IN VITRO CYTOTOXICITY ASSAYS. POTENTIAL ALTERNATIVES TO THE DRAIZE OCULAR ALLERGY TEST*

ELLEN BORENFREUND[1] AND OLINA BORRERO

Laboratory Animal Research Center, The Rockefeller University, New York

A short-term cytotoxicity assay carried out in multiwell test plates and a supplementary colony forming assay are both useful for screening and range finding of toxic concentrations of test agents. The highest tolerated dose (HTD), a concentration at which only minimal morphological changes were observed, was designated as endpoint in the assay. Epithelial rabbit cornea cells, murine fibroblasts, Chinese hamster lung cells, human hepatoma cells and mouse macrophage cultures were used as targets. Several of the alcohols tested at HTD in the colony forming assay were found to inhibit colony formation. An ID50 of colony formation was used as a quantitative corroborating test. The ranking of 34 toxicants was found to be virtually the same with all cell types examined. This easily reproducible, rapid in vitro *test is cost-effective and can be used for preliminary large scale screening of potential toxicants.*

A need for the thorough testing of environmental chemicals of potentially toxic nature such as pharmaceuticals, cosmetics, food additives and pesticides is generally recognized. However, the manner in which to conduct such testing is unresolved. The large scale use of different species of animals for toxicity testing and the standard Draize rabbit eye irritancy test (Draize et al., 1944) have come under increasingly critical surveillance. Although, at this time, it seems neither prudent nor feasible to replace *in vivo* testing, a concerted effort to develop sensitive and meaningful short-term *in vitro* assays seems appropriate. These assays, at the outset, could be used for preliminary screening and range finding, thus drastically reducing the number of animals used and providing a more cost-effective test option. Initially, the decision was to develop assays which could serve as *in vitro* alternatives to the Draize rabbit eye irritancy test. Among the criteria to be met by such *in vitro* tests are sensitivity, dose response, ease of manipulation and scoring, reproducibility, cost effectiveness and good correlation with the considerably more complex *in vivo* system.

*Supported in part by Revlon, Inc.

1. Address correspondence to: E. Borenfreund, LARC, The Rockefeller University, 1230 York Avenue, New York, NY 10021.

2. Key words: toxicity screening, ocular irritancy, corneal cultures, cell lines, detergents, alcohols, ketones.

3. Abbreviations: HTD, highest tolerated dose; EGF, epidermal growth factor; FITC, fluorescein conjugated anti-guinea pig IgG; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

Cell Biology and Toxicology, 1:55-65, 1984.

The cytotoxic effects of a series of alcohols, ketones, ethers and surfactants were examined on several mammalian cell cultures of different organs and species of origin. These assays were designed to determine whether rabbit corneal cells are essential for an *in vitro* Draize rabbit eye irritancy test replacement, or whether readily available established cell lines could be used instead. A brief preliminary report of this work has been published. (Borenfreund et al., 1983.)

MATERIALS AND METHODS

Materials

Reagent grade chemicals including alcohols, ethers, esters, ketones, acids and amides were purchased from Sigma Chemical Co., USA, Aldrich, USA and Fisher Scientific, USA. Culture medium and fetal bovine serum were obtained from Gibco Laboratories (Grand Island, NY); epidermal growth factor (EGF) from Collaborative Research, USA; and fluorescein conjugated anti-guinea pig IgG (FITC) from Cappel Laboratories, USA. Benzalkonium chloride, benzethonium chloride and the Tweens were obtained from Sigma; the other surfactants came from sources indicated in the footnotes to Table 2. Corning 96-well tissue culture plates were purchased from Fisher Scientific (Pittsburgh, PA).

Cell Culture

Since long-term, established rabbit corneal epithelial cell lines are not available, it was necessary to isolate and grow such cells for these *in vitro* studies. To this end, corneas from female New Zealand white rabbits were removed under aseptic conditions after euthanasia, rinsed in Hanks solution without Ca^{++} and Mg^{++}, and processed by a modification of a method originally described by Stoker et al. (1958). The tissue was incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10mM Hepes, 1 μg/ml insulin, 0.4 μg/ml fungizone, penicillin and streptomycin and kept at 37°C in a humidified 5% CO_2 incubator. Epithelial cells readily grew out from the explant before fibroblasts started to move out from the stromal layer. Careful detachment of the tissue fragments about 6 days after incubation left primarily epithelial cells in the culture dishes. After an additional 6-8 days growth, areas of densely growing epithelial cells were recovered by trypsinization (0.1% trypsin-0.02% versene) in cloning cylinders and the cells transferred to fresh culture dishes. In some instances, early passage cells were also plated for cloning. Cells from passages 2 through 9 were used for the cytotoxic assays.

