In Vitro Alternatives for Ocular Irritation
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The necessity of using animals to test whether new chemicals and products are eye irritants has been questioned with increasing frequency and fervor over the last 20 years. During this time many new nonanimal methods have been proposed as reliable alternatives to the traditional rabbit (Draize) test. To date, however, none of these nonanimal (in vitro) tests have become universally accepted as a complete replacement for the Draize test. To understand why a complete replacement has not been found, one has to first understand the reasonably complex structure of the eye, the standard Draize scoring scale—which is based on a qualitative evaluation of three different tissues—the differences between human and rabbit eyes, the intrinsic variability of the animal test, and the details of the different in vitro tests that have been proposed as replacements. The in vitro tests vary from relatively simple assays using single cells to more sophisticated assays that use discarded animal tissue or artificially constructed human tissue. It is clear that appropriately designed in vitro tests will eventually give more useful mechanistic information about ocular injury from which we can more comfortably predict the risk of human eye irritation from new products and ingredients. — Environ Health Perspect 106(Suppl 2):485-492 (1998). http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-2/485-492 curren/abstract.html

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Introduction

The necessity of using animals to test whether new chemicals and products are eye irritants has been questioned with increasing frequency and fervor over the last 20 years. Admittedly the questions are very complex, and strong social, political, ethical, and scientific arguments have been raised on both sides of the issue. During this process, numerous nonanimal methods have been proposed as reliable alternatives to the traditional animal tests. However, before such tests come into common use, they must be carefully evaluated to determine if, in fact, these new methods can replace or reduce the use of whole animals. Such evaluation involves investigating the basic details of ocular irritation, reviewing what type of information is currently obtained from the animal tests, understanding how the design of in vitro systems relates to the animal model, and only then determining what real progress has been made in the search for alternatives to traditional ocular irritation testing.

Structure of the Eye

In interpreting the results from any toxicologic study, there must be some basic knowledge of the organ system being studied—at the very least an understanding of its morphology, cellular constituents, and normal function—that allows one to determine whether an injury has occurred and what the consequences of that injury are. Because there are many similarities between the animal and the human eye, and because the human eye is the organ we are trying to protect, the human eye will be used here as an example for the discussion of ocular structure. Where differences exist between the eyes of humans and rabbits (the usual target species of ocular irritation testing), they will be noted.

Figure 1 depicts a human eye in both a normal front view and in cross section. The latter view more clearly shows the tissues that are of concern to toxicologists and ophthalmologists. Perhaps the most important tissue is the cornea: the exterior surface that is exposed to the outside environment. The normally transparent cornea allows light to freely enter the eye and eventually be focused on the retina. If the cornea becomes cloudy (opaque)—as can happen after accidental exposure to strongly irritating chemicals—light can no longer pass easily into the eye and vision becomes impaired or even completely blocked. Although the eyelids offer the cornea some protection, it is still very susceptible to injury.

About 80% of the cornea's structure is the stroma—a regular array of macromolecules through which light can easily pass as a consequence of the stroma's high degree of order and exact level of hydration. Maintenance of this very important hydration level (75–80% water) is the responsibility of two active cell layers, a single-cell-thick endothelium covering the inside surface of the cornea and a much thicker epithelium that covers the outside surface. These cell layers work together to keep additional water from entering the cornea, which would result in swelling and opacity. The epithelium also has a second function of providing a physical barrier against the entry of foreign materials. If the epithelium is injured, corneal opacity can result. However, minor opacities can often be reversed because the epithelium can repair itself either by movement of surrounding cells to cover the wound or by the actual replacement of damaged tissue through new cell division. In contrast, the endothelium is generally not capable of repair. Therefore, if these cells suffer cytotoxic damage there can be significant consequences, e.g., permanent blindness. It is this relationship between the induction of cellular damage and resulting ocular irritation or other injury that is the basis for many of our current in vitro ocular irritation screening systems.

Another delicate tissue of the eye is the conjunctiva, the nonkeratinized squamous...
epithelium that lines the inner surfaces of the eyelids and much of the external surface of the ocular globe (it is continuous with the cornea). The conjunctiva is highly vascularized and may become quite inflamed after exposure to irritating material. Mildly irritating chemicals or other products often cause conjunctivitis without any associated corneal damage.

