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# Development of a method for assessing micronucleus induction in a 3D human skin model (EpiDerm$^{TM}$)

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**Abstract**

To meet the requirements of the EU 7th Amendment to the Cosmetics Directive, manufacturers of cosmetics products will need to ascertain the safety of ingredients using non-animal methods. Starting in 2009, *in vivo* genotoxicity tests for cosmetics ingredients will not be allowed. Skin is a target area of interest for many cosmetic products because of its relatively high exposure. Therefore, it would be beneficial to have a non-animal, skin-based genotoxicity assay, especially one that utilized human skin *in vitro*. In this paper, we describe the development of a reproducible micronucleus assay that uses EpiDerm$^{TM}$ engineered human skin constructs (MatTek Corp., Ashland, MA). We describe methods for isolating single cells from the 3D skin model and for processing the cells for microscopic analysis of micronuclei (MN). In addition, since little was known about the kinetics of the dividing keratinocytes in the EpiDerm$^{TM}$ model, we evaluated whether cytochalasin B (Cyt-B) could be used to distinguish the population of dividing cells allowing the development of a micronucleus assay in binucleated cells. We found that the frequency of binucleated cells increased both with time and with increasing concentration of Cyt-B. After a 48-h exposure, 30–50% binucleated cells were reproducibly obtained. Finally, we evaluated micronucleus induction using the model genotoxicants mitomycin C (MMC) and vinblastine sulfate (VB). The background frequency of MN is very low and reproducible in this model, and statistically significant increases in the frequency of micronucleated cells were induced by both MMC and VB. These are initial steps in developing a routine “*in vivo*-like” assay for chromosomal damage in human tissue. It is hoped that other investigators utilize these methods to further the understanding of this potentially valuable new non-animal method.
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*Keywords:* Non-animal skin-based micronucleus assay; *In vitro*; Genotoxicity; 3D human skin model

## 1. Introduction

The *in vitro* micronucleus assay is an established method for detecting potentially clastogenic and aneugenic chemicals [1]. Its acceptance has been furthered by the development of the cytokinesis-block methodology [2], which allows the identification of cells that have undergone one nuclear division, thereby allowing an accurate assessment of the appropriate cell population for quantifying micronuclei (MN) along with an easy assessment of alterations in cell division kinetics due to cytotoxicity. Results from an international validation study have recently been completed [3], a draft OECD guideline for the *in vitro* micronucleus assay has been prepared [4] and the *in vitro* micronucleus

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assay is increasingly being used in the risk-assessment process.

Because current *in vitro* genotoxicity assays induce a high level of false-positive results (75–95% of rodent non-carcinogens are positive in one or more *in vitro* genotoxicity tests) [5] chemicals that are genotoxic *in vitro* are often evaluated further using *in vivo* genotoxicity assays to determine whether the chemical is bioavailable and active in the whole organism. One of the most developed *in vivo* assays is the analysis of MN in bone marrow (see, e.g. [6]). However, the requirements of new regulations in Europe such as the 7th Amendment to the Cosmetics Directive, will make it very difficult, and in some cases impossible, to use animal models in safety assessments in the near future. In addition, large toxicity testing programs, like the REACH program in Europe, will likely not be successful unless *in vitro* methods are utilized for many endpoints previously measured *in vivo*. The inability to use *in vivo* methods to address the relevance of positive results from *in vitro* genotoxicity assays is particularly problematic in light of the high rate of false-positives for current *in vitro* genotoxicity assays [7]. Clearly new methods and approaches are needed.

There has been renewed interest in utilizing skin as a target organ for assessing genotoxicity [8–14] since it is a relevant tissue for many different environmental exposures. Early methods for assessing genotoxicity in rodent skin utilized *in vivo* exposures followed by *in vitro* cell culture to identify MN, e.g. [13,14], while current methods utilize only *in vivo* exposure [8–12], thereby avoiding the complications involved in *in vitro* cell culture. Both of these approaches utilize rat or mouse skin as a surrogate for human skin, and results from such models may not completely reflect what would occur in humans after dermal exposure.

Further, *in vivo* methods such as those described will not be acceptable for use with cosmetics in the future. A task force initiated by the European Commission and led by the European Center for the Validation of Alternative Methods (ECVAM) on which one of us (MJA) participated, recommended a new approach for the safety assessment of dermally applied cosmetics with regards to genotoxicity. This approach would include an *in vitro* assay using skin models [15]. To this end, we have investigated whether human three-dimensional skin constructs can be used as a target system for micronucleus studies. Theoretically such models may better reflect the complexities typical of *in vivo* exposures, e.g. absorption, tissue specificity, metabolism, etc., and at the same time reflect human-specific responses in these parameters. In this paper, we describe the development of methods for isolating and processing cells from the 3D EpiDerm™ human skin model (MatTek Corp., Ashland, MA) and provide initial results with the model genotoxicants, mitomycin C (MMC) and vinblastine sulfate (VB). Preliminary data have been presented previously [16,17].

## 2. Materials and methods

### 2.1. Test chemicals and reagents

MMC, VB, cytochalasin B (Cyt-B), DMSO, methanol, acetic acid, KCl (0.075 M; Cat# P9327), and trypan blue were obtained from Sigma (St. Louis, MO). Acetone was obtained from Aldrich Chemical Co., and ethanol (absolute) was obtained from Pharmco (Brookfield, CT). Acridine orange (AO) solution (10 mg/ml) was obtained from Sigma (Cat# A8097) and used at a final concentration of 40 μg/ml in calcium/magnesium-free Dulbecco's phosphate-buffered saline (DPBS) (MatTek, Ashland, MA). EDTA (1 g/l) was obtained from Quality Biological (Gaithersburg, MD) (Cat# 118-090-060). Trypsin (0.25%)–EDTA (0.02%) was obtained from JRH Biosciences (Lenexa, Kansas) (Cat# 59228-100M).

### 2.2. Cell culture media

New Maintenance Medium (Cat# EPI-100-NMM; NMM) was obtained from MatTek Corporation (Ashland, MA). Its exact composition is proprietary to the manufacturer, but the formulation is based on Dulbecco's Modified Eagle's Medium (DMEM) and contains Keratinocyte Growth Factor (personal communication; MatTek Corporation). DMEM (Quality Biological, Gaithersburg, MD) or equivalent containing 10% FBS and 1% L-glutamine was used as a trypsin neutralization solution.

### 2.3. Tissue constructs

EpiDerm™ tissue (EPI-200) was obtained from MatTek Corporation. The EpiDerm™ construct is a multilayered, differentiated tissue consisting of basal, spinous, granular and cornified layers (Fig. 1) resembling the normal human epidermis. The tissue is constructed from normal epidermal keratinocytes (foreskin-derived), which are cultured on chemically modified, collagen-coated, 9-mm (i.d.) cell culture inserts (e.g. Millicell CM or Nunc polycarbonate cell-culture inserts). Differentiation is induced by air-lifting the growing cultures so that the cell inserts sit just on the surface of the medium and the apical surface of the tissue is exposed to the atmosphere (Fig. 2). The tissues are shipped cold overnight in 24-well plates.

