells from the toxic effects of the test compounds during longer exposure times, the differences between the EC50 values of serum-containing exposures and serum-free exposures were not statistically significant. Variations in the results between different laboratories were noted in every test. The coefficients of variation, calculated for all EC_{50} values at each time, were generally high (31 to 121%, mean 58%). The high CV% values can partly be

attributed to the use of serum. In serum-free exposures, the CV% was notably smaller than in serum-containing exposures (39% and 76%, respectively). Exposure to the cationic surfactant BAC caused lower variability than exposure to PSE, the non-ionic surfactant (53% *vs.* 63%). The variability was also found to be lower after one-hour exposure than after 15-minute and 5-minute exposures (43%, 72%, and 58%, respectively).

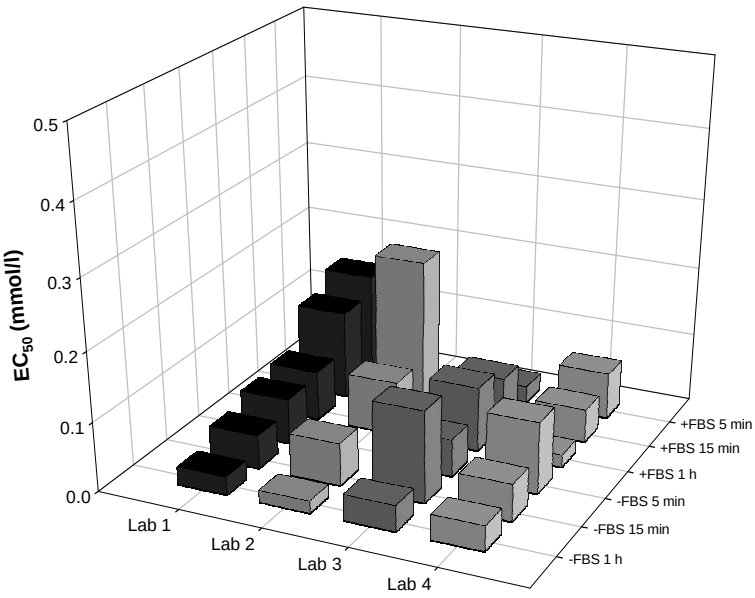

Figure 6. EC_{50} values of BAC in four laboratories evaluated by the WST-1 test in the absence of serum (-FBS) and in the presence of serum (+FBS).

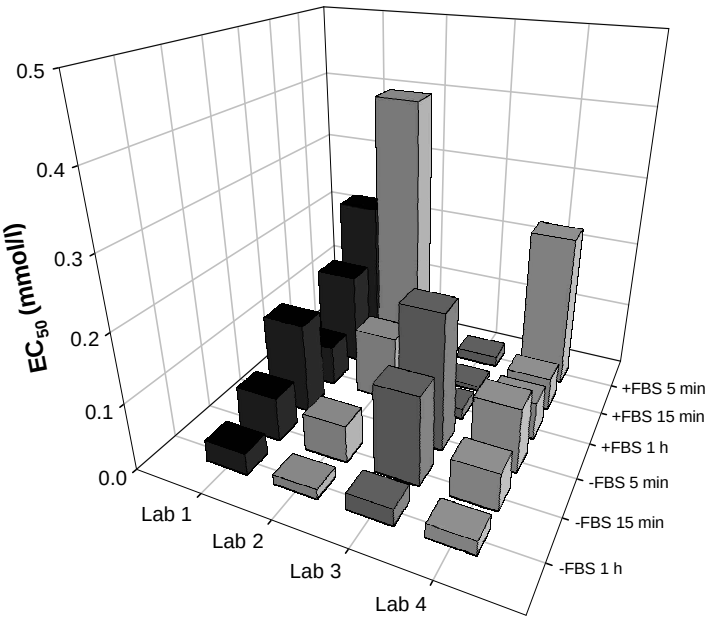

Figure 7. EC_{50} values of PSE in four laboratories evaluated by the WST-1 test in the absence and presence of serum.

5.4. Long-term Adverse Effects by the HCE-WST-1 and HCE-LDH Tests (IV)

The performance of the HCE cell line and the WST-1 and LDH tests for short-term acute cytotoxicity was investigated in earlier studies (II). In the short-term exposures, cell cultures were exposed to test compounds for 5 min to one hour, and in the long-term exposures for 24 hours or longer. Therefore, the terminology used in the present study differs from that used in the clinical and animal test situation. A common antiproliferative agent, 5-fluorouracil (5-FU), was used to study the application of these tests for the evaluation of long-term adverse effects. Special attention was paid to differentiate the effects on cell proliferation and viability. The HCE cells were treated with 5-FU for 1, 24, 48, and 72 hours in serum-free medium, and in medium containing 15% (v/v) FBS.

Treatment with as little as 0.0005 mg/ml (0.00005% (w/v), 0.0038 mmol/l) 5-FU for 48 hours hindered cell division (Figure 8). Exposure for a longer time (72 hours) in this dose did not kill the cells but hindered cell proliferation. A hundred times higher concentration (0.005% (w/v), 0.38 mmol/l) decreased cell proliferation after 24-hour treatment in a similar way, *i.e.* decreasing cell number. Treatment with the highest 5-FU concentration

studied (5 mg/ml, 38.43 mmol/l) for 24 hours decreased cell numbers to about 50%, while treatment with the same concentration for 72 hours resulted in complete cell death. When the cells were exposed to 5-FU for 24 hours, the protective effect of serum could be observed. After longer exposure times the protective effect of serum was lost. The estimated EC_{50} value after 24-hour serum-free exposure was about 0.5 mg/ml (3.84 mmol/l), and in the serum-containing medium about 5 mg/ml (38.43 mmol/l).

Exposure to 5-FU for as long as 24 hours did not increase LDH leakage, compared to controls, whether serum was used or not (Figure 9). The loss of cell membrane integrity and the resulting LDH release was at its highest after 48 hours of treatment with more than 0.05 mg/ml (0.005 % (w/v), 0.38 mmol/l) 5-FU in serum-free medium. The leakage was smaller in serum-containing medium, but the difference was not statistically significant.