Established cell lines selected for additional *in vitro* studies were Balb/c 3T3 murine fibroblasts (American Type Culture Collection), HepG2 human hepatoma cells (provided by Dr. B. Knowles, Wistar Institute, USA), Chinese hamster V79 lung fibroblasts (provided by Dr. E. Huberman, Argonne National Laboratory, USA) and RAW 264.7, a murine macrophage cell line (Ralph and Nakoinz, 1977) (American Type Culture Collection).

Characterization of Intermediate Sized Filaments of Corneal Cells

Rabbit corneal epithelial cells at passage 1, 2, 6 and 9 were grown on coverslips, fixed for 10 min at −20° with methanol, postfixed for 30 sec in acetone and then treated as previously described (Borenfreund et al., 1981). Cells were incubated either with guinea pig antisera prepared against bovine hoof prekeratin or with antisera against murine vimentin. (We are grateful to Dr. W. W. Franke, German Cancer Research Center, Heidelberg, Germany, for a gift of these specific antisera.) Cells were then washed with phosphate buffered saline, stained with fluorescein-isothiocyanate conjugated anti-guinea pig IgG and mounted with Elvanol for viewing under a Nikon photomicroscope equipped with epifluorescent illumination.

Cytotoxicity Assay

Cells were seeded to 96-well flat bottom tissue culture microtest plates in DMEM supplemented with 10% FBS and antibiotics and kept at 37°C in a humidified 5% CO_2 incubator. The number of cells seeded was adjusted to yield a subconfluent cell layer at the time when test agents were added, usually 24 hr later. Actual cell numbers varied with the size of the particular cell type. Fresh medium containing the toxicants over a wide range of concentrations was added to quadruplicate wells for preliminary screening. In subsequent runs, the concentrations of the agents were tested over a very narrow range (usually in 10% or 20% increments) for a more precise determination of the cytotoxic endpoint. In preparing dilutions of the test substances, Hamilton micro-syringes (Hamilton, USA) were used and special care was taken, with the aid of a vortex mixer, to assure solubilization. All test substances were soluble at the concentrations used. Cell cultures were examined under an inverted phase microscope, after 6, 24 and 48 hr of incubation. Identity and dilutions of the agents were coded. Cells were scored for degree of cytoplasmic granularity, vacuolization, contraction, detachment from the substratum, and lysis. A rating system based on minimal morphological changes was developed. In this test the highest tolerated dose (HTD) of a given agent which produced only minimal changes when compared to control cultures was defined as the assay endpoint. Such changes were manifested by cytoplasmic granularity and a small reduction in cell density due to inhibition of cell growth or by a change in cell shape. This stage was further defined by the next higher concentration of agent (a 10% or 20% increase) which resulted in detachment of a few cells and vacuolization or contraction of others. The previous, lower concentration was defined as HTD. Since a 6 hr observation timespan was found to be too short and the 48 hr results were essentially similar to those after 24 hr, a 24 hr standardized incubation with the toxicants was subsequently adhered to. Cells which received only normal medium served as controls.

Colony-forming assay

Balb/c 3T3 cells were seeded at 200 or 250 cells per 60 mm plastic culture dish in DMEM with 10% fetal bovine serum and allowed to attach for 24 hr at 37°C in a 5%

humidified CO_2 incubator. Culture fluids were then replaced by fresh medium containing test agents and incubation was continued for an additional 24 hrs. Appropriate concentration ranges were selected from previous microtest plate assays. Each concentration was run in quadruplicate dishes. Toxicants, consisting of a series of alcohols, were removed after 24 hr of incubation, fresh normal medium was added and cells were allowed to replicate for 7 days to form colonies. At this time, cultures were fixed, stained with 1% crystal violet in 95% ethanol and colonies were counted. Control dishes received normal medium throughout. Results were expressed as percent (%) colony formation of untreated controls.