Cultures were received from MatTek on Tuesdays in 24-well trays packed in insulated shipping containers. Within the containers, the tissue-containing tray was on top of shipping agar and a wet gauze pad covered the top of the tissues. On arrival the gauze pad was removed, the tissue inserts lifted from the agar, and each tissue observed for any gross morphologi-

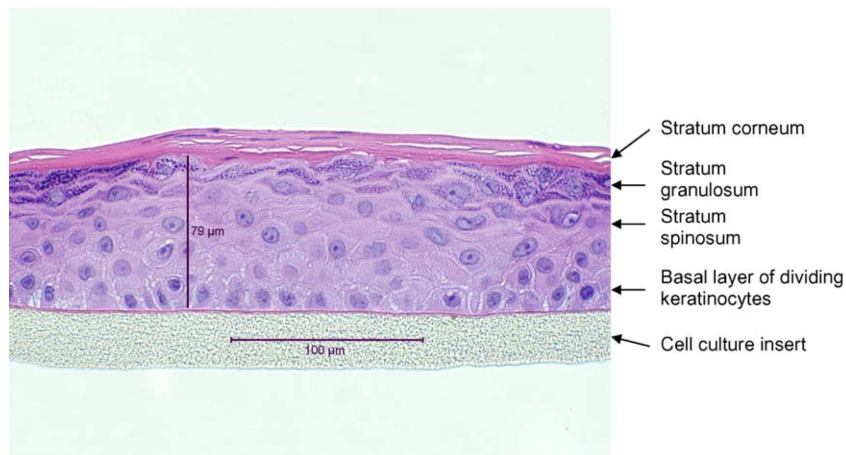


Fig. 1. Cross-section of a representative EpiDerm™ tissue on arrival at the laboratory. H&E. Photo courtesy of J. Harbell, IIVS.

cal defects. The inserts containing the tissue were then placed into six-well tissue culture plates containing one ml of fresh, warmed medium (generally NMM). The tissues were incubated for approximately 24 h (unless otherwise noted) at $5 \pm 1\%$ $CO_2$ and $37 \pm 1$ °C. To maintain the most favorable conditions, cultures were refed with fresh media approximately every 24 h.

All experiments described here were conducted with EpiDerm™ tissue grown with cells from a single donor, a newborn male. To do this, the manufacturer expanded the original monolayer keratinocyte culture and cryopreserved the resulting cells in multiple stock vials. Individual vials from this freeze lot were then used to establish new three-dimensional cultures before each use. In our laboratory tissues were maintained in medium NMM that contains Keratinocyte Growth Factor (technical communication from MatTek Corporation). This medium maintained acceptable differentiated morphology (as evidenced by only minor thickening of the stratum corneum [<2-fold increase], and the presence of easily recognizable granular, spinous and basal layers) of the EpiDerm™ tissue for at least 5 days.

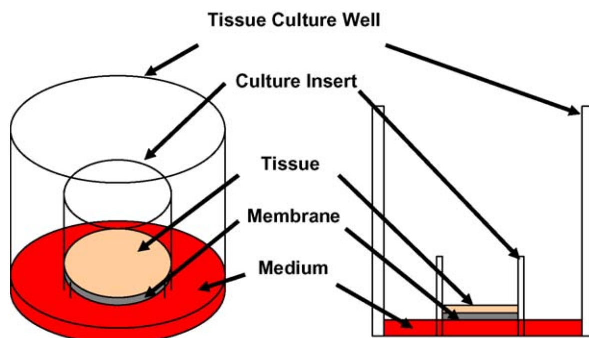


Fig. 2. A diagrammatic representation of the EpiDerm™ tissue as used in these experiments. The air-exposed skin tissue is fed by medium diffusing through the support membrane to the basal cells. Diagram courtesy of MatTek Corporation.

### 2.4. Treatment conditions

Tissues were treated with test chemical either topically or via the culture medium. Dosing via the medium was accomplished by diluting the test material to its final concentration in the culture media immediately before refeeding. For topical dosing, the test material was diluted to the desired concentration in the appropriate solvent (typically acetone or ethanol) and 10 µl of this dosing solution was applied with a micropipette directly to the surface of the EpiDerm™ tissue. The dosing solution was then very gently spread across as much of the surface of the tissue as possible with gentle tilting of the plate. Cyt-B was present in the culture media from the time of first chemical dosing until the tissue was harvested.

As shown in Fig. 3, there were generally two exposures of the tissues to the test chemical; the first at $T = 0$ h (24 h after receipt) and the second 24 h later. Cells were always harvested from the 3D constructs ∼24 h after the last exposure to the test chemical. This was generally 48 h after the initial exposure, unless otherwise described.

### 2.5. Cell harvesting and slide preparation

Cells were isolated from the EpiDerm™ tissue and further processed using the following methods (carried out at room temperature unless otherwise noted):

(1) Tissue-containing inserts were removed from the treatment plates, their bottoms blotted to remove excess medium and placed into individual wells (each containing 5 ml DPBS) of a 12-well plate.
(2) After 15 min, the insert was transferred (after blotting) to another well (containing ∼5 ml EDTA [0.1%], at room temperature) in a 12-well plate and allowed to sit for 15 min.
(3) The insert was once more blotted and transferred to another well of a 12-well plate, which contained ∼1 ml

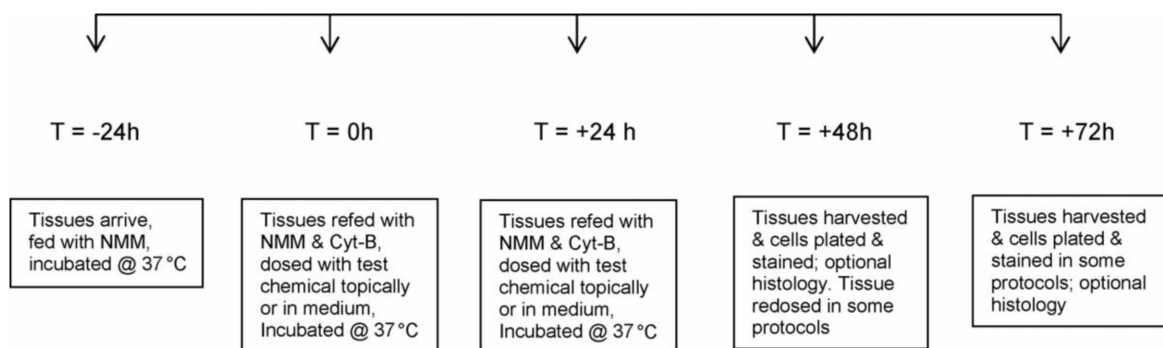


Fig. 3. Typical treatment protocol. EpiDerm[TM] tissues were allowed to recover in NMM for 24 h after receipt before exposure to the test chemical.

warm (~37 °C) trypsin-EDTA solution. An additional ~0.5 ml of the same solution was added to the top surface of the tissue, and the tissues were allowed to sit for ~15 min at room temperature.