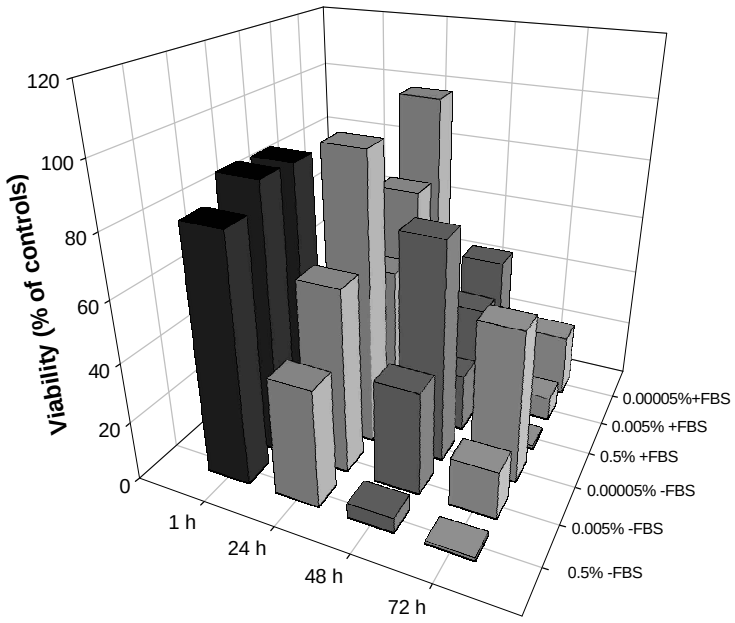

Figure 8. Viability of HCE cells exposed to 5-fluorouracil for 1-72 hours in the absence or presence of serum.

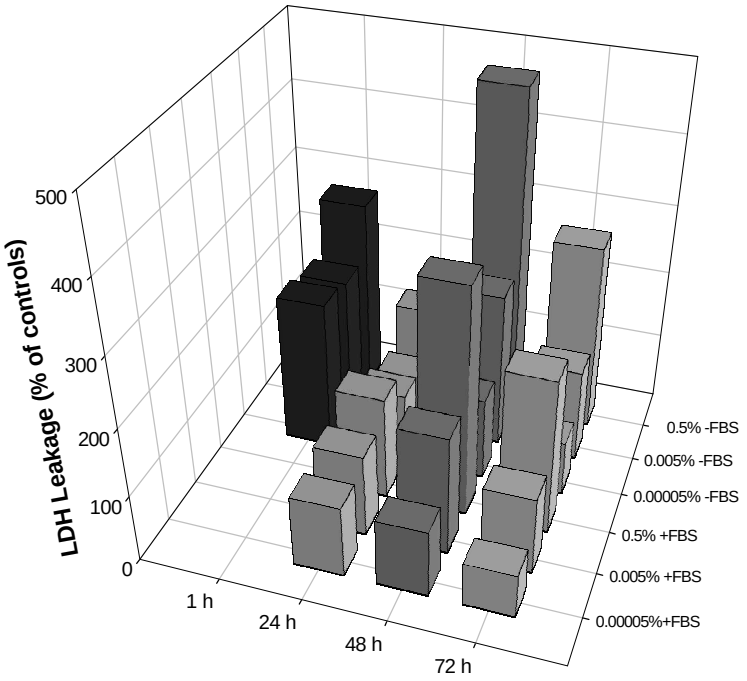

Figure 9. LDH leakage in HCE cells exposed to 5-FU for 1-72 hours.

5.5. RPE Cell Cultures in the WST-1 Testing (V)

The WST-1 test was used with pig primary RPE cell cultures and the human RPE cell line D407 as potential cytotoxicity tests for evaluating the cytotoxicity of the selected systemic and intravitreally dosed drugs tamoxifen, toremifene, chloroquine, gentamicin, ganciclovir, and 5-FU. The cytotoxicity of these drugs was evaluated in serum-free and serum-containing medium after 24, 48, 72, and 96 hours of exposure, depending on the drug. To achieve 90% confluence before the drug exposure pig primary RPE cells were seeded at the cell density of 130,000 cells/cm^2 and D407 cells at the cell density of 50,000 cells/cm^2. In part of the experiments, several cell densities were used to study the effect of cell density on cytotoxicity.

The EC_{50} values of tamoxifen, toremifene, and chloroquine in 24-hour exposures were found to increase with the amount of cells plated (Figure 10). In D407 cells in medium containing 3% FBS, only the highest concentrations of tamoxifen and toremifene (25 µmol/l) induced cell death (V, Fig. 1a). Tamoxifen and toremifene did not cause any cytotoxic effects to RPE cell cultures in medium containing 10% FBS across the whole

concentration range tested up to 20 µmol/l (V, Figs. 1b and 2b). In chloroquine exposures, pig RPE cells were more sensitive compared to D407 cells (Figure 11). The presence of serum had no effect on cell viability.

In gentamicin serum-free 24-hour exposures with D407 cells, plated at the cell density of 50,000 cells/cm^2, the number of the cells in culture reduced starting from 10 mmol/l (V, Fig. 5a), while with pig RPE cells plated at the same cell density reduction started at 40 mmol/l (V, Fig. 5b). The use of serum, 3% for D407 cells and 10% for pig RPE cells had only a slightly protective effect. Surprisingly, in ganciclovir 24-hour exposures with D407 cells at the plating density of 50,000 cells/cm^2, there was initially an increase in cell numbers, which then decreased at 25 mmol/l in the presence of serum and at 40 mmol/l in serum-free medium (V, Fig. 6a). In pig RPE ganciclovir-exposed cell cultures at the plating density of 130,000 cells/cm^2, there was a small dose-dependent decrease in cell numbers in serum-free cultures, but at the presence of 10% FBS the effect was minor (V, Fig. 6b).

In 5-FU 24-hour exposures, concentrations of up to 1 mmol/l decreased the cell numbers of both cell types to about 50%, D407 cells plated at 50,000 cells/cm^2 and pig RPE cells plated at 130,000 cells/cm^2, whether serum was used or not (V, Figs. 4a and 4b). A smaller plating density (10,000 cells/cm^2) was used when D407 cells were exposed to 5-FU for 48, 72, and 96 hours. After initial decrease in cell numbers, they reached a plateau (V, Fig. 4c). After 48-hour exposure, the cell number decreased to about 70% at 1 mmol/l and to about 60% with the highest concentration tested (5 mmol/l). After 72-hour exposure, cell numbers decreased to about 25-30% at 1 mmol/l and remained on that level also with the highest test concentration. After 96-hour 5-FU exposure, the cell number compared to the control was less than 20% starting from 40 µmol/l.