A third important ocular tissue is the iris (the colored part of the eye), which, by constricting or dilating, controls the amount of light that enters the eye and is eventually focused on the retina. The iris lies under the cornea within the aqueous humor. In some cases foreign materials penetrate completely through the cornea and interact with the iris. The iris may then become very inflamed and may lose its ability to react to light, seriously damaging the ability to see.

Observations of the degree of injury to each of these tissues in the animal model are incorporated as part of the scoring system of most common eye irritation protocols. The details of these scoring systems will be discussed in “In Vivo Ocular Irritation Testing.”

**In Vivo Ocular Irritation Testing**

It is important to understand how manufacturers assure themselves that their products will not pose an unacceptable risk to the eyes of their customers. Generally the process consists of several steps. First, the maximum potential hazard of the ingredient or formulation to the ocular tissue is determined. Second, the actual use of the product is considered, estimating the probability that it may inadvertently enter the eye. Third, a final safety assessment takes into account benefits, risks, and the impact of the instructions for use that generally accompany the product. Although the entire process is important, it is the first stage of this process—generally termed hazard identification—and the development of improved in vitro systems to detect such hazards that are important to this discussion.

For obvious ethical reasons, tests using animals, rather than volunteer human subjects, have generally been used to assist toxicologists in determining the degree of danger a material poses to the eye. Although it is possible to expose human eyes to dilute forms of materials whose chemical properties are well known and generally regarded as safe, it is obvious because of the risk of severe injury that this cannot be done with novel materials whose toxic properties are as yet unknown.

The albino rabbit has historically been the animal of choice for testing potential eye irritants, primarily because its large eyes make it easy to observe damage. In addition, it has a large conjunctival sac (accommodated by loose lids) that easily accepts test material and holds it against the eyes. However, because of several striking differences, the rabbit is far from a perfect model for humans. One difference is the presence of a nictitating membrane, or third eyelid, in the rabbit. This membrane moves laterally across the eye, likely causing the kinetics of removal of many test materials to differ from humans. Another difference is that the conjunctival sac of the rabbit is much larger than in humans, meaning that more test material can be placed in a rabbit’s eye than would be likely to ever get into the human eye during an accidental exposure. Additionally, the rabbit cornea is somewhat thinner than that of humans and there is less tear production to aid in washing out a foreign material. For these and other reasons, the rabbit is generally considered an overly sensitive model for humans. Although this may be considered a positive aspect of the rabbit model because it adds a margin of safety to the risk assessment, it also presents the problem of inappropriate hazard assessment and suggests that a more predictive model would be beneficial.

The conduct of the animal test now needs to be examined in detail to help us understand the subjective nature of the test and appreciate the difficulties faced in developing and validating in vitro models. To test a material for potential ocular irritation, the lower lid of the animal is pulled away from the eyeball, and 100 µl of a liquid (100 mg of a solid) is placed in the resulting conjunctival sac. The lids are then held together for a few seconds to ensure contact between the test material and the ocular tissues. The animal’s eyes are carefully observed, first at 1-hr and then at 24-hr intervals for up to 14 days. It is important to highlight again a major difference between the structure of a rabbit’s eye and that of a human eye. Because 100 µl will not fit into the human eye, the animal’s eyes are being exposed to much more material than might actually enter the human eye from an accidental exposure. The low-volume eye test (1), which uses one-tenth of the material normally applied to the rabbit eye, is reported to better predict the response of human eyes and to be less hazardous to the animal.

The time that the test material is in contact with the eye is not controlled during the Draize test because the material is only removed by the natural processes of tearing and blinking. Therefore, time of exposure may differ with each test material, which makes it difficult to develop an in vitro model.

At various standard time periods after instillation, the three major tissues of the eye (cornea, conjunctiva, and iris) are observed macroscopically for injury. Each tissue is observed for different signs and the degree of injury is recorded according to a standard scale. For the cornea, the degree of opacity and the area of the eye involved are recorded. The iris is examined for inflammation and the conjunctiva—a mucus membrane—is examined for redness, chemosis, and any exudate. Generally mild responses of the conjunctiva alone are not serious unless the test material is designed to be applied to or around the eyes.

**Draize Scoring Scale**

The fact that three ocular tissues can be affected by chemical treatment makes simple scoring and evaluation of ocular damage difficult. Draize (2) proposed in 1944 what has become a solution to the problem. He devised an individual numerical scoring system for each of the three ocular tissues of interest and then proposed a special weighting system to combine the scores into a single eye irritation score. Table 1 shows specifically how Draize reduced the evaluation of a very complicated type of injury to a single number.