(4) Each tissue was carefully separated from the supporting membrane by gently lifting the edge of the tissue with fine forceps, transferred to a new well in a 12-well plate, and exposed to fresh ~1 ml of trypsin-EDTA for ~5 min.
(5) To capture cells still adherent to the membrane of the insert, the insert (containing any remaining trypsin-EDTA) was placed in the same well as its matching tissue.
(6) After ~5 min the insert was thoroughly rinsed (four to six times) to collect the trypsinized cells. These washes remained with the matching detached tissue. The tissue was then agitated to release any remaining attached cells, and the resulting cell clumps and tissue additionally disrupted by repeatedly drawing into a pipette and gently expelling the solution. The single cell suspension (~1.5 ml) was transferred to a 15-ml conical tube containing 8.5 ml of warm DMEM with 10% FBS to neutralize the trypsin.
(7) A 1-ml sample of cells was diluted with 1 ml trypan blue solution (or less, depending on sample volume) and counted using a hemocytometer.
(8) The remaining cell suspension was centrifuged at $100 \times g$ for 5 min.
(9) After the centrifugation, the supernatant was carefully removed, the cell pellet loosened with gentle agitation, and 1 ml of warm (~37 °C) KCl solution was slowly added in several small increments.
(10) After ~3 min, 3 ml of cold methanol/acetic acid (3:1) was added to fix the cells, and the solution gently mixed.
(11) The cell suspension was centrifuged at $100 \times g$ for 5 min.
(12) The supernatant was removed, the pellet loosened, and 4 ml of cold methanol/acetic acid (3:1) was added.
(13) The cell suspension was stored at 2–8 °C for at least 72 h and then centrifuged at $100 \times g$ for 5 min.
(14) All but a small portion of the supernatant was then removed, the cell pellet loosened, and a drop of the cell suspension was gently pipetted onto a clean, dry or FBS-coated microscope slide. One slide was prepared from each tissue.
(15) Once completely dry, slides were immersed in AO solution for 3 min.
(16) Slides were then rinsed three times with DPBS, allowed to dry, and scored using a fluorescence microscope.

### 2.6. *MN scoring*

In general, 500 total cells were scored per tissue to determine the percentage of cells with two, three or more nuclei. The percentage of binucleated cells at each concentration of test chemical was compared with the solvent or untreated control as a measure of cytotoxicity. The highest concentrations of a test chemical reported here for analysis of MN produced no more than a 70% decrease in binucleated cells compared with controls. All experiments reported here are also in agreement with the draft OECD guidelines for the *in vitro* micronucleus assay [4], which suggests a top dose, which produces at least 60% toxicity. One thousand binucleated cells (if possible) per tissue were scored to determine the frequency of micronucleated cells in the binucleated cell population. The criteria of Fenech [18] were used to identify MN. Fisher's exact test was used to determine the statistical significance of differences between solvent control and chemical-treated cultures.

### 2.7. *Histology*

Tissues to be processed for histology were fixed for at least 24 h in 10% buffered formalin by immersing the entire tissue insert in the fluid. The insert (tissue plus supporting membrane) was then cut from the cup and re-immersed in formalin. In cases where histology and MN induction were compared in the same tissue, the membrane and tissue were bisected before harvest, one half of the tissue was fixed in formalin and processed for histology, and the other half was subjected to a cell-isolation procedure similar to that previously described. Formalin-fixed tissues were sent to a commercial histology lab (generally HistoTech, Springfield, OH) for embedding, sec-

tioning and staining (H&E). All slides were evaluated at the Institute for In Vitro Sciences Inc.

## 3. Results

### 3.1. Cell isolation

The enzymatic dissociation procedure described in Section 2.5 was chosen because it provided reasonable processing speed (one set of four tissues could be processed in ∼60 min), while supplying the high level of cell integrity required for effective microscopic determination of MN, The average cell yield (for 87 preparations from untreated or solvent-treated control cultures) was $2.3 \times 10^5$ cells per EpiDerm™ tissue with an average viability (trypan blue exclusion) of 93%.

Following enzymatic digestion of the EpiDerm™ tissue and the collection of the resulting cell suspension, a small "pad" of tissue remained. To assess both the quality of the digestion method and to determine whether this pad contained any of the cell types that might have MN, we isolated representative pads and had them sectioned and stained (H&E). Fig. 4 shows that a typical "pad" consisted only of stratum corneum with one or two layers of stratum granulosum, although a few cells from the stratum spinosum were sometimes observed. This indicates that the cells of interest for micronucleus analysis – the basal cells and cells immediately superficial to them – were collected by our procedure, and there was no significant contamination with the more highly differentiated, non-dividing cells of the tissue.

### 3.2. Determination of optimal Cyt-B concentration and exposure time

EpiDerm™ tissues were exposed to various concentrations of Cyt-B in the medium to determine: (a) whether the rate of cell division in the tissue was sufficiently high to give a reasonable number of binucleated cells for the analysis of MN, and (b) the optimal concentration of Cyt-B for the production of binucleated cells.

Specifically we examined the induction of binucleated cells at time periods up to 72 h in medium NMM with two concentrations of Cyt-B (2 and 3 μg/ml). We report the results of multiple experiments to demonstrate the reproducibility of the induction. As shown in Fig. 5, a concentration- and time-dependent increase in the percentage of binucleated cells was observed. At 24 h, 2 μg/ml Cyt-B induced ∼10% binucleated cells, which increased to ∼20% at 48 h and 35–50% at 72 h. At 24 h, 3 μg/ml Cyt-B induced ∼30% binucleated cells, which increased to 40–55% binucleated cells at both 48 and 72 h. As a result of this and other data, we chose 3 μg/ml as the standard concentration of Cyt-B to be used in subsequent experiments. From the 48-h data obtained in five experiments conducted with 3 μg/ml Cyt-B (see Fig. 5), it is clear that the induction of binucleated cells was quite reproducible in this system. Although the binucleation frequencies induced were lower than the frequencies (∼70–80%) obtained in some monolayer cell-culture models, this was a higher level than expected, and it is adequate for the analysis of MN.

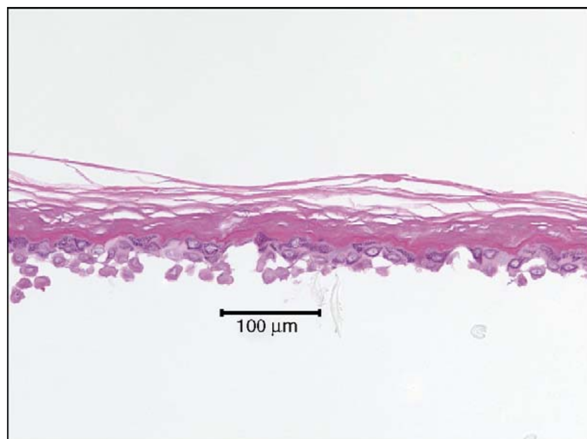


Fig. 4. A cross-section through the "pad" of material that always remained after cells were isolated from the EpiDerm™ tissue. Generally, only *stratum corneum* and *stratum granulosum* were observed in this material. H&E stain.

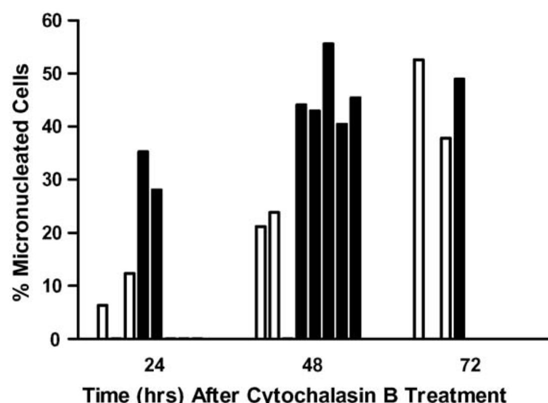


Fig. 5. Effect of Cyt-B. EpiDerm™ tissues were re-fed every 24 h with medium containing 2 μg/ml Cyt-B (open bars) or 3 μg/ml Cyt-B (solid black bars). Results from nine different experiments are shown. Each bar represents the results from a single tissue.