RESULTS

The cytotoxic effect of 34 test agents, which included alcohols, ethers, esters, ketones, acids, amides and detergents, on five different mammalian cell cultures was studied. The highest doses tolerated (HTD) by human hepatoma cells (HepG2), murine fibroblasts (Balb/c 3T3), Chinese hamster lung fibroblasts (V79) murine macrophages (RAW 264.7) and epithelial rabbit cornea cells were compared and ranked. Although this corneal cell culture could be carried for about 12 passages, it was noted that at about passage 6, some cells tended to lose their typical epithelial morphology. Since one interest was to determine whether corneal epithelial cell cultures respond differently to toxic agents than established cell lines from other sources, it was important to characterize the cell type we were studying. A determination of the type of intermediate-size filaments of these corneal cells at passage 1 and 2 with specific antisera raised against either prekeratin or vimentin type intermediate filaments showed fluorescent stained with anti-prekeratin sera only (Fig. 1). This result is characteristic of epithelial cells, whereas vimentin type intermediate-sized filaments are seen in mesenchymal cells (for a review, see Lazarides, 1980). Cells examined in passage 6-9, however, were all positive against both types of antisera.

The highest tolerated dosages (HTD) determined for each test compound (in mM) were compared and ranked according to their toxic potency. As can be seen in Table 1, there is, in general, very good agreement in the ranking of the toxicants, although there is some variation in the absolute concentration tolerated by the different cell types, with corneal cells showing greatest sensitivity. The rank correlation coefficients calculated from the data are HepG2 vs. 3T3=0.990.; HepG2 vs. V79=0.997; HepG2 vs. rabbit cornea=0.982 and HepG2 vs. RAW 264.7=0.994. The HTD data are based on a minimum of three independent experiments. All cell cultures maintained for 24 hr in this concentration of toxicant containing medium readily retained their ability to reproduce when subsequently incubated with normal medium for long-term studies. Higher concentrations resulted in an increasing extent of cell death, in direct proportion to the concentration of the toxic agent. The last column in Table 1 refers to the published rabbit ocular irritancy data (Grant, 1974; Clayton and Clayton, 1982) which compare well in ranking with our *in vitro* results. In Table 2, HTD data (in μg/ml) of a series of surfactants, screened with three

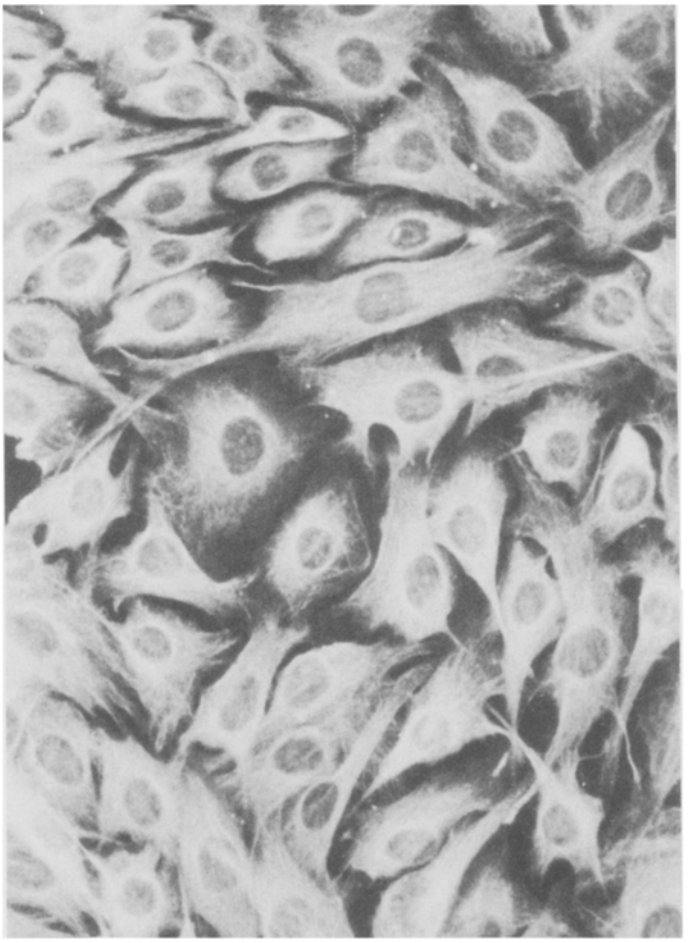

FIGURE 1. Indirect immunofluorescence of intermediate type filaments in rabbit cornea cells. Epithelial rabbit cornea cells propagated *in vitro* were stained at passage 2 with specific antiserum to prekeratin type filaments. Photographed with epifluorescence photomicroscope, magnification × 100.

different mammalian cell lines, are given. A good correlation in the toxicity ranking between the different cell lines can again be demonstrated. Cationic surfactants appear to be more toxic than the anionic or non-ionic compounds tested.