This awareness of the various ocular tissues and the ways they respond to injury is very important because we need to understand exactly what score an in vitro eye irritation assay is supposed to predict. However, the complete Draize scoring system is not generally used to classify materials for regulatory purposes. The European Union classification scheme, for example, uses only discrete categories such as R36 (irritating to eyes), and R41 (risk of serious injury), which are not integers.

| Eye tissue | Maximum |
|------------|---------|
| Cornea     | opacity (0–4) x area (0–4) x 5 | 80 |
| Iris       | grade value (0–2) x 5 | 10 |
| Conjunctiva| (redness (0–3) + edema (0–4) + discharge (0–3)) x 2 | 20 |
| Total score| 110 |

*An illustration of how individual subjective observations of injury to three ocular tissues are converted into a single numerical score estimating total eye injury.*
damage to eyes) to define the amount of danger that exposure to a material represents (3). However, a continuous scale like that of Draize gives considerably more information about the severity of the hazard than does an abbreviated classification system, and this prediction of many levels of severity is what most people envisage as being supplied by a replacement in vitro test.

However, the single number (Draize score) presented as the eye irritation potential of a chemical or formulation is not exact (4). The subjective nature of the gross observations made during the scoring of the test, plus normal animal-to-animal variability, make it virtually impossible to routinely reproduce the final Draize score, especially for midrange irritants. This does not mean that Draize scores are completely meaningless. Repeated trials will generally generate scores within an acceptable range. However, a single Draize score should not be viewed as an exact predictor of eye irritation potential. Bruner et al. (5) presented a summary of the effect of this in vitro variability on the evaluation of in vitro tests.

With this degree of variability as the norm, it becomes very difficult to have confidence that a Draize test will be able to reveal any real differences between two mild materials. Yet product developers and toxicologists often need to be able to detect with confidence small differences between candidate products. Thus the need for alternative tests that may provide more precise data springs not just from concerns for animal welfare, but also from the imprecision of the animal test and the desire of toxicologists for better and more accurate tools.

**Development of Alternative Models**

The need to develop alternative in vitro tests for eye irritation has been apparent for some time. These alternative assays have used a diverse set of human and animal cells, tissues, and even biochemical matrices (Table 2). What is the strategy that researchers have used to develop in vitro assays that model the animal assay? First, only a few in vitro assays actually attempt to model the entire eye. In fact, most in vitro tests that have been proposed are reductionist, i.e., they tend to model only one small part of the complex process of eye irritation. This has led to situations where an in vitro test that may measure only one specific type of damage is compared to a Draize score that covers several types of damage in several tissues. Success at this type of comparison is more than might reasonably be expected from a single in vitro test; thus it makes sense to think of an eventual test battery with several in vitro tests, each one capable of detecting a different type of damage. This type of approach has recently been used by the U.S. Interagency Regulatory Alternatives Group (IRAG). This group led an extensive international evaluation of the state-of-the-art of in vitro eye irritation tests based on a comparison of in vitro scores to individual animal tissue scores, not to the total combined Draize score (6).

**Ex Vivo Models**

Figure 2 illustrates the continuum of reductionist relationships between the

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**Table 2. Characteristics of common in vitro assays.**