### 3.3. Tissue morphology over time and effect of Cyt-B

Since EpiDerm™ is a living, continually differentiating tissue, we investigated how well tissue morphology was maintained over the 3–4-day period that would be required for an experiment. In addition, because Cyt-B inhibits cytokinesis (but not mitosis), and no new cells are generated to replace those in the EpiDerm™ tissue that are differentiating, we investigated the effect of Cyt-B on tissue morphology. Tissues were fed with medium NMM immediately after receipt. Twenty-four hours later half the tissues were re-fed with NMM and the other half re-fed with NMM containing 3 μg/ml Cyt-B. Each set of tissues was then re-fed 24 h later either without or with Cyt-B, as appropriate. Individual tissues were harvested every 24 h and processed for histology.

Fig. 6 shows that at receipt (Fig. 6a; $T = -24$ h from Fig. 3) the EpiDerm™ tissues had 6–7 readily discernable cell layers. Twenty-four hours later (Fig. 6b; $T = 0$ h from Fig. 3) when chemical treatment would normally begin, the tissues grown in NMM were still similar in appearance to those at receipt with only a slightly heavier stratum corneum. At this time half the cultures were refed with media containing Cyt-B and the other half with media without Cyt-B. After an additional 48 h (Fig. 6c; $T = 48$ h from Fig. 3), the tissues without Cyt-B had 5–6 cell layers and a noticeably thickened stratum corneum, suggesting that differentiation was proceeding slightly faster than new cells could be produced. As expected, tissues maintained an additional 48 h (Fig. 6d; $T = 48$ h from Fig. 3) in media containing Cyt-B showed an even greater reduction in the number of cell layers (four to five layers) since essentially no new cells were produced from cell division in the basal layer while the upper layers continued to differentiate. The effect of the Cyt-B exposure in this later group was evidenced by the presence of numerous binucleated cells in the basal layer.

Since these studies showed that the EpiDerm™ tissue maintained reasonable morphology after 48 h in Cyt-B, and our previous studies had shown a good percentage of binucleated cells (40–55%) after 48 h, we chose 48 h as the standard harvest time for the experiments described here.

### 3.4. Effect of solvents

We evaluated some common solvents including ethanol, acetone, acetone:olive oil (4:1), DMSO and saline to determine if they would affect either cell cycle (as evidenced by changes in the percentage of binucleated cells) or the morphology of the tissue after direct application. Table 1 shows that two applications of 10 μl or 20 μl ethanol, acetone or a 4:1 mixture of acetone and olive oil appear not to decrease the percentage of binucleated cells. In addition, the morphology of

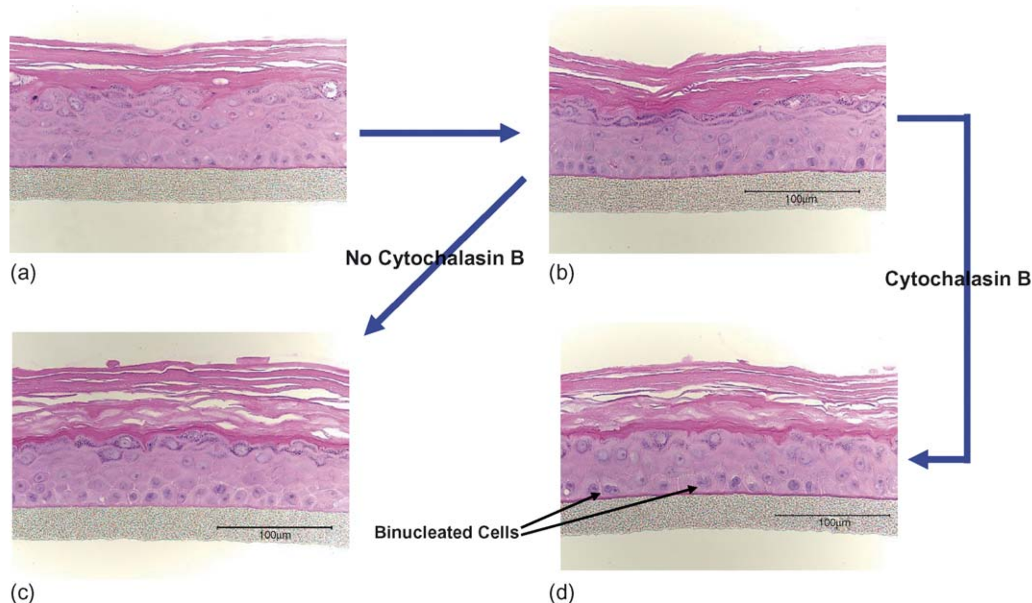


Fig. 6. Changes in EpiDerm™ morphology during a typical 48-h total testing period in the presence and absence of Cyt-B: (a) tissue at arrival ($T = -24$ h); (b) tissue 24 h after receipt ($T = 0$ h) just before addition of Cyt-B and test material; (c) tissue after 48 additional hours ($T = 48$ h) in medium without Cyt-B; (d) tissue after 48 additional hours ($T = 48$ h) in medium containing 3 μg/ml Cyt-B. Note the presence of binucleated cells in the basal layer of condition (d).

Table 1
EpiDerm™ cultures received two topical applications (one at time $T=0$, when 3 μg/ml Cyt-B was added, and a second application 24 h later; see Fig. 3) of the indicated amount of solvent

| Solvents tested | % Binucleated cells |
|---|---|
| Untreated | 41.0 |
| 10 μl ethanol[a] | 40.4 |
| 20 μl ethanol[a] | 38.5 |
| 10 μl acetone[a] | 41.2 |
| 20 μl acetone[a] | 41.3 |
| 10 μl DMSO[a] | 21.9 |
| 10 μl acetone:olive oil (4:1)[a] | 50.9 |
| 20 μl acetone:olive oil (4:1)[a] | 44.8 |
| 10 μl saline[b] | 43.6 |
| 100 μl saline[b,c] | 17.8 |

Tissues were re-fed with fresh 3-μg/ml Cyt-B-containing medium immediately before the second application of solvent. Cells were harvested 24 h after the final solvent application (48 h after initiation of treatment) and the percentage of binucleated cells was determined after scoring at least 300 cells. One tissue was sampled for each condition. Results are the average of two separate lots of EpiDerm™.

[a] Post-treatment histology was equivalent to that of the untreated tissue.

[b] One lot of EpiDerm™ was tested for these conditions.