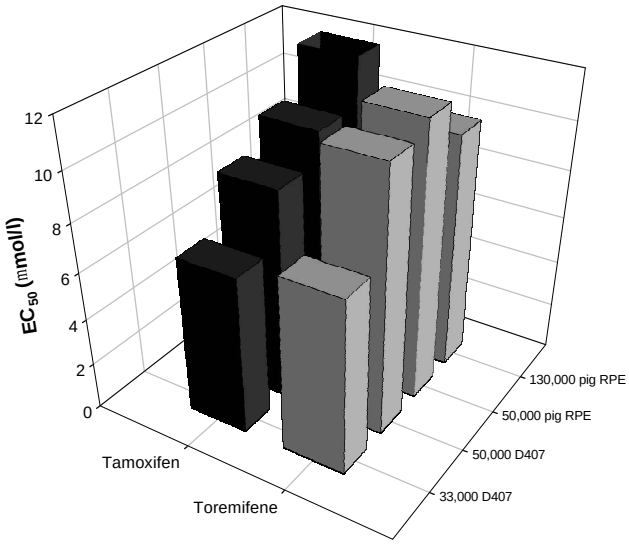

Figure 10. EC_{50} values in RPE cells exposed in the absence of serum to tamoxifen and toremifen for 24 hours. The number of cells Plated varied from 33,000 to 130,000 cells/cm^2.

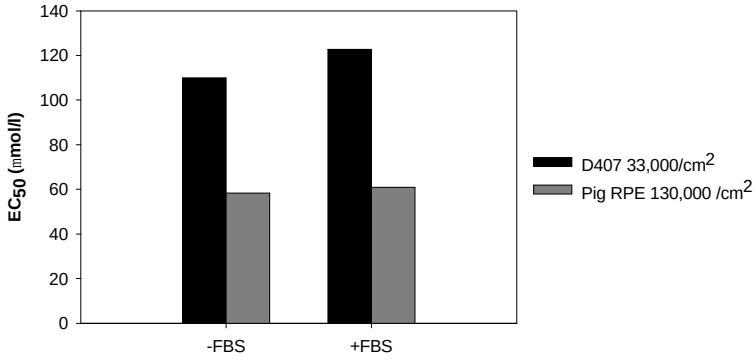

Figure 11. EC_{50} values of RPE cells exposed to chloroquine for 24 hours.

5.6. Summary of the Cytotoxicity Results

The primary RCE cultures and the HCE cell line yielded comparable results in the WST-1 test and the LDH leakage assay. Both primary corneal cells and the HCE cell line gave quite similar responses to the test compounds studied, BAC and EDTA. Furthermore, the LDH test was found to show great variations in the cytotoxicity results, especially when serum was not used. The use of serum in exposure medium resulted in lower toxicity in both tests. In the interlaboratory HCE-WST-1 study, variations were found in toxicity results, which however were not dependent on the cell culture experience of the participating laboratories. The most reproducible results were obtained when the cells were exposed to the test substances BAC and PSE for one hour in the absence of serum. When the long-term adverse effects of 5-FU were evaluated with the HCE cell line using the WST-1 and LDH tests, 24-hour and longer incubation times induced time-dependent inhibition in cell proliferation. The serum was found to protect the cells for 24 hours, but after longer exposure times the protective nature of serum was lost. According to the EC_{50} values in the culture conditions used, the order of the decreasing cytotoxicity of the compounds tested with corneal cells was BAC ≅ PSE > EDTA > 5-FU.

After 24-hour exposure time, studies with pig primary RPE cell cultures and the human RPE cell line D407 using the WST-1 test showed radical losses in cell viability with three test drugs. The order of decreasing cytotoxicity was tamoxifen ≅ toremifene > chloroquine. The EC_{50} values of 5-FU, gentamicin and ganciclovir were in the range of millimolar, and thus they were far less cytotoxic than tamoxifen, toremifene, and chloroquine. With all compounds tested, except for ganciclovir, the pig primary cultures and the D407 cell line showed quite identical toxicities in the culture conditions used.

6. DISCUSSION

6.1. Need of *In Vitro* Methods for Ocular Toxicity Testing

In vitro methods are valuable tools for drug development in pharmaceutical industry, because they are rapid and economical means for ocular toxicity screening and mechanistic studies. The safety assessment of ocular drugs intentionally administrated to the eye requires detailed toxicity studies. *In vitro* toxicity screening can be used at the early stage of drug development for selecting the safe molecules for further development. *In vitro* tests are also good at distinguishing even small differences between slightly irritating materials and products. The Draize test is not capable of measuring these differences and, above all, it is not a convenient model for studying the mechanisms that are responsible for the formation of eye irritation. A set of well-chosen *in vitro* tests can provide more comprehensive toxicity results than the traditional Draize test. Moreover, *in vitro* tests are most valuable in pharmaceutical and cosmetic industry for studying the toxic effects of the mixtures of drugs and other chemical compounds. Moreover, there are no specific tests for the evaluation of retinal safety in the OECD guidelines (Tähti et al., 1999). The addition of an *in vitro* retinal toxicity test into generally used guidelines would considerably improve the safety assessment of drugs and other chemicals with oculotoxic effects.

6.2. Test Compounds in the Present Study

The test compounds currently studied were chosen based on the previous knowledge about their ocular toxicity. BAC, a cationic surfactant, is known as a severe eye irritant. Its eye irritancy has been determined with the Draize test and a number of diverse cytotoxicity tests (III). In the Draize test, out of the possible 110 points, 1% BAC produces 56 points, 5% BAC 84 points, and 10% BAC 108 points (Bagley et al., 1992a; Bagley et al., 1999b). BAC is used as a preservative in most topically used eye drops to reduce microbial contamination. Furthermore, BAC is used as a "penetration enhancer" for improving the permeability characteristics of the corneal epithelium (Hochman and Artursson, 1994). Disodium EDTA is a known calcium chelator and used in ophthalmic preparations for stability purposes. EDTA (0.1 %) has been shown to be non-irritating *in vivo* by the rabbit blinking count test (Sasaki et al., 1995). PSE (polyoxyethylene-20-stearyl ether, Brij®78), a nonionic surfactant is used as a solubilizer for poorly water-soluble ophthalmic drugs. 5-FU is used in ophthalmology for suppressing fibroblast activity after glaucoma filtering surgery. However, there are complications associated with the 5-FU therapy, especially as CE defects.

