

bis-(β -Chloroethyl)sulfide (BCES)-Induced Changes in Epidermal Cell Homeostasis *in Vitro*¹

WARREN W. KU* AND ISADORE A. BERNSTEIN*†²

*Departments of Environmental and Industrial Health and †Biological Chemistry,
The University of Michigan, Ann Arbor, Michigan 48109-2029

Received December 11, 1987; accepted May 4, 1988

bis-(β -Chloroethyl)sulfide (BCES)-Induced Changes in Epidermal Cell Homeostasis *in Vitro*. KU, W. W., AND BERNSTEIN, I. A. (1988). *Toxicol. Appl. Pharmacol.* **95**, 397-411. A rat cutaneous keratinocyte culture system was developed to study the effects of the vesicant bis-(β -chloroethyl)sulfide (BCES) on the homeostasis of cell proliferation and differentiation. Lectins were used to reveal cell surface carbohydrate changes as the keratinocytes differentiate. In the newborn rat epidermis, the isolectin, *Griffonia simplicifolia* I-B₄ (GS I-B₄), binds to basal cell surfaces. *Ulex europeus* agglutinin I (UEA) binds to the surfaces of spinous and lower granular cells and is therefore considered an indicator of keratinocyte differentiation. A fluorometric assay was developed which determines the ratio of bound UEA to bound GS I-B₄ (the UEA/B₄ ratio) in primary monolayer cultures of rat cutaneous keratinocytes maintained in low Ca²⁺ medium. The UEA/B₄ ratio was found to be a representation of the relative sizes of the differentiating and proliferating cell compartments in the monolayer cultures, respectively (W. W. Ku and I. A. Bernstein, 1988, *Exp. Cell Res.*, **175**, 298-316). Monolayer cultures exposed for 1 hr to BCES at Day 1 exhibited a dose-related increase in the UEA/B₄ ratio at Day 7 when compared to solvent controls. The results from the analysis of lectin binding sites showed a decrease in GS I-B₄ binding with little or no change in UEA binding as a result of BCES exposure, contributing to the increase in the UEA/B₄ ratio. BCES-exposed monolayers also showed early perturbations in replicative DNA synthesis as revealed by autoradiography. Subsequent to the perturbations in replicative DNA synthesis was an inability of BCES-exposed cultures to produce cells into the monolayer through mitosis. In addition to an increase in the UEA/B₄ ratio, BCES-exposed monolayers also showed a dose-related loss of DNA, with the appearance of enlarged cells at Day 7. These enlarged cells failed to show evidence of DNA synthesis, with groups of these cells showing intense UEA staining with only faint GS I-B₄ staining. Overall, exposure to low concentrations of BCES appeared to disrupt the normal homeostasis of cell proliferation and differentiation in this monolayer culture system. This disruption was primarily through a reduction in the fraction of germinative (basal) cells with concomitant retention of some early differentiated cells, presumably early spinous or spinous cells. © 1988 Academic Press, Inc.

¹ This investigation was supported in part by Grant 5 RO1 AM 15206 from the National Institutes of Health, U.S. Public Health Service, Department of Health and Human Services, and by the United States Army Medical Research and Development Command, Contract DAMD 17-86-C-6124. The views, opinions, or findings contained in this report are those of the authors and should not be construed as position, policy, or decision of the agency supporting this research.

² To whom reprint requests should be addressed.

bis-(β -Chloroethyl)sulfide (BCES) is a potent alkylating agent and a severely cytotoxic vesicant (Papermeister and Davison, 1965). Exposure of human skin to BCES results in an initial erythema followed by blistering (Warthin and Weller, 1919; Sinclair, 1949; Stoughton and Bagatell, 1959). The pathological process of cutaneous chemical blistering involves a complex response from a host of systems (e.g., intact vascular supply and

immune system) in the whole organism. The initial molecular events associated with the vesicating actions of BCES are unknown. However, it is known that BCES reacts rapidly with cellular DNA, RNA, and protein, thus possibly leading to many disruptions in normal cellular metabolism (Wheeler, 1962).

The response of the cutaneous epidermis to most environmental stimuli is controlled by a negative feedback loop which assures, in normal circumstances, that cells which are lost through desquamation or as a result of some environmental insult are replaced through cell proliferation (Weiss and Kavanaugh, 1957). This phenomenon is classically observed in epidermal wound healing (for a review see Wright and Alison, 1984). Exposure of the skin to a cytotoxic chemical which exerts preferential damage to the lowermost nucleated epidermal cell layers may alter the regulation of cell proliferation and the commitment to differentiation and thus disrupt this steady state. This would ultimately lead to alterations in the outermost layers and thus compromise the skin's primary function as a protective barrier against the external environment.