| Assay, reference | General description | Method of applying test material |
|------------------|---------------------|---------------------------------|
| Neutral red release (16), neutral red uptake (17) | Target cells (primary or continuous; fibroblasts or epithelial-like) are grown in submerged monolayer culture | Generally increasing dilutions of test material are added to growth medium until a predetermined end point (generally cytotoxicity) is reached |
| Fluorescein leakage (14,15) | Target cells (primary or continuous; fibroblasts or epithelial-like) are grown in submerged monolayer culture; medium may be removed for dosing | Either increasing dilutions of test material are added to growth medium or cell surface for a set time, or a single concentration is added for varying times. End point is induction of permeability of the monolayer |
| BCOP (10) | Living bovine corneas are treated with test material and changes in opacity and permeability are measured by instrument | Test materials are applied neat or at in-use concentrations directly to the epithelial surface of the cornea |
| HetCam (25) | Chorioallantoic membrane of a chicken egg is treated | Test materials are applied neat or at in-use concentrations directly to the membrane and damage to the membrane is recorded |
| Tissue equivalent assay (11) | Three-dimensional reconstructed tissue (often human) is grown with top surface exposed to air | Test materials are applied neat or at in-use concentrations directly to the tissue construct and cell killing is measured |
| Enucleated chicken eye (23) | Isolated eye of a chicken is treated and subsequent damage recorded | Test materials are applied neat or at in-use concentrations directly to the tissue construct |
| Cytosensor microphysiometer (23) | Cells held over or on a coated sensor are treated and changes in cellular metabolism are recorded in real time | Generally increasing dilutions of test material added to growth medium until a predetermined end point (decrease in metabolism) is reached |
| Irritation (Eytex) (23) | End point is precipitation of protein in a nonwivable commercially supplied matrix. Meant to mimic opacity formation in the cornea | Either dilutions or neat test material is added to a membrane bulleted over a responding protein matrix |
| Pollen tube growth (26) | Tobacco pollen is allowed to germinate in the presence of test material | Dilutions of test material used; end point is inhibition of pollen tube elongation |
| Red blood cell (19) | Red blood cells are exposed to test material | Dilutions of test material used; lysis (release of hemoglobin) and hemoglobin denaturation are monitored |
| SIRC (27) | Target cells (continuous cell line derived from rabbit cornea) grown in submerged culture at clonal densities | Generally increasing dilutions of test material added to growth medium until a predetermined end point (generally cytotoxicity) is reached |

Abbreviations: HetCam, hen’s egg test on the chorioallantoic membrane; SIRC, Staatsen Seruminstitute Rabbit Corneal—a fibroblastlike cell line derived from a rabbit cornea. *Molecular Devices Corporation, Menlo Park, CA. †InVitro International, Irvine, CA.
whole animal and the in vitro model. Some assays focus on a first stage of reduction, i.e., looking just at the isolated eye without any associated conjunctiva that would be present in the animal. In this type of assay, test material is applied directly to the excised eye and any resulting injury is recorded. Such assays are the enucleated rabbit eye test (7) and the enucleated chicken eye assay (8). These tests have been developed using the normally discarded eyes from food production species such as chickens or from laboratory animals that have been used for other purposes.

A second group of in vitro tests models only the cornea, which is a logical approach because maintenance of an intact transparent cornea is the major concern of ocular safety studies (severe damage to the cornea can lead to permanent blindness), and because damage to the cornea contributes more to the Draize score than does damage to any other ocular tissue. In fact, one recent study indicated that corneal score alone is an excellent predictor of total Draize score (9). An example of an assay that focuses primarily on detecting corneal damage is the bovine cornea opacity and permeability (BCOP) test (10). This assay uses corneas isolated from cattle used for meat production. With this model the amount of corneal opacity that has been induced by a test material can be quantitatively measured with an optical instrument, as opposed to the subjective estimation of opacity made by gross observation in the animal test. Damage to the cornea's barrier function can also be measured with the BCOP assay.

Another in vitro model designed to mimic corneal response is the tissue equivalent assay (TEA) (11). This assay uses a reconstructed, nonkeratinized epithelial-like tissue made of human cells, upon which test materials can be directly placed. Such tissue is commercially available (MatTek Corporation, Ashland, MA and Skin Ethic, Nice, France), although it can also be produced by the individual investigator (12). The structure of this three-dimensional model is meant to simulate the epithelial covering of the cornea. Damage is estimated by measuring the viability of the human cells after treatment. Because this model is only a reconstructed tissue, it is not clear how closely it mimics the response of the epithelial layer of a normal cornea, but the model has been reasonably predictive of ocular irritation (Draize data) in recent studies (13).

Cell-Based Assays

One step further in the reduction of the animal eye into less complicated in vitro models is accomplished using single cell or monolayer culture assays, which generally use epithelial cells similar to those that make up the outer surface of the cornea. If these cells are injured or killed in the animal, chemicals can more easily penetrate into the stroma of the cornea and cause additional damage. This penetration phenomenon is modeled by an in vitro assay called the fluorescein leakage test (14,15), in which a single layer of cells acts as a barrier to a common dye, fluorescein. If the cells are damaged or killed by a test chemical they lose their ability to act as a barrier to fluorescein. The subsequent movement of this dye through the cell layer can be measured and is an indicator of the amount of cell damage.

