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# TOXICITY DETERMINED IN VITRO BY MORPHOLOGICAL ALTERATIONS AND NEUTRAL RED ABSORPTION

(Surfactants; highest tolerated dose; in vitro alternative; spectrophotometric analysis)

ELLEN BORENFREUND and JAMES A. PUERNER

*Laboratory Animal Research Center, The Rockefeller University, New York, NY 10021 (U.S.A.)*

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SUMMARY

A method is described which combines the use of a visual morphological cytotoxicity assay with a quantitative neutral red (NR) spectrophotometric test, for the assessment of the effect of toxic agents on 3T3 cells in culture. These sensitive and reproducible assays lend themselves to a screening procedure of potential toxicants which can help reduce the use of animals for toxicity testing.

## INTRODUCTION

We are attempting to develop reliable, reproducible and inexpensive in vitro assays which can be used for screening of potentially toxic substances. At this time, our attentions focus primarily on a possible replacement of the Draize rabbit eye irritancy assay [1] although such research can undoubtedly have broader applications. As previously reported [2, 3] a large series of diverse chemical agents including organic solvents, emulsifiers, diluents, surfactants and more recently shampoos and metals have been tested with the assays developed in our laboratory. The designated endpoint for one of these assays, a morphological cytotoxicity test, was the HTD of toxicant by a given cell population as compared to untreated controls [2]. In this paper we are reporting a supplementary component to the HTD assay in which a neutral red dye absorption procedure has been standardized and adapted to enhance quantitative assessment of this in vitro toxicity screening.

Abbreviations: DMEM, Dulbecco's minimum essential medium; HTD, highest tolerated dose; NR, neutral red (3-amino-*m*-dimethylamino-2-methyl-phenazine hydrochloride)

MATERIALS AND METHODS

The BALB/c 3T3 mouse fibroblast cell line was obtained from the American Type Culture Collection and NR from National Aniline Div., Allied Chemical and Dye Corp., New York). A Dynatech microtiter reader Model MR600 (Dynatech Lab Inc., Alexandria, VA) and a Perkin-Elmer spectrophotometer were used for absorption analysis. The 96-well flat-bottom tissue culture trays were obtained from Corning (Corning, NY). DMEM from GIBCO Laboratories (Grand Island, NY) was supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 $\mu$g/ml streptomycin and 2.5 $\mu$g/ml fungizone (complete medium). Stock cultures of 3T3 cells were trypsinized with 0.05% trypsin – 0.02% versene, and $9 \times 10^3$ cells were routinely seeded to each of the 96 wells. Cells were allowed to attach over a 24-h period at which time 0.2 ml of test agents in fresh medium, covering a broad range of concentrations, were added to the semiconfluent cultures. Each concentration was tested in triplicate wells and cells were incubated for 24 h. Cultures were then scanned with an inverted microscope equipped with phase optics and cells scored morphologically for cytotoxic effects as previous reported [3]. In a second test series a narrow range of toxicant concentrations was added to semiconfluent 3T3 cells in 96-well trays. One 8-well lane on each tray was used for control cells propagated in normal medium. Treated and control cells were compared after 24 h with an inverted microscope and the HTD, a dose causing only minimal morphological changes, was determined [3]. The medium was then removed and each well rinsed with Hanks balanced salt solution before the NR assay was performed.

*Neutral red assay*

An 0.4% aqueous stock solution of the dye was prepared and an aliquot added to complete DMEM medium to a final concentration of 50$\mu$g/ml. It was found advantageous to preincubate NR-containing medium overnight at 37°C to remove fine precipitate and dye crystals which form when NR is mixed with medium. Deposit of such precipitated crystals onto the cell cultures during incubation would interfere with the assay. The NR-medium was centrifuged for 10 min at $1500 \times g$ before use to facilitate removal of crystals. Addition of 0.2 ml of the NR-medium to the wells and incubation for 3 h at 37°C resulted in the uptake of the vital dye into viable cells. The dye-medium was taken off and the cells were washed rapidly with 40% formaldehyde–10% $CaCl_2$, to remove extraneously adhering, unincorporated dye and simultaneously promote adhesion of the cells to the substratum. The formaldehyde should be left only briefly in contact with the cells since longer exposure will result in extraction of the dye. Removal of the formaldehyde and addition of 0.2 ml of a mixture of 1% acetic acid–50% ethanol to each well then results in the extraction of the NR into the solution. After 20 min the trays are placed for a few seconds on a microtiter plate shaker and the absorbances are measured with a Dynatech microplate reader equipped with a 540-nm filter. The readings, usually in