The maintenance of cellular homeostasis in the cutaneous epidermis is highly dependent on the replacement of desquamated cells by cell proliferation. Since mitosis appears to be the physiological function which is most sensitive to BCES, it is reasonable to consider that the reaction of BCES with cellular DNA might be the major toxicologic phenomenon in both a disruption in epidermal cell homeostasis and possibly in the macromolecular events associated with chemical blistering (Fox and Scott, 1980). It has also been noted that the blistering process associated with BCES initially involves damage to the basal and lower spinous cell layers of the epidermis (Warthin and Weller, 1919; Sinclair, 1949).

A rat cutaneous keratinocyte culture system was developed to model the effects of BCES on the homeostasis of cell proliferation and the commitment to an early stage of

differentiation. Specifically, lectins were used to estimate changes in the relative sizes of the proliferating (germinative or basal) and differentiating (early spinous/spinous) cell compartments in monolayer cultures of rat keratinocytes maintained in low Ca^{2+} medium and exposed to BCES.

Lectins are carbohydrate-binding proteins or glycoproteins of non-immune origin which, when fluorescently labeled, have been used to reveal cell surface carbohydrate changes during epidermal differentiation (Nieland, 1973; Hashimoto *et al.*, 1974; Nemanic *et al.*, 1979; Brabec *et al.*, 1980). In the newborn rat epidermis, the isolectin *Griffonia simplicifolia* I-B₄ (GS I-B₄), specific for terminal, nonreducing α -D-galactosyl residues, binds to the surfaces of basal cells. The lectin *Ulex europaeus* agglutinin I (UEA), specific for terminal nonreducing α -L-fucose, binds to the surfaces of spinous and lower granular cells and shows no binding to basal cell surfaces. The presence of UEA binding is therefore considered a marker of keratinocyte differentiation (Brabec *et al.*, 1980; Brown *et al.*, 1987).

A spectrophotofluorometric assay was developed which determines the absolute quantity of UEA and GS I-B₄ bound to primary monolayer cultures of rat cutaneous keratinocytes by monitoring two separate fluorescent analogs simultaneously (Ku and Bernstein, 1988). It was postulated that the ratio of bound UEA to bound GS I-B₄ (the UEA/B₄ ratio) would be a representation of the relative sizes of the differentiating and proliferating cell compartments, respectively. It was predicted that a high UEA/B₄ ratio would reflect a greater proportion of cells in the monolayer at some early stage of differentiation. A low ratio would reflect a higher fraction of germinative (basal) cells in the monolayer.

Initial studies by Ku and Bernstein (1988) demonstrated that primary monolayer cultures of rat keratinocytes maintained in low Ca^{2+} medium exhibited a characteristic unimodal pattern in the UEA/B₄ ratio over a cul-

ture period of 7 days that was consistent with (a) the progression of a proportion of the cells in the monolayer to an early spinous or spinous cell stage, the ultimate fate of which is desquamation into the medium (increase in the UEA/B₄ ratio between Day 2 and Day 4) and (b) a consequent proliferative response by the monolayer (decrease in the UEA/B₄ ratio between Day 5 and Day 7). It was suggested that this system would be useful for studying environmental influences on the homeostasis of cell proliferation and differentiation in the cutaneous epidermis (Ku and Bernstein, 1988).

In the present study, the effects of low concentrations of BCES on the homeostasis of cell proliferation and differentiation was examined using the described culture system. It was found that low concentrations of the vesicant BCES disrupts the normal homeostasis of cell proliferation and differentiation in this monolayer culture system. This disruption was primarily through a reduction in the growth fraction (fraction of germinative or basal cells) consequent with a retention of some early differentiated cells, presumably early spinous or spinous cells.

MATERIALS AND METHODS

Chemicals. The isolectin, *Griffonia simplicifolia* I-B₄ (GS I-B₄), fluorescein (FITC)-labeled, and the eluting sugar methyl- α -D-galactopyranoside were purchased from Sigma Chemical Co. (St. Louis, MO). *Ulex europaeus* agglutinin I (UEA), rhodamine-labeled, was purchased from Vector Laboratories (Burlingame, CA). The eluting sugar α -L-fucose was obtained from U.S. Biochemical Corp. (Cleveland, OH). Kodak NTB-2 Emulsion for autoradiography was obtained from Eastman Kodak Co. (Rochester, NY). 3,5-Diaminobenzoic acid dihydrochloride used for DNA determination was purchased from Aldrich Chemical Co. (Milwaukee, WI). Calf thymus DNA was obtained from Sigma. The toxic vesicant and potent alkylating agent bis-(β -chloroethyl)-sulfide at a stock concentration of 10 mg/ml in methylene chloride was obtained from the U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, Maryland. Crystal violet (gentian violet) was from Allied Chemical and Dye Corp. (New York, NY) and nuclear fast red (Kernechtrot) was obtained from Chroma-Gesellschaft Schmid and Co. (Stuttgart, Ger-

many). Methyl-³H-thymidine (³H-TdR) was purchased from NEN (Boston, MA). Crude trypsin, 1:250, was purchased from Difco Laboratories (Detroit, MI). Calcium-free Eagle's minimum essential medium (MEM) and Dulbecco's phosphate-buffered saline (DPBS) in powdered form, and fetal bovine serum were obtained from KC Biologicals (Lenexa, KS).