[c] Post-treatment histology indicated extreme differentiation of tissue with only one or two viable cell layers. This response to high volumes of saline was confirmed in subsequent studies.

the H&E-stained tissue after treatment with these solvents was equivalent to that of the untreated cultures. However, when applied in 10 μl applications DMSO appeared to depress the cell cycle as evidenced by an ∼45% decrease in the percentage of binucleated cells compared with the untreated control. Interestingly, the histology of the DMSO-treated cultures had not noticeably changed. Saline, when used at a volume of 100 μl (two 100-μl applications), induced a strong inhibition in the cell cycle as evidenced by the reduction in the percentage of binucleated cells from 41% to ∼18%. In addition, saline (100 μl) had a negative effect on tissue morphology, resulting in a tissue with only one or two viable cell layers after 48 h. In contrast, a smaller volume (10 μl) of saline showed little effect on either the percentage binucleation or histology. Based on these results, ethanol, acetone and acetone:olive oil (all limited to 10-μl application volumes) have been used as solvents in our studies.

More recent studies have indicated that "blistering" – separation of EpiDerm™ tissue from the underlying support membrane – can sometimes occur after 10-μl applications of solvent (more frequently with ethanol than with acetone or acetone:olive oil mixtures). The "blistering" has also been seen on occasion in cultures not receiving solvent treatment. "Blistering" generally involves less than 10% of the tissue but can involve up to 50%. Although it is sometimes accompanied by cell death in the overlying tissue (Fig. 7), it generally does not noticeably affect cell yields or percent micronucleated cells. We are currently working with the tissue manufacturer to better understand the causes of this phenomenon so that it can be reduced or prevented in the future.

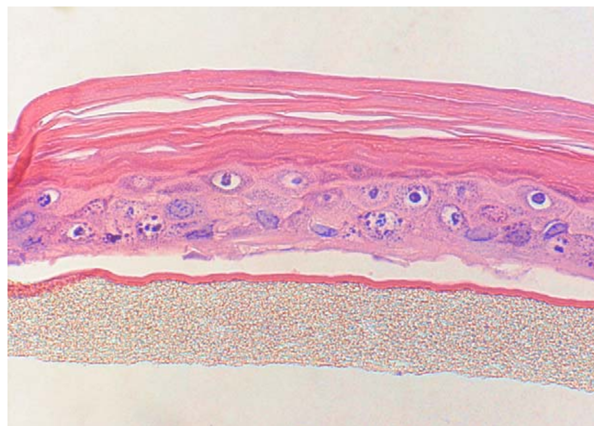

Fig. 7. Cross-section through a representative "blister" (tissue separation from the underlying insert) which sometimes occurred in EpiDerm™. In this example, the cells above the separation appear necrotic, although in many cases the separated cell layer contained what appeared to be normal cells. H&E stain.

### 3.5. Background MN frequency

A very important aspect in developing a new model is determining the spontaneous background response. A low, reproducible MN response in controls is optimal for a robust assay. Fig. 8 demonstrates that the background level of micronucleated cells in this model is indeed low

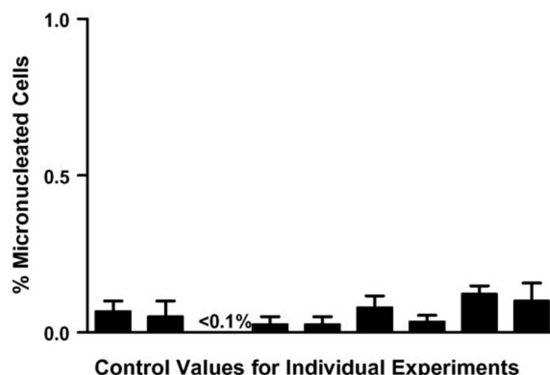


Fig. 8. Frequency of MN in untreated or solvent-control EpiDerm™ cultures. Results for 35 individual tissues from nine separate experiments are shown. The average is 0.05% micronucleated cells with a range of 0–2 micronucleated cells/1000 binucleated cells analyzed.

and reproducible. In 35 different untreated or solvent control cultures, the percentage of micronucleated, binucleated cells was 0.05% with a range of 0–2 MN/1000 cells.

### 3.6. Induction of MN: exposure via medium

#### 3.6.1. Mitomycin C

To determine if the conditions used for the culture of EpiDerm™ tissue were sufficient to allow induction of MN, the known micronucleus-inducing agent MMC was tested in the model. We first exposed the EpiDerm™ tissue to MMC by direct application to the underlying maintenance medium. This allowed the MMC to diffuse along with the maintenance medium through the membrane support and into the tissue where the basal epidermal cells were directly exposed. We believed this method would maximize exposure of the cells to the test chemical (relative to a topical exposure protocol) and allow us to evaluate whether the cells in this model are responsive to genotoxic treatment under optimal experimental conditions. Exposure of up to 0.03 μg/ml MMC did not induce toxicity as evidenced by lack of significant reduction in the percentage of binucleated cells in three different studies (Table 2). At concentrations of 0.06–0.1 μg/ml MMC, the percent binucleated cells were reduced by 22%, 23%, and 23% (experiments 10, 12 and 25, respectively). At 0.3 μg/ml MMC, toxicity was high: 80% in experiment 10, 65% in experiment 12, and 73% in experiment 25. The percentage of MN induced by MMC concentrations that did not exceed a 70% decrease in binucleated cells relative to control are shown in Fig. 9. There was no significant increase in MN frequency up to 0.03 μg/ml MMC, however, a statistically significant ($p < 0.05$) dose-responsive increase in the percentage of micronucleated, binucleated cells was observed at 0.06, 0.1, 0.3 μg/ml MMC (Fig. 9). Photographic examples of representative MN found in these experiments are shown in Fig. 10.

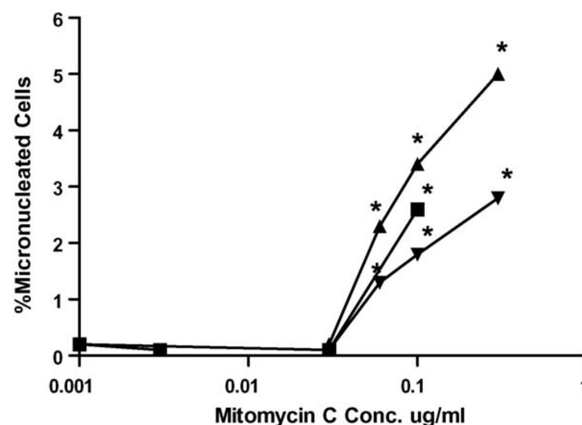


Fig. 9. MN induction in EpiDerm™ tissue after exposure to the indicated concentrations of MMC in the medium. Cells were harvested 24 h after the last re-feeding ($T = 48$ h). The results from three independent experiments are shown. Each experiment consisted of a single tissue per dose except that duplicate control cultures were used in one experiment. *Significant at $p < 0.05$.