Colony-forming assay

For a preliminary evaluation of this assay, a series of alcohols was tested, using Balb/c 3T3 cells as targets. Table 3 shows a comparison of the results from this test with data obtained from our HTD microtest plate assay. The colony forming assays lend themselves to an examination of the toxicants over a concentration range of less than one log when butanol, pentanol, ethanol and isopropyl alcohol were tested. The concentration which gave an endpoint designated as highest tolerated dose (HTD) in the cytotoxic microtest plate assay was found to inhibit colony formation by about

TABLE 1
Toxicity of Selected Agents to Different Cell Types Highest Tolerated Dose (mM)**

Agent	Mouse 3T3	Hamster CH V79	Rabbit Cornea	Human HepG2	Mouse RAW 264.7	Ocular Irritancy*
Benzalkonium Chloride	0.003	0.003	0.0008	0.003	0.002	Severe
Silver Nitrate	0.008	0.01	0.007	0.01	0.008	Severe
Sodium Dodecyl Sulfate	0.28	0.28	0.21	0.31	0.28	Severe-Moderate
Sodium Hypochlorite	1.06	0.71	0.28	2.13	0.50	Severe
1-Octanol	6.4	5.1	3.2	9.6	5.1	Severe
Heptanol	8.2	16.5	12.4	16.5	16.1	Severe
Trichloroacetic Acid	12.1	12.1	9.1	12.2	12.2	Severe-Moderate
Hexanol	12.6	21.4	17.4	23.7	21.4	Severe
1-Pentanol	12.9	36.8	12.9	50.6	23.9	Severe-Moderate
Allyl Alcohol	14.7	58.8	14.7	82.5	32.2	Severe
Solketal	40.0	32.0	20.0	40.0	32.0	Moderate
Tetrahydrofurfuryl Alcohol	61.8	41.2	20.6	66.9	41.2	Moderate
1-Butanol	65.4	76.3	43.6	76.3	76.3	Moderate
Isopropyl Alcohol	104.0	104.0	78.0	91.0	104.0	Mild-Moderate
Methylethyl ketone	112.0	89.6	67.2	168.0	112.0	Moderate-Mild
Ethylene Glycol Monomethyl Ether	177.4	190.0	88.7	253.4	177.4	Mild-Moderate
N-Methyl Formamide	307.8	273.6	205.2	307.8	273.6	Moderate-Mild
Dimethylsulfoxide	310.2	310.2	280.2	352.5	280.2	Mild
Ethanol	428.2	478.8	342.0	428.2	513.0	Mild-Moderate
Propylene Glycol	435.2	435.2	353.6	435.2	435.2	Mild
Ethyl Acetone	571.2	550.8	499.8	612.0	550.8	Mild
Methanol	864.5	1037.4	617.5	111.5	1037.4	Mild-Moderate

*Based on *in vivo* Draize ocular irritancy tests with pure substances as reported (Clayton, G.D. and Clayton, F.E., 1982), (Grant, M.E., 1974).
**Highest tolerated dose determined as described in Methods.

TABLE 2
Toxicity of Surfactants to Cell Lines Tested Highest Tolerated Dose (μg/ml)*

Agent	Type	Cell Lines 3T3	HepG2	RAW 264.7
Benzalkonium Chloride	Cationic	1.0	1.0	0.66
Benzethonium Chloride	Cationic	5.0	4.0	4.0
Emcol E 607S[1]	Cationic	8.0	20.0	20.0
Emcol E 607L[2]	Cationic	50.0	50.0	35.0
Emery 6748A[3]	Amphoteric	70.0	70.0	80.0
Sodium Lauryl Sulfate	Anionic	80.0	80.0	80.0
Brij 35[4]	Non-Ionic	100.0	100.0	100.0
Bioterge AS40[5]	Aionic	150.0	200.0	150.0
Tween 40	Non-Ionic	150.0	200.0	150.0
Richonol T[6]	Anionic	200.0	250.0	200.0
Tween 60	Non-Ionic	220.0	250.0	220.0
Tween 80	Non-Ionic	280.0	300.0	300.0