Tamoxifen is an antineoplastic drug used in breast cancer therapy and also preventively, but it has been shown to cause changes in the retina, while toremifene, developed by Orion Pharma in 1997, has shown no oculotoxic effects *in vivo*, although it is structurally very close to tamoxifen. Chloroquine is used in the treatment of malaria and rheumatoid

arthritis. It has been shown to accumulate in the retina and to degenerate photoreceptors after chronic exposure. The intravitreally applied drugs gentamicin and ganciclovir are used for their antibacterial and antiviral properties, respectively. There are controversial reports about their retinal toxicity (D'Amico et al., 1985; Talamo et al., 1985; Brown et al., 1990; Hines et al., 1993; Saran and Maguire, 1994; Hashizoe et al., 1994).

The concentrations of the compounds tested were within the relevant range, considering their concentrations in topically applied ocular drugs, or the serum and tissue levels of the systemic and intravitreally administrated drugs. In this study, the comparison of the *in vitro* cytotoxicity results to the *in vivo* situation was limited for a number of reasons. First of all, only one of the studied compounds, BAC, has been tested in the Draize test. Secondly, validation studies have shown that the interpretation of the *in vitro* results is problematic, not only due to the variability of the Draize test, but also to the problems of selecting the appropriate algorithms to convert the *in vitro* data into predictions of the *in vivo* exposure. These statistical means were not within the scope of this study.

6.3. HCE Cell Cultures *Vs.* the Human Cornea *In Vivo*

Single cell-type cultures of both target and non-target cells have been extensively used in ocular toxicology. Assays for eye irritation studies with corneal cell cultures are adequate since the cornea is directly exposed to topically dosed test agents. The target cells most utilized in the eye irritancy studies have been rabbit primary CE cells (Lazarus et al., 1988),(Grant and Acosta, 1990; Grant et al., 1992; Tripathi et al., 1992; Grant and Acosta, 1994; Yang and Acosta, 1994; Yang and Acosta, 1995; Grant and Acosta, 1996a; Grant and Acosta, 1996b; Grant and Acosta, 1997) and the rabbit SIRC cell line (North-Root et al., 1985; Demetrulias and North-Root, 1987; Conduzorgues et al., 1989; Adams et al., 1992; Ohno et al., 1998; Tani et al., 1999). Other non-ocular fibroblastic cell lines have also been extensively used, such as Balb/c 3T3, 3T3-L1, and L929 mouse fibroblasts (Shopsis and Sathe, 1984; Borenfreund and Puerner, 1985; Riddell et al., 1986; Decker and Lohmann, 1988; Clothier et al., 1988; Spielmann et al., 1993; Nishi et al., 1995; Clothier et al., 1997; Pinto et al., 2000). Ocular cells originating from the human eye have not yet been widely used for toxicology studies. Until today, only a few human corneal cell lines have be developed (Kahn et al., 1993; Araki-Sasaki et al., 1995; Kruszewski et al., 1997; Griffith et al., 1999) Only CE cell lines are also commercially available for all investigators from the American Type Cell Collection (Kahn et al., 1993; Kruszewski et al., 1997).

In the present study, the recently developed SV40-immortalized HCE cell line (Araki-Sasaki et al., 1995) was used for cytotoxicity studies. We characterized the CK pattern of this cell line in culture conditions that have most often been used in ocular toxicology as pre-confluent, confluent, and post-confluent cell cultures in culture medium (I). An important question, dealing with the reliability of this kind of simplified culture test, is how well the immortalized epithelial corneal cells in culture resemble those of the human cornea *in vivo*. Comparison of these cells to the human cornea *in vivo* is relevant, since the safety testing of topically applied ocular drugs and other related compounds is primarily carried out to avoid the adverse effects on the human eye. Because CKs are a

part of the intermediate filament family and characteristic of epithelial cells, they are valuable tools for this kind of a comparison and characterization (Moll et al., 1982; Osborn, 1983).

The characterization of the HCE cells is even more important, because immortalization has been reported to cause changes in the expression of CKs. We found that confluent and post-confluent HCE cells, after they started to pile up, expressed cornea-specific CK3, but they also expressed simple-epithelium specific CKs 7, 8, 18 and 19, which are not expressed in the normal conditions in the human cornea *in vivo* (Moll et al., 1982). The expression of the simple epithelium-specific cytokeratins 7, 8, 18 and 19 has earlier been reported in the SV40-immortalized human keratinocyte cell lines originating from the epidermis (Banks-Schlegel and Howley, 1983; Bernard et al., 1985; Brown and Gallimore, 1987; Kamalati et al., 1989). Our results are in accordance with earlier studies, in which normal human primary CE cells have been found to express CK3 after the cells have piled up in a similar way as in the current study (Araki-Sasaki et al., 1995). The difference between the patterns of the cytokeratin expression in the corneal epithelium *in vivo* and in the HCE cell line grown in culture medium may therefore be due to the SV40-immortalization process and/or to the culturing conditions used. The synthetic plastic substrate used in this study forced the cells to adjust to an artificially flat and rigid surface. However, in the authentic environment *in vivo*, CE cells form a multilayered epithelium supported by a stromal layer, a complex three-dimensional extracellular matrix (ECM) through which CE cells are influenced by various complicated cell-to-cell and cell-to-ECM interactions (Cukierman et al., 2002). When HCE cells are grown on a collagen matrix with keratocytes at the air-liquid interphase to form a three-dimensional, stratified multilayer similar to that found in the normal human cornea *in vivo* (Ward et al., 1997; Orwin and Hubel, 2000), the CK pattern may be more like that of the normal cornea. As far as the author knows, there are no published results of this subject yet and thus the validity of this hypothesis needs to be studied further.