quadruplicate, are averaged and the results expressed as absorbance observed as % of control cultures, or as μg dye extracted, calculated from a standard curve of NR dissolved in 1% acetic acid–50% ethanol. NR-90 is an extrapolation of the dose-response curve to a value representing 90% of the absorbance observed with control cells.

## RESULTS

The first part of the assay consisted of the screening of a broad range of concentrations of the test agents followed by a narrow range. The determination of the toxic endpoint defined as the characteristic HTD based on morphological alterations has previously been described [3]. To standardize the assay, the linearity of absorbance of NR in acetic acid–ethanol over a range of 0.25 μg–15 μg/ml with a peak at 540 nm was determined (see Fig. 1). For comparison the same NR solution was read both on a Perkin-Elmer spectrophotometer and a Dynatech reader (Fig. 1) Both systems give a linear absorption, though the Dynatech reader, due to a shorter light path, is about 50% less sensitive than the spectrophotometer. To examine whether the amount of dye extracted is proportional to the number of cells exposed to the dye, various densities of 3T3 cells were incubated with NR-medium for 3 h and then extracted as described. A 3-h incubation period, which resulted in a 50% increase of dye uptake over a 2-h period, was found to be optimal. The results of the absorbance of the dye extract are shown in Fig. 2. There is good linearity with cell populations ranging from $1 \times 10^3$ to $4 \times 10^4$ cells per well, in an assay in which 8 wells per cell concentration were examined. Standard deviation was $< 5\%$, except with a concentration of $4 \times 10^4$ cells when it was 7.5%.

We tested the applicability of this procedure under experimental conditions in-

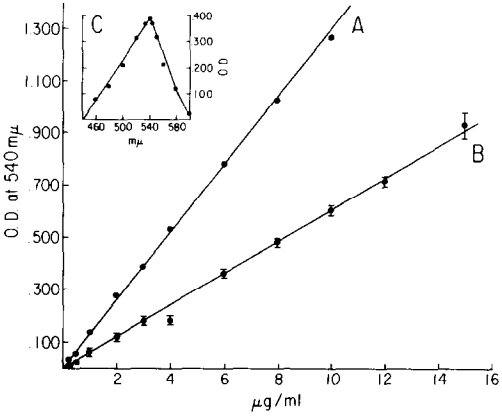


Fig. 1. Absorbance, at 540 nm, of neutral red in 1% acetic acid–50% ethanol. (A) Perkin-Elmer spectrophotometer; (B) Dynatech reader (MR600); (C) inset: absorption spectrum of 3 μg/ml neutral red in 1% acetic acid–50% ethanol, read on a Perkin-Elmer spectrophotometer.

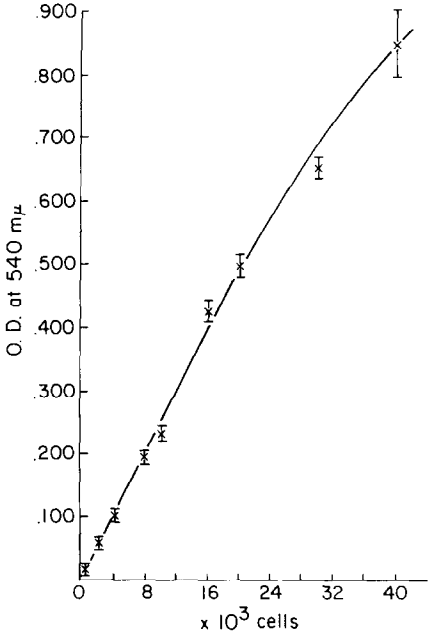


Fig. 2. Cells were seeded to 96-well microtiter plates at concentrations ranging from $1 \times 10^3$ to $4 \times 10^4$ cells/well, 8 wells/concentration. 0.2 ml of 50 μg/ml neutral red-containing medium was added to each well 4 h later. Cells were incubated for 3 h, then washed and extracted as described in METHODS. Bars indicate mean ± S.D.