Additional studies (Fig. 11) indicated that the frequency of MMC-induced MN per binucleated cell in the absence of Cyt-B was dramatically less than the frequency of micronucleated cells found in the presence of Cyt-B. For instance, at 0.1 μg/ml MMC, a six-fold higher induction of MN was observed in the presence of Cyt-B compared with that in the absence of Cyt-B. At 0.3 μg/ml MMC, the difference was 18-fold. This difference is as expected since only a part of the population

Table 2
Cytotoxicity of MMC to EpiDerm™ tissue as evidenced by reduction in binucleated cells after 48 h exposure

| MMC concentration (μg/ml) exposure via growth medium | % Binucleated cells (% decrease compared to control) | | |
|---|---|---|---|
| | Experiment 10 | Experiment 12 | Experiment 25 |
| 0.0 (solvent control) | 50 | 44.1 | 41.5[a] |
| 0.003 | 57.8 | NT | NT |
| 0.01 | 47.4 (5) | NT | NT |
| 0.03 | 52.2 | 38.9 (12) | 40.3 (3) |
| 0.06 | NT | 33.0 (25) | 41.6 |
| 0.10 | 39.1 (22) | 33.8 (23) | 31.8 (23) |
| 0.30 | 9.9 (80)[b] | 15.5 (65) | 11.2 (73)[b] |
| 0.60 | NT | 4.9 (89)[b] | 6.2 (85)[b] |

The percentage of binucleated cells at each concentration is shown along with the percentage decrease from solvent controls in parentheses. Tissues were re-fed with MMC-containing medium 24 h after the original exposure and harvested 24 h after the last re-feeding. Data are from individual tissues except where noted. NT = not tested.

[a] Average of two tissues.

[b] Results for those concentrations that exceeded 70% toxicity are not included in the graph in Fig. 8.

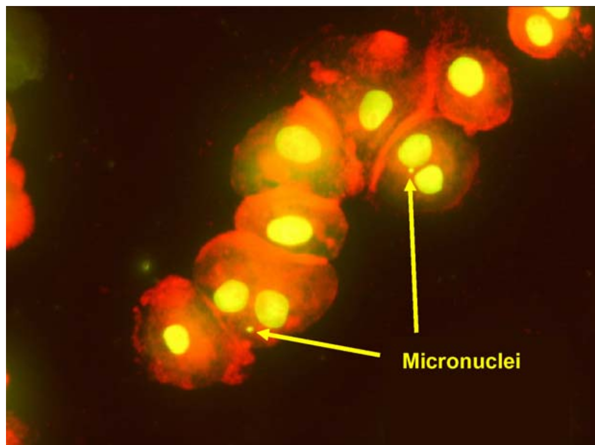


Fig. 10. Examples of MN identified in binuclear human epidermal cells isolated from EpiDerm™ tissues after treatment with MMC. Cells stained with acridine orange and photographed with UV-illumination.

of cells in the EpiDerm™ culture is dividing. Based on this, the use of Cyt-B in this model is recommended.

### 3.6.2. Time course of MN induction

The time course for micronucleus induction by MMC added to the medium was investigated by exposing cultures to increasing concentrations of MMC in the presence of Cyt-B for 24, 48, or 72 h (Fig. 12). Micronucleus frequencies increased with both time of exposure and dose of MMC with maximum induction of MN found at 48–72 h and 0.06–0.1 μg/ml MMC. Results are not given for the 24 and 72-h time points for 0.3 μg/ml MMC because high toxicity (a very low percentage of binucleated cells were present) resulted in too few cells from each tissue (≪1000 binucleated cells) being available for counting.

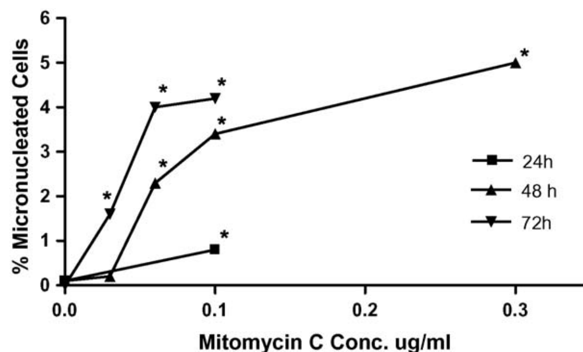


Fig. 12. Effect of length of exposure to MMC in the medium on MN induction in EpiDerm™ tissue. One tissue per concentration per time point was exposed to increasing concentrations of MMC in the presence of 3 μg/ml Cyt-B. *Significant at $p < 0.05$.

### 3.6.3. Vinblastine sulfate

We investigated another model genotoxin, vinblastine sulfate (VB), with a different mechanism of genotoxicity. VB induces genotoxicity by interfering with microtubule formation, thus is considered an aneugen. A stock solution of VB was prepared and aliquots were added to the culture medium to make appropriate dilutions for the assay. Table 3 shows that the cytotoxicity dose-response was quite steep. Toxicity went from 0% at 0.003 μg/ml to greater than 90% at concentrations above 0.005 μg/ml. Similarly, the frequency of MN per binucleated cell (Fig. 13) increased significantly from control

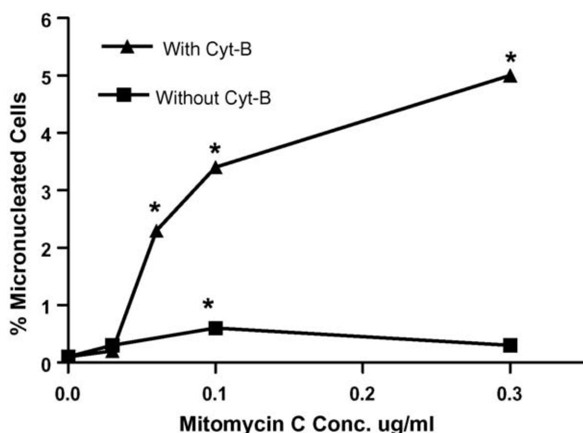


Fig. 11. Comparison of MN induction in EpiDerm™ tissue in the presence (▲) and absence (■) of Cyt-B. Cells were harvested at 48 h. The results from single EpiDerm™ tissues are shown. *Significant at $p < 0.05$.

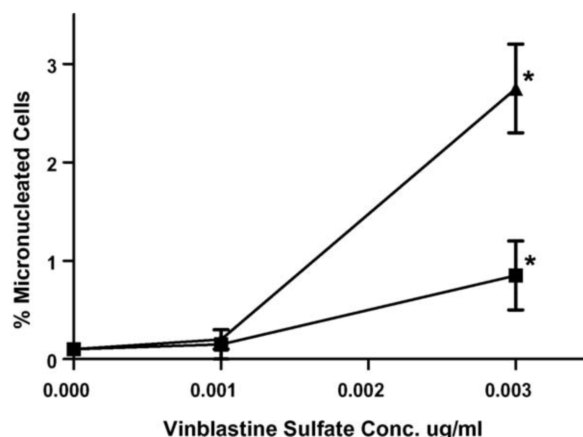


Fig. 13. MN induction in EpiDerm™ tissue after exposure to the indicated concentrations of vinblastine sulfate in the medium. Cells were harvested 24 h after re-feeding ($T = 48$ h). The results from two independent experiments (2–4 tissues/treatment) are shown. *Significant at $p < 0.05$.

Table 3
Cytotoxicity in EpiDerm™ tissue exposed to VB via the growth medium

| VB concentration (μg/ml) | % Binucleated cells (% decrease compared to control) | |
|---|---|---|
| | Experiment 28 | Experiment 30 |
| 0.0 (solvent control) | 38.9 | 31.2 |
| 0.001 | 45.5 | 33.5 |
| 0.003 | 49.6 | 31.3 |
| 0.005 | NT | 2.1 (93)[a] |
| 0.007 | NT | 1.4 (96)[a] |
| 0.01 | 2.2 (94)[a] | NT |
| 0.03 | 2.1 (95)[a] | NT |

The percentage of binucleated cells at each concentration is shown along with the percentage decrease from solvent controls in parentheses. VB was added immediately after a change of medium 48 and 24 h prior to tissue harvest. All values represent the average of two or more individual tissues. NT = not tested.
[a] Results for those concentrations that exceeded 70% toxicity are not included in the graph in Fig. 13.

levels at 0.001 μg/ml VB, to 0.85% (experiment 28) or 2.8% (experiment 30) at 0.003 μg/ml.