It is important to note that in the current study immortalized HCE cells were used as pre-confluent cultures for cytotoxicity testing. Our preliminary cytotoxicity experiments with different cell densities and growth times had shown more reproducible results with pre-confluent cell cultures than with confluent and post-confluent partly stratified cell cultures. Besides, the cells in the exponential phase, before confluence is exceeded, can be used not only to measure the toxic effects of test substances, but also to evaluate the antiproliferative effects of test compounds. In addition, HCE cells were originally grown in a culture medium that also contained DMSO (0.5% (v/v)) and cholera toxin (0.1 µg/ml). During our preliminary experimental test development, we also used these culture supplements, but we eliminated them from the normal growth medium to simplify the culture conditions for rapid, large-scale toxicity screening. Cholera toxin is used to prevent the outgrowth of fibroblasts when establishing cell cultures from heterogenous tissue samples. DMSO is widely used in the cryopreservation of the cells. The elimination of these culture supplements was found to slightly reduce the growth rate of the cells, but no changes in the cell morphology were noticed as a response to the changed culture conditions. Therefore, the stage of the culture and other culture conditions used in our cytotoxicity studies is a compromise of biological facts and a methodology that is, according to our experience, the most suitable for the intended purpose in cytotoxicity

screening. However, the best stage of culture and methodology might be different when the HCE cell line is used for other purposes.

6.4. Primary RCE Cell Cultures *Vs.* HCE Cells in Cytotoxicity Testing

Primary cultures have been thought to be better candidates for toxicity screening than cell lines, because primary cells have been considered to be more sensitive in toxicity testing than the respective cell line (Borenfreund and Borrero, 1984). To the best of our knowledge, to support this assumption, there were no earlier studies comparing the cytotoxicity results of primary cell cultures to the cell line cultures of the same study in similar test conditions. In the present study, rabbit primary CE cells were compared to the HCE cell line by evaluating the cytotoxicity of BAC and disodium EDTA with both WST-1 and LDH leakage tests (II). Moreover, pig RPE cell cultures were compared to the human RPE cell line D407 in the WST-1 testing for assessing the cytotoxicity of selected systemic and intravitreally administrated drugs (V). Statistically, we did not find any differences between primary RCE cell cultures and the HCE cell line. RPE cell cultures were also in most cases equally sensitive to the compounds tested.

The WST-1 test (Ishiyama et al., 1993) belongs to the microculture tetrazolium assay (MTA) family that has been developed for the measurement of cell viability and proliferation to overcome the problems associated with cytochemical quantification, such as counting the cells after the inclusion or exclusion of a dye or a radioactive label (Mosmann, 1983; Marshall et al., 1995). Tetrazolium salts are reduced to their respective intensely colored formazans by metabotically active cells. When used as a microtiter plate format, thousands of assay points can be processed daily. MTAs are a convenient method for high throughput screening and a preferable non-radioactive alternative *e.g.* for the earlier widely used method that is based on the uptake of ^{3}H-thymidine (Marshall et al., 1995). MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide}, the mitochondrial tetrazolium test, was first described by Mosmann in 1983 (Mosmann, 1983), and it is the most commonly used tetrazolium salt test. The yellow MTT tetrazolium salt ring is cleaved in the active mitochondria of living cells by dehydrogenase enzymes, mostly succinic dehydrogenase, to blue formazan crystals. A wide range of solubilization methods have been used for the elution of the MTT-formazan, such as acidified isopropanol, DMSO, SDS, or propanol/ethanol solution (Marshall et al., 1995). The MTT test is a well-known cytotoxicity test for ocular toxicity. It has been used with a wide range of corneal cellular systems, such as rabbit primary CE cell cultures (Grant et al., 1992; Sina et al., 1992; Grant and Acosta, 1994; Yang and Acosta, 1994) and the SIRC cell line (Vian et al., 1995). More recently, the MTT assay has been used with the presently studied HCE cells (Saarinen-Savolainen et al., 1998), as well as with corneal models constructed from fetal pig corneal cells (Schneider et al., 1997), bovine corneal cells (Parnigotto et al., 1998), and another immortalized HCE cell line (Ward et al., 1997; Clothier et al., 2000).

Unlike the MTT tetrazolium salt, alternative tetrazolium salts, such as XTT {sodium (2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carbanilide} and MTS {3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulpholphenyl)-2H-tetrazolium},

generate charged water-soluble formazans, due to the introduction of sulphonate groups into their structures (Scudiero et al., 1988; Roehm et al., 1991; Cory et al., 1991; Marshall et al., 1995). This advantage eliminates the error-prone solubilization step required in the MTT assay. The most recently developed tetrazolium salt WST-1 {4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate} with two sulphonate groups forms highly water-soluble formazan upon bioreduction by activated cells. WST-1 has been reported to be at least as sensitive as XTT (Ishiyama et al., 1993) and more sensitive than MTT (Takamatsu, 1998). Furthermore, WST-1 is more stable compared to XTT and MTS. WST-1 reagent is used as a ready-to-use solution, and it can be stored at 4ºC for several weeks without significant degradation. Because no volatile organic solvent is needed for solubilization, the WST-1 test procedure is simpler and more beneficial than that of the most commonly used MTT, especially when used in high throughput screening.

Among the cytotoxicity tests based on the release of intracellular enzymes into culture medium, the LDH release test has been the most commonly used (Korzeniewski and Callewaert, 1983). In corneal toxicity studies, the LDH test has mostly been used with rabbit primary CE cultures (Grant et al., 1992; Grant and Acosta, 1994; Yang and Acosta, 1994; Yang and Acosta, 1995). A very practical aspect of the current study was that the WST-1 and LDH cytotoxicity tests were designed for use in combination with the same cells. The LDH enzyme released by the cells was assessed from the exposure medium removed from the microtiter plate. After that the cells were incubated with the WST-1 reagent to assess the effect of the test compound on the mitochondria. When evaluating the acute short-term cytotoxicity for 5 min to one-hour exposures in HCE cells, the WST-1 test was not performed immediately after the exposure but after a one-hour recovery period to produce more reproducible results. With the combined use of the WST-1 test and the LDH test, at the same time valuable information is gathered simultaneously of two different physiological endpoints, mitochondrial function and membrane permeability. When WST-1 test is used with the cells in the exponential growth phase, before the confluence is exceeded, both the toxic effects and the proliferative effects of the substances on the cells can be evaluated.