*Highest tolerated dose determined as described in Methods.
[1]Emcol E 607S = 1-((2-Hydroxyethyl) carbomoyl) methyl pyridinium chloride stearate (Witco Chemical Corp., USA).
[2]Emcol E 607L = 1-((2-Hydroxyethyl) carbamoyl) methyl pyridinium chloride laurate (Witco Chemical Corp., USA).
[3]Emery 6748A = Cocoamidopropyl dimethyl glycine (Emery Ind. Inc., USA).
[4]Brij 35 = Polyoxyethylene (23) lauryl ether (ICI Americas, USA).
[5]Bioterge AS40 = Sodium C14-16 olefine sulfonate (Stepan Chemical Co., USA).
[6]Richonol T = Triethanolamine lauryl sulfate (Witco Chemical Corp., USA)

20-40% (see Table 3 and Fig. 1). When an ID50 (50% inhibition) of colony formation was used as endpoint, the ranking of the various test agents was found to be the same as that determined by HTD in the cytotoxicity assay. This assay can, thus, be used as an objective confirmation and supplement to the more rapid morphological cytotoxicity test.

DISCUSSION

Mammalian cells propagated *in vitro* have been used for toxicity testing of a broad spectrum of compounds by a variety of techniques; for review see Eckwall, 1980a and Paganuzzi-Stammati et al., 1981. Some compounds require metabolic activation and others are genotoxic, causing mutagenic alterations. Aware of these metabolic and physiological complexities inherent in toxicity testing in live animals, it was decided to initially focus on compounds used primarily in topical applications, which are currently screened by the Draize test.

The use of cultured cells as targets for the testing of acute, general cytotoxicity effecting basic cellular processes, and the validation of the observed toxic dosages with results observed *in vivo* has also been proposed by Eckwall (1980a, 1983). Using HeLa cells primarily, this investigator studied a large series of drugs by a 7-day metabolic inhibition assay (pH changes) supplemented by a 24 hr toxicity study

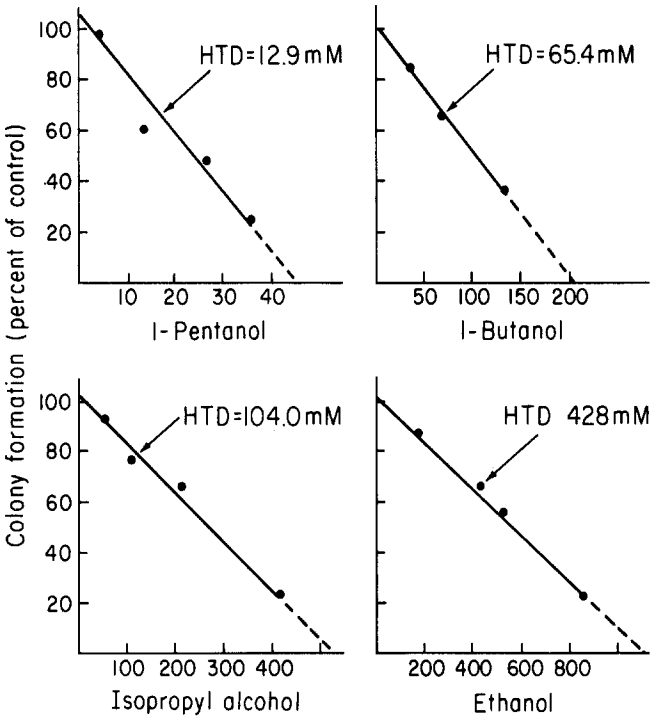

FIGURE 2. Survival of colonies of 3T3 cells. Cells seeded at a density of 250 per dish were incubated for 24 hr with various concentrations of alcohols and then allowed to grow in normal medium for an additional 7 days. Plotted as percent of untreated controls. Each point represents an average of three independent experiments.

referred to as the MIT-24 test. He analyzed the results for relevance to the effects reported in man by calculating toxic ratios. (Eckwall 1980b). These studies indicated that basic cytotoxicity was a useful endpoint in toxicity assays using established cell lines as targets. Our own data are in agreement with these findings. Regardless of the cell type examined in our HTD cytotoxicity assay, the ranking of 22 chemical agents and 12 surfactants was practically the same. Constant comparison with control cultures makes a mild change in the effect of the toxicant easily discernable to even an inexperienced examiner.