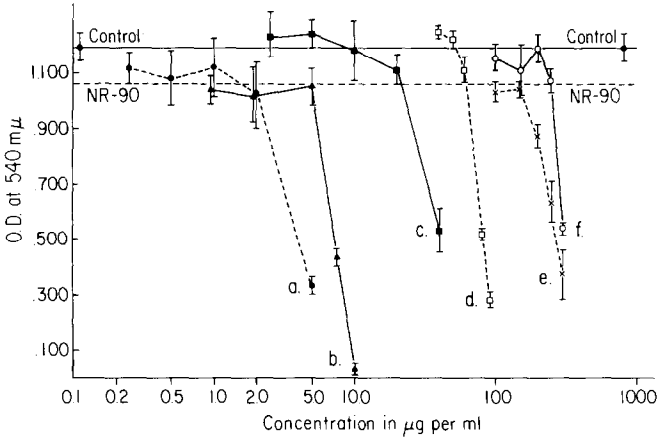


Fig. 3. Neutral red absorption at 540 nm. 3T3 cells were incubated for 24 h with surfactants; medium was removed and fresh medium containing 50 μg/ml neutral red was added and incubation continued for 3 h, followed by extraction of the incorporated dye as described. (a) ● – ● – ●, benzalkonium chloride; (b) ▲ – ▲ – ▲, benzethonium chloride; (c) ■ – ■ – ■, Triton X-155; (d) □ – □ – □, sodium lauryl sulfate; (e) ● – ● – ●, Tween-60; (f) □ – □ – □, triethanolamine lauryl sulfate. Absorbance was plotted against concentration of toxicant. Bars indicate mean ± S.D. of four experimental points.

TABLE I

COMPARISON OF HTD AND NR-90 AS CYTOTOXIC ENDPOINTS (in μg/ml)

| Agent | Type | HTD[a] | NR-90[b] |
|---|---|---|---|
| Benzalkonium chloride | cationic | 1.0 | 1.5 |
| Benzethonium chloride | cationic | 5.0 | 5.0 |
| Triton X-155 | non-ionic | 10.0 | 21.0 |
| Sodium lauryl sulfate | anionic | 70.0 | 62.0 |
| Triethanolamine sulfate | anionic | 200.0 | 240.0 |
| Tween-60 | non-ionic | 200.0 | 160.0 |

[a]Concentration of toxicant eliciting only minimal morphological alterations when compared to control cultures.

[b]Concentration of toxicant extrapolated from the dose–response curve giving 90% of the neutral red absorbance compared to the control culture.

volving the screening of a series of surfactants which were scored with the HTD cytotoxicity and the NR assays. As can be seen in Fig. 3, there is excellent agreement between the visually designated HTD and the measured decrease in dye absorption at 540 nm compared to controls. As determined, the presence of cells in the wells does not interfere with the absorbances. Thus an HTD-NR-90 based on two assays, where HTD represents the dose observed to elicit only minimal morphological changes and NR-90 which stands for 90% of the NR absorbance observed with control cultures, constitutes a good index for standardization of experimental toxic agents (Table I).

The observed ranking was benzalkonium chloride (cationic) > benzethonium chloride (cationic) > Triton X-155 (non-ionic) > sodium lauryl sulfate (anionic) > Tween-60 (non-ionic) > triethanolamine lauryl sulfate (anionic). As has been reported previously for the HTD assay [3] the NR test also indicates that cationic surfactants are more toxic than those belonging to the anionic or non-ionic groups. This is in agreement with in vivo observations [4].