Although the LDH test as an index of membrane permeability and the WST-1 test as an index of cell viability/proliferation and mitochondrial function measure different physiological endpoints, the tests yielded comparable results, especially in EDTA-treated corneal cells. Most of the drugs are lost from ocular tissues and fluids within a few hours, while anionic and cationic surfactants show retention (Green et al., 1987). When HCE cells were exposed to BAC for 5 min, and the WST-1 test was performed immediately or after one-hour recovery time, less cytotoxic effects appeared in the cells that were not allowed to “recover” after the BAC treatment, indicating that BAC was in fact retained in the corneal epithelium and acted as a reservoir for further penetration. In the case of EDTA, this kind of phenomenon was not observed. In the case of ionic surfactants, possible penetration into the cells should therefore be taken into consideration. However, to generate more reproducible test results, the one-hour time period after the exposure and before the addition of the WST-1 reagent was included in our further studies.

In both tests and corneal cell types, the use of serum resulted in lower toxicity. Therefore, the use of serum in cytotoxicity testing is not recommended. In the case of BAC, the WST-1 test appeared to be an earlier indicator of cytotoxicity. Mitochondria may

therefore be more sensitive to toxic effects than the plasma membrane. Bearing in mind also the reproducibility, the WST-1 test seemed to be a better choice than the LDH assay. The large variations in LDH results may be due to the adaptation of the one-day cultured cells to new culture conditions and to changes in enzyme levels, as observed in earlier studies with rabbit primary CE cells (Grant et al., 1992). In BAC exposures with more than 0.05 % (w/v), 2.78 mmol/l BAC, a decrease in LDH activity was observed. This could be partly due to the inactivation of LDH by BAC, because cationic and other ionic surfactants can inactivate LDH isoenzymes (Sanford et al., 1981). The possible interactions of ionic surfactants with LDH isoenzymes should therefore be taken into consideration in the LDH leakage test. In concordance with the current study, earlier studies with rabbit primary CE cell cultures have shown that the mitochondrion-based MTT assay is a more sensitive method than the membrane-based LDH leakage test (Grant and Acosta, 1994; Yang and Acosta, 1994) and another membrane-based cytotoxicity test, the propidium iodide assay (Grant and Acosta, 1994). In HCE cell cultures, the MTT test also appeared to be more sensitive than the propidium iodide assay (Saarinen-Savolainen et al., 1998).

The comparison of the EC_{50} values to those in other studies is difficult, due to different cytotoxicity tests and variations in cell densities and exposure times. When the results of BAC exposure are compared to the earlier studies conducted with primary RCE cultures and to the MTT test, our EC_{50} value evaluated by the WST-1 was only slightly higher (II, Tables 1 and 2). On the other hand, our EC_{50} value for HCE cells was about four times smaller than the value obtained in an earlier study using the same cell line and the MTT test (Saarinen-Savolainen et al., 1998). These differences can be attributed not only to the different cytotoxicity test, which is possibly more sensitive than the MTT test, but also to different cell culture conditions and a different exposure system. In the current study, pre-confluent HCE cells were exposed to test substances in culture medium with or without serum. After the exposure, the cells were washed once with basal DMEM/F12 medium and then incubated in the normal growth medium containing 15% serum. In the other study, confluent HCE cells were exposed to BAC in HBSS, after which the test solution was removed, and basal medium without serum was added with the MTT solution. When our results are compared to another study using the same HCE cells, the WST-1 test and practically the same test procedure (Burgalassi et al., 2001), the EC_{50} values for one-hour BAC exposure in serum-containing medium are in the same range (0.055 mmol/l in our study and 0.078 mmol/l in the other study). The results of the multilaboratory HCE-WST-1 study are also within the same range after one-hour exposure to BAC.

6.5. Interlaboratory Evaluation and Reproducibility of the HCE-WST-1 Test

The interlaboratory HCE-WST-1 study was undertaken to investigate how to perform it and, ultimately, to find the best possible conditions for its use (III). The study substances BAC and PSE gave quite similar dose- and time-dependent responses. However, variations in test results (from 31% to 121%, mean 58%) were found between laboratories. This was partly due to the low number of data, to short exposure times, and to the use of serum. Although all the laboratories used the same mutually agreed test protocol, the same test conditions, and the test substance from the same lot, we used a serum from a different lot. The use of different serum lots was necessary due to the extent of the research. Short exposure times (5 min to one hour) were chosen to simulate the normal acute *in vivo* exposure in a clinical situation. It was apparent in the study that longer exposure times and the lack of serum resulted in more reproducible results. Also, when the results from the HCE and RCE cell studies are compared to the interlaboratory study, more corresponding results are achieved with longer exposure times. Therefore, the use of one-hour exposure time and the elimination of serum in the exposure medium is our recommended test protocol for the use of HCE-WST-1 test.

6.6. Evaluation of Long-term Adverse Effects on HCE Cells

The cytotoxicity of 5-FU has been investigated by using several ocular cell types, mainly originating from rabbit (IV, Table 1), but there are no earlier reports on its cytotoxicity in HCE cells *in vitro*. Depending on the nature of the test compound and the purpose intended, the test conditions to detect the selected properties in the evaluation of toxicity may need to be modified. In our earlier studies, the HCE cell-based cytotoxicity tests were used to study the acute short-term (5 min to one hour) cytotoxicity simulating the clinical situation when ocular drug is topically administrated to the cornea. When 5-FU, a well-known antiproliferative agent widely used in ophthalmology to suppress the growth of fibroblasts after glaucoma surgery, is tested in these conditions, it does not show adverse effects in HCE cells. However, 5-FU is known to induce CE side effects.

In the current study investigating the adverse CE effects, HCE cells were exposed to 5-FU for 1-72 hours. In an exposure lasting for more than 24 hours, 5-FU caused time-dependent reduction in the cell number. After initial decrease in cell viability, especially in serum-containing exposures, cell numbers reached a plateau and decreased only slightly with the increasing 5-FU concentrations. 0.05 mg/ml (0.38 mmol/l) 5-FU appeared to be the threshold concentration of cytotoxicity for HCE cells. When HCE cells were exposed to less than 0.05 mg/ml, the cell proliferation was inhibited. Higher 5-FU concentrations, especially when serum was not used, induced cytotoxic effects rather than antiproliferative effects. 5-FU induced only minor effects on the LDH leakage compared to BAC. Serum was found to protect the cells against the loss of membrane integrity, as seen in the earlier studies.