On a simpler level, one can simply observe the amount of cell killing that occurs in a single layer of human or animal cells (cytotoxicity assay) and use this information to infer damage that might occur to the eye. Examples are the neutral red release test (16), which uses short exposure times (such as might occur with an accidental splash to the eye followed by a quick rinse), and the neutral red uptake assay (17), which looks at longer exposure periods. An extensive review of these assays has been published as part of the U.S. IRAG evaluation (18).

Eye irritation can be reduced further by looking at damage to only the cell membranes. An example of this type of test is the red blood cell assay, where red blood cells are exposed to test material and membrane lysis is quantitated by measuring the amount of hemoglobin released (19). However, we should remember that the more reductionist the in vitro assay is, the more likely it will only respond to certain classes of chemicals that are likely to cause eye irritation by the same mechanism. Thus only certain types of eye damage will be predicted by each in vitro test.

Performance of the in Vitro Tests

Practical experience with the performance of various in vitro tests either in validation trials or in everyday use has revealed that considerable care must be taken when using the tests in a routine safety testing program. Each test seems to exhibit a slightly different level of sensitivity to correctly predict only a specific range of chemical classes. To consistently give a correct prediction, an in vitro test must do at least two things. First it must appropriately model the exposure kinetics, i.e., it must accept the test material in the same physical form as the animal test; it must be able to be exposed to the same concentration as in the animal test; and it must remain in contact with the test material for the same amount of time. Second, the endpoints that are developed for the in vitro assay must be predictive of the underlying in vivo tissue responses and this relationship must be clearly understood.

Currently, not all (or even the majority) of the in vitro tests fully meet these criteria. Nonetheless, if the use and interpretation of the tests is approached in an empirical fashion and attention is paid to certain key factors, the results can provide significant information for toxicologic evaluations. These key factors include type of product(s) to be used, physical characteristics of the product(s), expected level of toxicity, resolution required, intended use of the resulting data, and resources available.

An example of how to apply the above considerations is provided by examining the second factor, physical characteristics
of a product. Because there are two general forms of in vitro assays—those in which the substrate is completely immersed in growth medium (e.g., the neutral red uptake assay) and those in which the target surface is available for direct application of the test material (e.g., TEA)—the water solubility of a test material should first be considered. If a material is not water soluble, it would be fruitless to attempt to test it in a neutral red uptake assay because the test material will likely never actually come in contact with the target tissue. However, a topical application assay would be the logical choice because in this situation the test material will be applied directly to the surface of the target cells, ensuring exposure similar to what would occur in vivo. Examples of which assays are most suitable depending on the water solubility and form of the test material are as follows:

Water-soluble formations
- BCOP assay
- Fluorescein leakage assays
- Neutral red uptake/release assays
- Chiroallantoic membrane (CAM)-based systems
- Cytosensor microphysiometer Hydrophobic formulations
- Topical application assays
- BCOP assays
- Fluorescein leakage assays
- CAM-based systems

Also to be considered is whether to use dilution-based assays, i.e., assays in which serial dilutions of the test material are applied to the target tissue and the end point is the concentration that causes a certain response, or assays in which test material is only applied undiluted (neat) or at its in-use concentration.

Both types of assays have strengths and weaknesses, as can be seen in Tables 3 and 4.

Table 3. Advantages and disadvantages of dilution-based assays.

| Advantages                                    | Disadvantages                                      |
|-----------------------------------------------|---------------------------------------------------|
| Rapid to execute                             | Cannot be used with water-insoluble materials     |
| Most are machine scored                      | Dilution effects may mask toxicity of neat material|
| Generally very cost effective; materials are often batched (grouped together) | Change in the physical form, e.g., solids to solutions |
| Seem to work well with surfactants           | Buffering effects of the medium may effect toxicity significantly |
| Often differentiate well between very mild materials | Possible reaction of the test material with the solvent |

In these assays serial dilutions of the test material are applied to the test system and the end points are the concentrations of test material that cause a selected response.

Table 4. Advantages and disadvantages of topical application assays.

| Advantages                                    | Disadvantages                                      |
|-----------------------------------------------|---------------------------------------------------|
| Material is tested in its native form, i.e., as an in vivo exposure | Test substrate can often be expensive |
| Exposure of the target tissue can be assured  | Exposure times may be inconveniently long |
| In some models, exposure time can be selected to match expected in vivo exposure | |

In these assays only the neat or in-use concentration test material is applied to the test system.