### 3.7. Induction of MN: topical application

#### 3.7.1. Mitomycin C

The preferred method of exposing the test system in this model is topical because it more closely reflects the exposure situations that would occur *in vivo*. We evaluated whether MN could be induced in EpiDerm™ tissue by topical application by applying 10-μl doses of MMC in ethanol directly to the surface of the EpiDerm™ tissue with a micropipette. We chose this volume for application to the surface area (approximately 0.64 $cm^2$) of the EpiDerm™ cultures (resulting in 16 $\mu l/cm^2$), since it is almost identical to the dosing parameters (200 μl/12 $cm^2$; equals 17 $\mu l/cm^2$) reported by others [8,11] for *in vivo* micronucleus studies in rats and mice.

Cytotoxicity results for three dose-response studies with topically applied MMC are shown in Table 4. We observed increasing toxicity to the EpiDerm™ tissue in all studies with increasing concentrations of MMC (Table 4). Some variability was observed between experiments, which used different lots of EpiDerm™. A 60% reduction in the percentage of binucleated cells was observed in experiment 21 at 10 μg/ml (total dose of 200 ng MMC from two 10-μl applications of 10 μg/ml MMC), however, in experiments 13 and 14 toxicity was not evident until 30 μg/ml (total dose of 600 ng, two 10 μl applications of 30 μg/ml MMC).

The results for micronucleus induction in the experiments described above are shown in Fig. 14 along with data from three other studies where MMC was used

Table 4
Cytotoxicity of topically applied MMC in ethanol (10 μl) to EpiDerm™ tissue as evidenced by reduction in the number of binucleated cells after 48 h exposure

| MMC concentration (μg/ml) for each of two topical exposures in ethanol (10 μl) | % Binucleated cells (% decrease compared to control) | | |
|---|---|---|---|
| | Experiment 13 | Experiment 14 | Experiment 21 |
| 0 | 42.3 | 42.2 | 35.9[a] |
| 0.6 | NT | 38.4 (9) | NT |
| 1.0 | 55.4 | 28.1 (33) | NT |
| 3.0 | 48.1 | 37.4 (11) | NT |
| 6.0 | NT | 38.6 (9) | NT |
| 10.0 | 32.2 (7) | 40.7 (3) | 14.3 (60) |
| 30.0 | 19.9 (53) | 21.8 (48) | 11.6% (68) |
| 60.0 | 9.9 (76)[b] | 7.9 (82)[b] | 8.4[a] (77)[b] |
| 100.0 | NT | 8.7 (79)[b] | 9.8[a] (73%)[b] |

The percentage of binucleated cells at each concentration is shown along with the percentage decrease from solvent controls in parentheses. MMC was applied to the tissue twice, 48 and 24 h prior to tissue harvest. Data are from individual tissues except where noted. Results for experiments 13, 14, 21 are shown in Fig. 14, along with data for three other experiments conducted with one or two concentrations. NT = not tested.
[a] Average of two tissues.
[b] Results for those concentrations that exceeded 70% toxicity are not included in graph in Fig. 14.

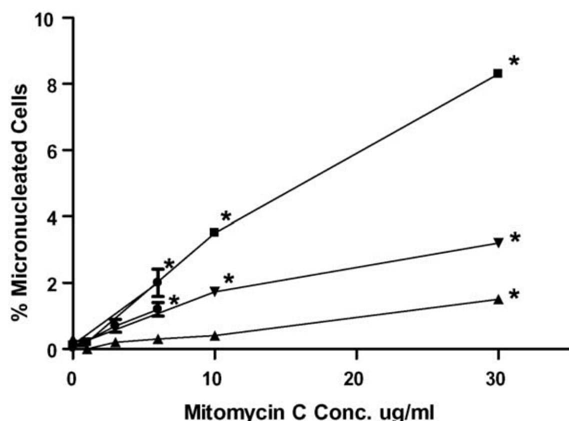


Fig. 14. MN induction in EpiDerm™ tissue after exposure to MMC in ethanol by topical application. Results from six experiments (1–4 tissues/treatment) are shown. *Significant at $p < 0.05$.

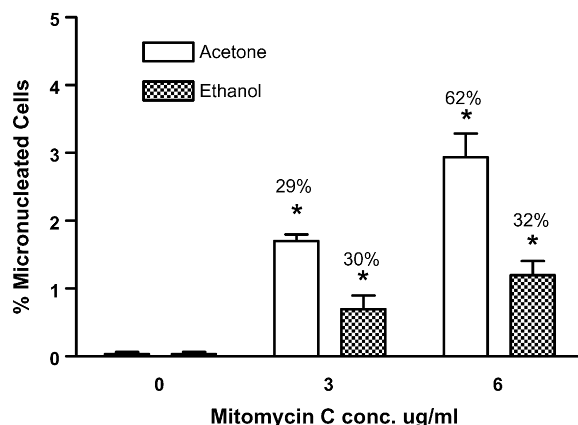


Fig. 15. Comparison of treatment with MMC in ethanol *vs* acetone on induction of MN in EpiDerm™ tissue by topical application. Numbers above the bars are the decrease from the appropriate solvent control in percentage of binucleated cells. Results are the average of triplicate cultures. *Significantly different from controls at $p < 0.05$.

as a positive control at one or two concentrations. A dose-responsive increase in micronucleus frequency was seen with MMC applied topically (Fig. 14). As observed previously, a low frequency of MN was present in controls and at low doses of MMC. Again, some variability between experiments was noticed. Statistically significant increases in the percentage of micronucleated cells were observed starting at 3–6 μg/ml MMC in all studies except for experiment 14 where such increases were only seen at 30 μg/ml. There appeared to be more variability in these studies than in the experiments where MMC was added directly to the media. This may be due to the probability of lot-to-lot variability in the barrier properties of the EpiDerm™ cultures, as well as to the intrinsic difficulty in evenly exposing the tissue when using a topical dosing procedure. Importantly, a reproducible increase in MN was obtained with MMC applied topically.

The lowest observable effect level (LOEL) of around 6 μg/ml MMC per dose corresponds to a total dose of 0.2 ng/cm$^2$ for the two 10-μl topical applications to the 0.64-cm$^2$ EpiDerm™ cultures used in our studies. This is around 10-fold lower than the results from Nishikawa et al. [7] that demonstrated a LOEL for MMC in the *in vivo* skin MN assay in rats of less than 3 μg/cm$^2$.

We also investigated the micronucleus induction with MMC in acetone compared with ethanol since there appeared to be more “blistering” of the EpiDerm™ tissue after application of ethanol than with acetone or acetone:olive oil (see Section 3.4). As shown in Fig. 15, a larger increase in the percentage of micronucleated cells was observed at two dose levels when using acetone compared with ethanol as a solvent. There also appeared to be a change in the toxicity of the MMC treatment as evidenced by a 62% reduction in the percentage of binucleated cells at 6 μg/ml MMC in acetone compared with a 32% reduction at 6 μg/ml MMC in ethanol. Based on these results, we are now using MMC in acetone as the positive control for micronucleus induction studies in this model.