The comparison of our HCE results to the previously published *in vitro* results with other cell types is problematic, due to differences in cell culture systems, exposure procedures,

and cytotoxicity tests (IV, Table 1). In most cases, cells have been exposed to 5-FU in complete growth medium, and the EC_{50} values have been estimated by counting the cells after the cell cultures have reached confluence. The estimated inhibitory EC_{50} values for both fibroblasts and epithelial cells are in the range of 0.0005 mg/ml (0.0038 mmol/l). In a clinical situation, the repeated postoperative injections of 5-FU as adjunctive treatment in filtration surgery in glaucomatous eyes may cause similar prolonged exposures to 5-FU as presented in this study. However, caution in conclusions is necessary, as always when extending the *in vitro* cytotoxicity data to *in vivo* situation.

6.7. Primary RPE Cell Cultures *Vs.* the RPE Cell Line in Cytotoxicity Testing

RPE cells form the outermost layer of the retina, which carries out several functions that are crucial for the normal function of the visual system (Newell, 1996). RPE is responsible for the phagocytosis of photoreceptor outer segments, and it provides enzymes and substances for photoreceptor renewal. Systemic and intravitreally administrated drugs can induce ADRs. Intravitreally dosed drugs have a straight contact with retinal cells, and some systemic drugs can penetrate the blood-retinal barrier. There are no generally accepted guidelines for the evaluation of retinal toxicity either *in vivo* or *in vitro*.

The RPE cell line D407 and pig primary RPE cell cultures were sensitive to tamoxifen and toremifene. Even thought oculotoxic side effects have been reported *in vivo* with tamoxifen treatment only, we found that tamoxifen and toremifene were equally cytotoxic *in vitro*. Chloroquine was far less toxic than tamoxifen or toremifene, but clearly more cytotoxic than the other compounds tested. As with corneal cell cultures, 5-FU had antiproliferative rather than acute toxicity effects on RPE cells. After initial decrease in cell viability, the cell numbers reached a plateau. This is in accordance with the previous *in vitro* studies in RPE cells, but *in vivo* no 5-FU toxicity in the retina has been detected. Gentamicin and ganciclovir did not show any cytotoxicity in micromolar concentrations, *i.e.* the concentrations that cover the therapeutic range. Our results supported the previous results concerning the eye toxicity of gentamicin and ganciclovir, although there are also controversial reports about this toxicity. Our study shows that retinal cells, the RPE cell line D407 and the pig primary RPE cell cultures, are good, reliable models for the evaluation of general RPE toxicity. However, the establishment of RPE primary cell cultures is a laborious procedure. Since both cell types were in most cases equally sensitive to the tested substances, the retinal cell line is a more appropriate model for toxicity screening.

6.8. Advantages and Difficulties of Ocular Cytotoxicity Tests

In vitro tests are not only valuable means for toxicity screening, but a well-defined set of *in vitro* tests can provide very comprehensive test results regarding the mechanisms responsible for adverse ocular effects. *In vitro* cytotoxicity tests have also been reported to have several other advantages: they are cost-effective and sensitive assays, and often easy to perform, repeat, manipulate, and score (Borenfreund and Borrero, 1984). In ocular toxicity, corneal cells, the target cells of topical ocular irritation, have mostly been used as primary cultures, though they have many drawbacks. First, animals may need to be sacrificed to obtain cells for the establishment of primary cultures, which however become senescent after a few passages. Rabbits have been the most common tissue source for primary cultured CE cells. In contrast to skin keratinocytes, human corneal tissue is not as readily available, and therefore primary cultured human corneal cultures are not likely to replace the animal primary cultures (Davila et al., 1998).

In toxicology, the potential cellular variability of primary cells can also make an interlaboratory comparison of the results difficult (Guillot, 1992). The use of established cell lines as *in vitro* systems is therefore well-founded, because cell lines are homogenous and practical due to their easiness of handling (Pasternak and Miller, 1995). Most often cell lines are well-defined and reliable models that are readily available to most investigators (Guillot, 1992). However until recently, ocular toxicology has suffered from lack of human-based ocular cell lines, which would also be valuable means to investigate the various mechanisms of ocular irritation. Moreover, since the Draize eye test has been designed to take into account primarily the adverse effects of the topically administrated substances on the outer parts of the eye, the possible adverse effects on the inner parts of the eye, such as retina, are ignored in the scoring. Consequently, retinal cells have been largely ignored in the development of *in vitro* methods for ocular safety assessment. In drug development, the use of retinal cells would be especially useful for studying the ADRs.

Besides the shortage of human-derived cell lines, there are some other disadvantages in cytotoxicity testing. Pharmacokinetic aspects are difficult to take into account in *in vitro* experiments. Corneal cell cultures lack protective clearing mechanisms, such as blinking and the precorneal tear film *in vivo* (Herzinger et al., 1995; Davila et al., 1998). The tear film has several important functions; it maintains moisture for the epithelial cells of the cornea and conjunctiva, it has bactericidal properties, it transports oxygen and carbon to and from the cornea, and it dilutes and washes away toxic agents (Baeyens and Gurny, 1997). However *in vitro*, cells are directly exposed to toxic agents. The cells are normally grown in a nutritive medium, in which the test substance is dissolved. Validation studies have shown that the most critical factor appears to be the degree of water solubility of the substance tested. Cell culture systems function best with water-soluble compounds, but are not equally suited for assessing lipophilic compounds and hydroalcoholic formulations. If the test substance is insoluble in the aqueous medium, contact with cells may be irregular (Rasmussen, 1995). To overcome this problem, insoluble substances can be dissolved in DMSO or ethanol, but the effect of solution also needs to be taken into consideration in cytotoxicity results.