Table 3 and Table 4 illustrate the expected level of toxicity possessed by the test material. Ocular toxicity ranges from very slight conjunctival redness to full corrosive destruction of the three primary tissues. For a single in vitro test to address this full range, with the desired resolution, would be challenging. Most in vitro assays are designed to balance resolution with dynamic range. Dilution-based assays rely on the changes in concentration to provide sensitivity and dynamic range. However, they are limited in the types of materials that can be tested. In contrast, the topical application assays, in which the test materials are applied neat, use time of exposure (tissue constructs) or the robustness of the tissue (BCOP) to provide dynamic range.

Choosing the Appropriate Assay

The tissue constructs provide high resolution for assessing potentially very mild (e.g., eye area cosmetics) to moderately irritating materials by extending the exposure times. Their resolution declines for the more aggressive materials because very short exposures (often a few seconds) are sufficient to kill the tissue. In contrast, the bovine cornea, with many layers of epithelium, does not resolve very mild products without excessively long exposures. However, it has the robustness to discriminate at the medium to high end of toxicity (20) (Figure 3). The double end points of opacity and permeability help the assay span the range from shampoos to industrial cleaners.

Tissue constructs

| Degree of ocular irritation |
|-----------------------------|
| Extreme | Severe | Moderate | Mild | Very mild |

The tissue constructs provide high resolution for assessing potentially very mild (e.g., eye area cosmetics) to moderately irritating materials by extending the exposure times. Their resolution declines for the more aggressive materials because very short exposures (often a few seconds) are sufficient to kill the tissue. In contrast, the bovine cornea, with many layers of epithelium, does not resolve very mild products without excessively long exposures. However, it has the robustness to discriminate at the medium to high end of toxicity (20) (Figure 3). The double end points of opacity and permeability help the assay span the range from shampoos to industrial cleaners.

Choosing the Appropriate Assay

The foregoing discussion illustrates that the choice of an in vitro ocular irritation assay is not simple. Unless the appropriate test is chosen, the subsequent results may be poor predictors of the actual ocular irritation potential of a test material. The previously mentioned key factors are extremely important and must be considered every time an in vitro test is contemplated. Table 5 gives some additional information (such as laboratory resources required and the technical skill needed) that should also be considered before choosing an in vitro ocular irritation test.

Need for a Prediction Model

How will we eventually determine that an in vitro test produces valid information that is at least as good as that produced by animal tests? Generally the accuracy of an in vitro model is assessed by identifying a number of materials that have been tested for ocular irritation in animals and then retesting the same materials in an in vitro assay. Both sets of data are then graphed, plotting the in vitro data on one axis and the animal data on the other axis, as in Figure 4. Each point on this graph represents the eye irritation score of a single material tested both in vitro and in vivo. Knowing that both assays give variable results, we use error bars rather than a single point to represent the range of scores that might be expected if we repeated each test several times. The data in Figure 4 are from the U.S. Cosmetics, Toiletries, and Fragrance Association Phase III evaluation of surfactants and surfactant-based
Table 5. Further characterization of common in vitro assays

| Test, reference | End point | in vivo tissue or irritation scale modeled | Resources needed | Technician skill level |
|----------------|-----------|------------------------------------------|-----------------|-----------------------|
| BCOP (10)      | Opacity + permeability | Draize MAS | Specialized equipment, commercially available, spectrophotometer | General laboratory skills |
| (Total score)  | Opacity   | Cornea | General laboratory skills |
| (Opacity)      | Permeability | Cornea/conjunctiva? | General laboratory skills |
| (Permeability) | Permeability | Conjunctiva, cornea | Tissue culture skills |
| Fluorescein leakage (14,15) | Permeability | Conjunctiva, cornea | Specialized equipment | Tissue culture skills |
| HetCam (25)    | Vascular damage, coagulation | Draize MAS, conjunctiva | General lab equipment | General laboratory skills |
| CAMVA (28)     | Vascular damage, coagulation | Draize MAS, conjunctiva | General lab equipment | General laboratory skills |
| Tissue equivalent assay (11) | Cytotoxicity | Draize MAS, corneal epithelium damage | General tissue culture lab equipment | Some tissue culture skills |
| Neutral red uptake (17) | Cytotoxicity | Conjunctiva, corneal epithelium damage | General tissue culture lab equipment, 96-well plate reader | Tissue culture skills |
| Neutral red release (16) | Cytotoxicity/membrane damage? | Conjunctiva, corneal epithelium damage | General tissue culture lab equipment, 96-well plate reader | Tissue culture skills |
| Enucleated chicken eye (23) | Opacity, corneal swelling | Corneal damage | Specialized equipment | General laboratory skills |
| Cytosensor microphysiometer (23) | Cellular metabolism | Conjunctiva, Draize MAS | Specialized equipment (expensive) | Tissue culture skills |
| Irritation (Eytex)* (23) | Precipitation | Draize MAS | Specialized equipment | General laboratory skills |
| Pollen tube growth (26) | Cytotoxicity | Draize MAS | General lab equipment, spectrophotometer | General laboratory skills |
| Red blood cell (19) | Membrane lysis | Draize MAS | General lab equipment, spectrophotometer | General laboratory skills |
| SIRC (27) | Cytotoxicity | Conjunctiva | General tissue culture lab equipment | Tissue culture skills |