### 3.7.2. Vinblastine sulfate

VB dosed topically (two applications of 10 μl) in the EpiDerm™ model induced a steep dose-response in toxicity (Table 5). No reduction in the percent binucleated cells was observed at 0.3 μg/ml, but a 51% decrease was seen at 0.6 μg/ml. A dose-responsive increase in MN was observed with VB dosed topically, with statis-

Table 5
Cytotoxicity of topically applied VB in ethanol to EpiDerm™ tissue as evidenced by reduction in the number of binucleated cells after 48 h exposure

| VB concentration (μg/ml) in ethanol for each of two topical exposures (10 μl) | % Binucleated cells (% decrease compared to control) |
|---|---|
| 0 | 30.8 |
| 0.03 | 44.7 |
| 0.1 | 41.2 |
| 0.3 | 32.1 |
| 0.6 | 15.1 (51) |
| 1.0 | 7.8 (75)[a] |

The percentage of binucleated cells at each concentration is shown along with the percentage decrease from solvent controls in parentheses. VB was applied to the tissue 48 and 24 h prior to tissue harvest. Data are the average of duplicate tissues and are from the same experiment as shown in Fig. 16.

[a] Results for those concentrations that exceeded 70% toxicity are not included in graph in Fig. 16.

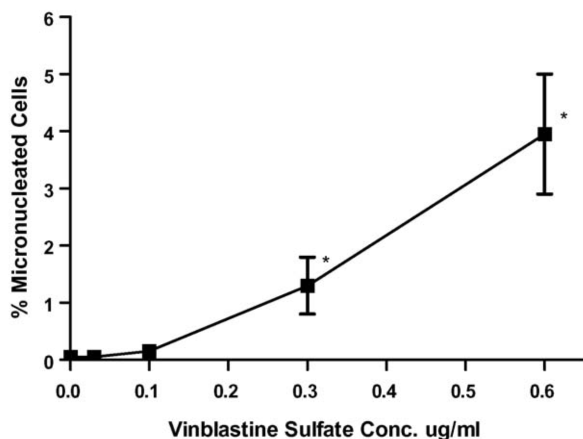


Fig. 16. MN induction in EpiDerm™ tissue after exposure to VB in ethanol by topical application. Results are the average of duplicate cultures. *Significant at $p < 0.05$.

tically significant increases found at 0.3 and 0.6 μg/ml (Fig. 16).

## 4. Discussion

We have described the first stages in the development of a novel micronucleus assay based on an *in vitro* three-dimensional human skin tissue model (EpiDerm™). There are several potential advantages to this system. First, the model is designed to represent a nearly normal epidermis, having clearly discernable basal, spinous and granular layers overlaid with a well-developed stratum corneum. As such, this model will more closely mimic human skin with barrier properties and may provide more relevant data than current *in vitro* genotoxicity assays which do not take into account the skin penetration of the test material as a potential limiting factor for target cell exposure. Although penetration in EpiDerm™ can still vary from normal human skin on a material-specific basis (MatTek, personnel communication, and our own unpublished observations), the MatTek™ barrier is generally more permeable, meaning that the model will provide a conservative estimate of exposure. Second, since the model is constructed of human keratinocytes, it is expected to have xenobiotic metabolism more relevant to normal human skin than the metabolism achieved in standard *in vitro* genotoxicity assays (generally that provided by a rat-liver S9 preparation). To explore this hypothesis, extensive work to characterize the metabolic capabilities of the EpiDerm™ model is underway.

A third important advantage to this model is that Cyt-B can be used to identify the dividing cells in the EpiDerm™ tissue. We show that not only were basal cells of the EpiDerm™ model subject to cytokinesis blockage by Cyt-B, but that sufficient cells were undergoing mitosis to result reproducibly in 30–50% binucleated cells after 48 h of Cyt-B treatment. The use of Cyt-B also allows a concurrent measure of cytotoxicity as seen by decreases in the percent binucleated cells that result with inhibition in the cell cycle caused by application of a test chemical. This measure of cytotoxicity is standard in the proposed OECD guideline for the *in vitro* micronucleus assay. Such a quantitative measurement has not been available for rodent *in vivo* skin models, which generally utilize more qualitative skin-irritation indices as measures of toxicity. Since only binucleated cells are scored for the presence of MN when using this EpiDerm™ model, the user is confident that the appropriate population of dividing cells are analyzed and that false-negative results due to a highly cytostatic response will not occur. We suggest that Cyt-B should be used routinely when performing an *in vitro* micronucleus assay in the EpiDerm™ model.

EpiDerm™ was shown to respond to two model genotoxicants – MMC and VB – by two different modes of exposure. MN could be induced in a dose-dependent fashion either by adding genotoxicants directly to the medium, resulting in exposure of the tissue from the basal surface, or by direct application of the genotoxicants to the apical surface of the tissue. We consider the ability to expose the tissue by this later method quite important since it closely mimics the way in which most individuals would be either accidentally exposed to chemicals or intentionally exposed to cosmetic or personal care products.

An additional feature of this model is that the background frequency of MN is considerably lower than that generally seen with cell cultures *in vitro*, in monolayer or suspension. We find that the average amount of micronucleated cells in control cultures is 0.05% of the binucleated cells, with a range of 0–2 MN/1000 cells. Importantly, the background frequency is very reproducible between cultures, making the model amenable to relatively easy statistical analysis for the detection of genotoxins.

We have developed methods for the reproducible treatment and isolation of high numbers of viable cells from single EpiDerm™ tissues for the assessment of MN. Our model provides a potentially novel *in vitro* system that can be used to detect genotoxic activity in an appropriate, *in vivo*-like, dermal target tissue of human origin. Although our initial results are encouraging, more work is needed to fully characterize the system including testing a wide range of chemicals with different modes of action and different genotoxic/carcinogenic

profiles in standard assays. Investigation of optimal treatment/harvest times for a range of chemicals is needed along with characterization of xenobiotic metabolism. The applicability of the methods described here to other human skin models also needs to be ascertained. When fully developed this model may provide useful data to add to the weight of the evidence for assessing the genotoxicity of cosmetics without the use of animals as necessitated by the EU 7th Amendment to the Cosmetics Directive. Initially, we envision this model as a second tier test to be used after a positive result has been found in a traditional *in vitro* genotoxicity assay. However, if the model is successful in providing more predictive results, i.e. high sensitivity (few false-negative results), with fewer false positive results than current *in vitro* genotoxicity assays, it may prove useful as a replacement for one or more of these less predictive tests.

We encourage others to join us in further investigations of this model so that a validated non-animal assay for skin genotoxicity can be available in the near future.

## Note added in proof

A recent manuscript (N. Flamand, L. Marrot, J-P. Belaidi, L. Bourouf, et al. Development of genotoxicity test procedures with Episkin$^{R}$, a reconstructed skin model: Towards new tools for in vitro risk assessment of dermally applied compounds?, Mutat. Res. in Press) describes a related methodology which detects MN in L5178Y cells co-cultured with a 3-dimensional skin model.

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