The buffer of the incubation medium can decrease the effect of acid or alkaline substances (Rasmussen, 1995). The toxicity can therefore be largely underestimated if the acidity or the alkalinity is the most important factor in the *in vivo* toxicity. In some cases, the toxicity can be overestimated if the solid form is in fact less toxic than the solution (Rasmussen, 1995). The toxic potential of the test substance may also be influenced and underestimated by the interaction of the test substances with the medium components, such as serum, which is supplemented to provide growth factors. In the present study, serum was found to protect the cells against the toxic effect. The protective effect of serum against the toxic effects of the test substances has been reported to be due to the binding of the toxic substances to serum proteins (Clemedson et al., 2003). The use of serum in culture medium can be thought to simulate the proteins in the normal tear film. In man, the total amount of proteins in tears is about 0.6-0.9% (w/v) (Baeyens and Gurny, 1997). The amount of serum proteins was comparable to this level in the exposure medium containing about 15% serum used in corneal studies. Nevertheless, the use of serum in exposure medium can result in the underestimation of toxicity. The interlaboratory HCE-WST-1 test study also showed more reproducible results when serum was not added to the exposure medium.

In the present study, it was found that animal primary cell cultures were, in most cases, as sensitive to the test compounds as the respective human cell lines. Because *in vitro* tests must exhibit practicability and reproducibility, these human cell-line-based assays are good models for toxicity studies. The assays can be easily automated with effective multiwell techniques and plate readers. Because of quantitative and objective measurements, the cell culture assays also permit an easy transfer between laboratories.

7. CONCLUSIONS

The present study aimed to develop novel cell culture assays for the evaluation of the ocular toxicity of pharmaceuticals and other industrial chemicals. The toxicity of selected test compounds as positive controls was investigated by using cytotoxicity assays based on CE and RPE cells. The main conclusions of the study are as follows:

- Pre-confluent cultures of the HCE cell line expressed cytokeratins 7, 8, 18 and 19, which are characteristic of simple epithelium. In addition to these simple epithelium-specific CKs, the confluent and post-confluent HCE cultures, after the cells piled up, also expressed cornea-specific CK3. In this respect, pre-confluent HCE cultures used for cytotoxicity testing were not identical to the normal human cornea *in vivo*.

- Primary cultures of rabbit corneal cell epithelium and the HCE line in the WST-1 cytotoxicity test as well as the LDH leakage assay yielded quite comparable responses to the topically applied ocular test compounds, BAC and disodium EDTA. The WST-1 test appeared to be an earlier indicator of toxicity compared to the LDH test, which also showed great variations in the cytotoxicity results, especially when serum was not used. The use of serum in the exposure medium was found to result in lower toxicity in both tests. Serum protected the cells from toxic effects to some extent.

- In the interlaboratory HCE-WST-1 study, the topically used ocular substances BAC and PSE (Brij®78) induced dose- and time-dependent toxicity results. Large variations were observed in the test results, which, however, were not influenced by the cell culture experience of the participating laboratories. The most reproducible results were obtained when the cells were exposed to the test compounds for one hour in the absence of serum. The serum in the culture medium protects the cells and may bind to drugs. Therefore, the use of serum in short-term cytotoxicity testing is not recommended.

- When the long-term adverse effects of 5-FU were evaluated with the HCE cell line using the WST-1 and LDH tests, only long incubation times, starting from 24 hours, induced time-dependent inhibition in cell proliferation. The serum was found to protect the cells for 24 hours, but after longer exposure times the protective nature of serum was lost. According to the EC_{50} values, the order of the decreasing cytotoxicity of the substances tested with corneal cells was BAC ≅ PSE > EDTA > 5-FU.

- Studies with primary cultures of pig retinal pigment epithelium and the RPE human cell line D407 with the WST-1 test showed, after 24-hour exposure time, radical losses in cell viability with three systemically used test drugs. The order of decreasing cytotoxicity was tamoxifen ≅ toremifene > chloroquine. The EC_{50} values of the intravitreally applied drugs 5-FU, gentamicin and ganciclovir were in the range of millimolar, and thus they were far less cytotoxic than tamoxifen,

toremifene, and chloroquine. Except for ganciclovir, all the test drugs in pig primary RPE cultures and the D407 cell line showed quite identical toxicities in the culture conditions used.

- As practical microtiter formats, the cytotoxicity tests studied, the WST-1 test and the LDH leakage assay are reliable, transferable, easy-to-perform cytotoxicity tests for ocular toxicity screening. More easily manageable human-based cell lines, the HCE and the RPE D407, can be used instead of animal primary cultures. As human-based cells, these cell lines are also valuable candidates for more detailed mechanistic studies. The WST-1 test and the LDH leakage assay have potential to be used as a part of a more comprehensive test battery. These tests can be performed in combination using the same cells. The WST-1 test provides information on the chemical substance's effect on mitochondrial function, while the LDH leakage test indicates its effect on the cell membrane. As screening tests they can be used for making preliminary decisions or establishing the direction for further studies.

8. FURTHER CONSIDERATIONS

- The effect of a chemical substance on a single component such as a single cell type culture system is likely to be different from the effects observed in the whole organ system. Since the eye is a complex organ composed of many tissues, a battery of *in vitro* tests that reflect the various aspects of eye damage is needed when considering the complete replacement of the Draize eye test. It has been argued that the replacement of the Draize test will not be possible until the mechanisms involved in eye irritation are adequately understood. A variety of mechanisms are responsible for the formation of ocular lesions. Serious lesions are produced by mechanisms different from those that cause moderate lesions. The use of retinal cells should also be taken into account in the *in vitro* evaluation of the ocular safety of drugs. Accordingly, a reliable and largely available test battery meeting all the requirements of the regulatory authorities, is still awaiting its breakthrough. Such a test battery requires *in vitro* methods that give complementary results of the relevant target sites and the various different mechanisms involved in ocular irritation, including information on the depth and time-scale of ocular injury, pain, recovery and repair from injury.

- The progress in human tissue engineering over the past ten years has made tissue constructs available for dermal toxicology. In recent years, corneal models have also been constructed for ocular studies. The establishment of reliable corneal models that mimic the entire human cornea *in vivo*, the outer multilayered corneal epithelium, the stromal layer with keratocytes and the inner monolayer endothelium, makes it possible to study in more detail the various cell-matrix and cell-to-cell contacts and interactions that are also present *in vivo*. Such models, especially when human cells are used, are valuable tools when studying the various mechanisms of eye irritation, such as inflammatory mediators extracted by the wounded cornea. A three-dimensional corneal model would also be a more sensitive test than the conventional animal test for studying the small differences between slightly irritating materials and products. In retinal toxicology, a three-dimensional coculture system of RPE cells and choroidal endothelial cells, and ultimately a perfusion culture system of the whole retina would be good models to study the retinal ADRs in greater detail.

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