Epithelial cells form the outermost layer of the cornea and, therefore, are most readily exposed to injury. We examined the effect of a series of toxicants on epithelial rabbit corneal cells propagated *in vitro* and compared the data with those observed with established cell lines derived from other tissues and species. Studies with specific antisera to prekeratin intermediate filaments indicated that our corneal cultures consisted of epithelial cells. The observation that these cells reacted in later passages also with antisera to vimentin type intermediate filaments is similiar to findings with other epithelial cultures after prolonged passage *in vitro* (Franke et al., 1979) and (Boren-

TABLE 3
Effects of Alcohols on Colony Formation

Agent	Colony Formation* Percent of Control			Alcohol Concentration (in mM) Inhibiting Colony Formation by 50%
1-Pentanol	Exp. 1	76		
(12.9 mM)	Exp. 2	60	Ave 67	23.5
	Exp. 3	65		
1-Butanol	Exp. 1	67		
(65.4 mM)	Exp. 2	57	Ave 58	102.0
	Exp. 3	50		
Isopropyl alcohol	Exp. 1	59		
(104.0 mM)	Exp. 2	76	Ave 70	270.0
	Exp. 3	74		
Ethanol	Exp. 1	66		
(428 mM)	Exp. 2	59	Ave 64	550.0
	Exp. 3	67		

*Survival of 3T3 colonies after 24 hrs incubation with various alcohols. See Methods for experimental details. The concentrations used are the highest tolerated doses (HTD) as determined in the cytotoxicity assay (see Table 1).

freund et al., 1980), and is believed to be due to adaptation to growth and attachment to the substratum. Although our data indicate that the corneal cultures were more sensitive to a given concentration of test agents than the established and transformed cell lines, the general ranking of the various toxicants was the same for all cell types examined (Table 1). Since a need for repeated establishment of fresh corneal cultures would be cumbersome, and would lead to great heterogeneity of starting material when prepared in different laboratories, the suitability of established cell lines for these studies is reassuring. Similar conclusions were arrived at by Litterst and Lichtenstein (1971) in their *in vitro* toxicity studies comparing HeLa cells and short-term cultures of normal human fibroblasts, and by Reinhardt et al. (1982), who used a baby hamster kidney cell line for quantification of cytotoxicity *in vitro*.

Our observation that cationic surfactants are more potent than those with an anionic or non-ionic chemical structure is in agreement with the ranking published by North-Root et al. (1982) who tested the same surfactants in a colony forming assay in which an established corneal fibroblast cell line was used. Similiarly, Muir et al. (1983) using an *in vitro* system in which a 50% block in spontaneous contractions of mouse or rabbit ileum were measured, recently reported greater potency for cationic than for the corresponding anionic surfactants. Draize rabbit eye irritancy scores also indicate that of the surfactants commonly present in shampoos and liquid household detergents, the cationic are most irritating *in vivo*, the anionic intermediate, and the non-ionic least irritating (Grant, 1974).

For a comparison with *in vivo* irritancy, the use of highest tolerated dose as an endpoint for *in vitro* cytotoxicity assays is more useful than cell death. Data obtained from such tests can be used to foretell pending lethal toxicities and allow for a more conservative estimate of acutely toxic higher concentrations. Cellular responses with respect to the highest tolerated dose, i.e. minimal cellular reactions, might be more uniformly expressed by different cell types than cell death, since it does not involve metabolic repair mechanisms which can vary from cell to cell. The short-term microtest plate assay, furthermore, does not require trypsinization of cells and lends itself to rapid scoring.

The colony forming assay, though longer and more laborious than the microtest plate assay (48 hr vs. 9 days), would allow for a more quantitative approach and could be used as a supplementary confirmatory study.

The data presented in this report indicate that mammalian cells propagated *in vitro* can be successfully used for preliminary screening and rangefinding of toxic concentrations of potentially irritant compounds. These assays are economical, easy to execute and can reduce the number of animals required for *in vivo* testing.

ACKNOWLEDGMENTS

We are grateful to Dr. J. Walberg for his help in removing the rabbit corneas and to Dr. C. Shopsis for valuable discussion.