## DISCUSSION

The need for acceptable alternatives to the use of whole animal testing for toxicity evaluation is generally recognized, as are the inherent problems posed by such in vitro approaches. It is most unlikely that any single test will be able to mimic the complexities of the in vivo system. A series of complementary assays will therefore have to be developed.

We set out initially to examine agents which naturally are encountered by topical contact and are currently screened by the Draize rabbit eye irritancy test [1]. When we tested and ranked a large series of surfactants and other agents with our morphological HTD cytotoxicity assay, we found good agreement with the Draize in vivo ranking reported in the literature [3] as well as with the uridine uptake inhibition assay also developed in our laboratory [5]. To add a quantitative parameter to our HTD assay, we now report a neutral red extraction and spectrophotometric ab-

sorption procedure, which is based on the uptake of a vital dye by a given population of viable cells. The standardized test can be used to score cell injury as well as to determine the number of remaining cells after toxic insult. The use of neutral red as a cytotoxic indicator in an assay modified from a viral cytopathogenicity test [6] has recently been reported [7]. NR is a weakly cationic dye which is believed to enter the cell by non-ionic diffusion through the cell membrane. The dye then accumulates in the lysosomes of living cells [8, 9]. Whether this step is due to an active or passive mechanism is not clear. We were able to demonstrate linearity of NR uptake by cultures ranging from $1 \times 10^3$ to $4 \times 10^4$ cells per well. We measured the absorption spectrum of supernatants containing NR extracted with a mixture of 1% acetic acid–50% ethanol from cells still present in the 96-well culture trays, in which they had been incubated with toxic substances. When cells were exposed to test agents such as a series of surfactants, we could demonstrate good agreement between the morphological HTD cytotoxicity test and the quantifiable NR assay. Both assays are fast, inexpensive, sensitive and reproducible and the NR-test lends itself to automated screening with a microplate reader. When preceded by the HTD cytotoxicity assay for a general broad range-finding of toxic endpoints, the combined approach of a morphological and NR-absorption assay over a second narrower range of concentrations of toxicants, constitutes a reliable and efficient in vitro screening procedure. We have, in preliminary experiments, applied both tests to agents other than surfactants, such as metals and solvents (to be published).

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REFERENCES

1 J.H. Draize, G. Woodard and H.O. Calvery. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes, J. Pharmacol. Exp. Ther., 82 (1944) 377–390.
2 E. Borenfreund, C. Shopsis, O. Borrero and S. Sathe, In vitro alternative irritancy assay: Comparison of cytotoxic and membrane transport effects of alcohols, Ann. N.Y. Acad. Sci., 407 (1983) 416–419.
3 E. Borenfreund and O. Borrero, In vitro cytotoxicity assays: potential alternatives to the Draize ocular irritancy test, Cell Biol. Toxicol., 1 (1984) 55–65.
4 M.E. Grant, Toxicology of the Eye, 2nd ed., Thomas, Springfield, IL, 1974.
5 C. Shopsis and S. Sathe, Uridine uptake inhibition as a cytotoxicity test: Correlations with the Draize test, Toxicology, 29 (1983) 195–206.
6 N.B. Finter, Dye uptake methods for measuring viral cytopathogenicity and their application to interferon assays, J. Gen. Virol., 5 (1969) 419–427.
7 A.P. Breau, L. Field and W.M. Mitchell, Thiono compounds, 4. In vitro mutagenic and antineoplastic activity of TEPA and thio-TEPA, Cell Biol. Toxicol., 1 (1984) 21–30.
8 A.C. Allison and M.R. Young, Vital staining in fluorescence microscopy of lysosomes, in J.T. Dingle and H.B. Fell (Eds.), vol. 2, Wiley, New York, 1969, pp. 600–626.
9 Z. Nemes, R. Dietz, J.B. Luth, S. Gomba, F. Hackenthal and F. Gross, The pharmacological relevance of vital staining with neutral red, Experientia, 35 (1979) 1475–1476.