Abbreviations: CAMVA, chorioallantoic membrane vascular assay; MAS, maximum average score. *InVitro International.

Figure 4. Surfactant formulations—BCOP permeability score. Graph showing the relationship between the Draize score of a number of surfactant-containing formulations with permeability measurements made in the in vitro BCOP assay. The error bars represent ±1 standard deviation. r = 0.79.

products (21) and illustrate how data obtained from the in vitro BCOP assay compare with the data obtained using animals. This relationship between in vitro and in vivo scores, i.e., the algorithm that allows one to predict an in vivo score from an in vitro score, is currently called the prediction model (22). Without such a known relationship it is impossible not only to use an in vitro test correctly but also to conduct a rigorous validation (22).

Notice the variability of in vivo data in Figure 4, especially when compared to the more easily reproducible in vitro data. However, if a relationship is found to be good enough, the model could be used in a validation study to test the validity of both the assay and its prediction model. However, the difficult question is how good does this relationship have to be? Bruner et al. (5) provide a discussion of what could be expected if one Draize test is used to predict the results of a second Draize test.

Recently two large international validation trials of in vitro ocular irritation assays have been completed (23,24). The results from these studies were mixed, as might be expected from previous discussion in this paper concerning the difficulties involved in validating against the Draize test. Whereas the results from the first study [sponsored by the European Commission and the British Home Office (23)] were not encouraging, the results from the second [sponsored by the European Cosmetic, Toiletry and Perfumery Association (24)] indicated that three of the assays tested satisfied one or
more of the predetermined success criteria. There were major differences in the scope of each study, especially in the range of chemical classes tested; the second study was limited to chemicals commonly used in the cosmetics industry. More validation studies of this type can be expected in the coming years. It is prudent for researchers and toxicologists searching for appropriate in vitro assays to keep abreast of the findings of current validation studies—and to take notice of all the key factors involved in assay selection before deciding which test to use with any given set of test materials.

Summary
Before a perfect, quantitative in vitro ocular irritation model is available, much basic work still needs to be done to understand mechanistically how injury happens in the human eye and how to model these mechanisms in vitro. However, several of the appropriate in vitro models may already be in development. The upcoming results from current and planned validation studies may tell us just how close any of these assays are to meeting our goals.

Several points need to be emphasized as we evaluate the state of readiness of in vitro eye irritation assays:

- The eye is a very intricate organ made up of multiple tissues, each of which responds differently to injury. Current animal tests for ocular irritation use a complex scoring system involving three important ocular tissues.
- In vitro tests have generally been designed to model only one—or just a few—ocular tissues, not the whole eye. This is very helpful in obtaining more detailed mechanistic information about the process of eye irritation. However, it then potentially leaves us in the position of having to replace a single animal test (a Draize test) with multiple in vitro tests. This is not necessarily undesirable, as we will likely learn more about the actual risk to humans from a chemical when multiple mechanistically understandable tests are used.
- Validation of in vitro ocular irritation assays will be difficult because the animal test is not very reproducible and because the animal test scores represent a combination of subjective observations of multiple ocular tissues.
- Different in vitro tests are suitable for different types of test materials and different ranges of toxicity. Careful consideration must be given to choosing the correct in vitro test for the required purpose.

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