REFERENCES

BORENFREUND, E., SCHMID, E., BENDICH, A. AND FRANKE, W.W. (1980). Constitutive aggregates of intermediate-sized filaments of the vimentin and cytokeratin type in cultured hepatoma cells and their dispersal by butyrate. Exp. Cell Res. **127**:215-235.

BORENFREUND, E., DEHARVEN, E. AND GARRA, L. (1981). Mallory body-like abnormalities in carcinomas induced by cultured transformed rat liver cells. Hepatol. **1**:408-415.

BORENFREUND, E., SHOPSIS, C., BORRERO, O. AND SATHE, S. (1983). *In vitro* alternative irritancy assays: Comparison of cytotoxic and membrane transport effects of alcohols. Ann. N.Y. Acad. Sci. **407**:416-419.

CLAYTON, G.D. AND CLAYTON, F.E. (Eds.) (1982). Patty's Industrial Hygiene and Toxicology, 3rd ed., John Wiley and Sons, New York.

DRAIZE, J.H., WOODARD, G. AND CALVERY, H.O. (1944). Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. J. Pharmacol. Exp. Ther. **82**:377-390.

ECKWALL, B. (1980a). Screening of toxic compounds in tissue culture. Toxicol. **17**:127-142.

ECKWALL, B. (1980b). Toxicity to HeLa cells of 205 drugs as determined by the metabolic inhibition test supplemented by microscopy. Toxicol. **17**:273-295.

ECKWALL, B. (1983). Screening of toxic compounds in mammalian cell cultures. Ann. N.Y. Acad. Sci. **407**:64-77.

FRANKE, W.W., SCHMID, E., OSBORN, M. AND WEBER, K. (1978). Different intermediate-sized filaments distinguished by immunofluorescence microscopy. Proc. Nat. Acad. Sci. U.S.A. **75**:5034-5038.

FRANKE, W.W., SCHMID, E., BREITKREUTZ, D., LEDER, M., BOUKAMP, P., FUSENIG, N.E., OSBORN M. AND WEBER, K. (1979). Simultaneous expression of two different types of intermediate-sized filaments in mouse keratinocytes proliferating *in vitro*. Differentiation **14**:35-50.

FRANKE, W.W., SCHMID, E., WINTER, S., OSBORN, M. AND WEBER, K. (1979). Widespread occurrence of intermediate-sized filaments of the vimentin type in cultured cells from diverse vertebrates. Exp. Cell Res. **123**:25-46.

GRANT, M.E. (1974). Toxicology of the eye. 2nd ed. Charles C. Thomas, Springfield, IL.

LAZARIDES, E. (1980). Intermediate filaments as mechanical integrators of cellular space. Nature **283**:249-256.

LITTERST, C.L. AND LICHTENSTEIN E.P. (1971). Effects and interactions of environmental chemicals on human cells in tissue culture. Arch. Env. Hth. **22**:454-549.

MUIR, C.K., FLOWER, C. AND VAN ABBE, N.J. (1983). A novel approach to the search for *in vitro* alternatives to *in vivo* eye irritancy testing. Toxicol. Lett. **18**:1-5.

NORTH-ROOT, H., YACKOVITCH, F., DEMETRULIAS, J., GRACULA Jr., M. AND HEINZE, J.E. (1982). Evaluation of an *in vitro* cell toxicity test using rabbit corneal cells to predict the eye irritation potential of surfactants. Toxicol. Lett. **14**:207-212.

PAGANUZZI-STAMMATI, A., SILANO, V. AND ZUCCO, F. (1981). Toxicology investigations with cell culture systems. Toxicol. **20**:91-153.

RALPH, R. AND NAKOINZ, I. (1977). Antibody-dependent killing of erythrocyte and tumor targets by macrophage related cell lines. J. Immunol. **119**:950-954.

REINHARDT, C.A., SCHAWALDER, H. AND ZBINDEN, G. (1982). Cell detachment and cloning efficiency as parameters for cytotoxicity. Toxicol. **25**:47-52.

STOCKER, F.W., EIRING, A., GEORGIADE, R. AND GEORGIADE, N. (1958). A tissue culture technique for growing corneal epithelial stromal and endothelial tissue separately. Am. J. Ophth. **46**:294-298.