Cell culture. Keratinocytes were isolated from newborn rats obtained from a randomly inbred colony of CFN albino rats (Carsworth Farms) maintained in this laboratory. The methods for the isolation of epidermal cells from newborn rat skins was a modification of the method described by Vaughan *et al.* (1981). The cells isolated from the lowermost band in an isotonic 38% Percoll density gradient (Pharmacia, Piscataway, NJ) ($\rho > 1.075$ g/cc) were resuspended to a final density of $1.6\text{--}1.8 \times 10^6$ cells/ml in low Ca²⁺ medium. This medium consisted of calcium-free Eagle's MEM supplemented with 10% Chelex-treated fetal calf serum (Brennan *et al.*, 1975), 10 μ g/ml insulin, 10 μ g/ml hydrocortisone, 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 0.5 μ g/ml fungizone, and was adjusted to 0.08–0.11 mM Ca²⁺ by the addition of calcium chloride. The cells were seeded in either Nunclon 16-mm four-well multidishes or Corning 24-well multiplates at $4\text{--}5 \times 10^5$ cells/cm². Cultures were maintained in low Ca²⁺ medium at 35°C in a humidified 5% CO₂:95% air environment. The medium was changed every other day. Percentage cell attachment at 22–24 hr postseeding was around 40–50%. Confluency was routinely achieved 2 days postseeding.

Exposure to BCES. At 22–24 hr after seeding, monolayer cultures were rinsed in DPBS with Ca²⁺ (0.10 mM) and Mg²⁺ (1.8 mM) added. BCES, at a concentration of 10 mg/ml in methylene chloride, was diluted in DPBS to a 10 μ M stock solution. This stock solution was further diluted in DPBS to provide the desired concentrations for exposure to the monolayer cultures. Cultures were then exposed to BCES in DPBS for 1 hr at 35°C. Cultures similarly exposed to an appropriate concentration of methylene chloride served as solvent controls. After 1 hr, the cultures were rinsed in DPBS, placed in low Ca²⁺ medium and returned to the incubator until harvesting.

UEA/B₄ ratio determination. Monolayer cultures were exposed to various concentrations of BCES (0.5, 1.0, 2.0, and 4.0 μ M) at Day 1. At 6 days postexposure (Day 7), the UEA/B₄ ratio was determined by a modification of the method of Monsigny *et al.* (1979) and as described previously (Ku and Bernstein, 1988). The modification permits the simultaneous determination of the quantity of UEA and GS I-B₄ bound to the same monolayer by spectrophotofluorometrically monitoring two separate fluorescent analogs (FITC-labeled GS I-B₄ and rhodamine-labeled UEA). Briefly, after incubation with the two lectins, specifically bound UEA and GS I-B₄ were extracted from the monolayer cultures by incubation with the appropriate eluting monosaccharides; methyl- α -D-galactopyranoside for GS I-B₄ and α -L-fucose for

UEA. The UEA/B₄ ratio was determined spectrophotometrically with a Perkin-Elmer Model 650-40 fluorescence spectrophotometer. FITC-labeled GS I-B₄ was excited at 496 nm and the emission detected at 520 nm. Rhodamine-labeled UEA was excited at 556 nm and the emission detected at 580 nm. All measurements were carried out at room temperature. The number of GS I-B₄ and UEA binding sites and their association constants for 5 μ M BCES-exposed and solvent control cultures was determined by the method of Scatchard (Scatchard, 1949).

DNA determination and cell production. Monolayer cultures were exposed to various concentrations of BCES or solvent at Day 1. At Day 7 (6 days postexposure), monolayer cultures used for the determination of the UEA/B₄ ratio were treated with ice-cold trichloroacetic acid and processed for DNA determination as described previously by Ku and Bernstein (1988). DNA content was determined spectrofluorometrically by a modification of the method described by Setaro and Morley (1976). One-tenth N NaOH rather than 1 N KOH was used to dissolve the TCA precipitate (Abler, 1985). Calf thymus DNA (Sigma) was used as the standard. To determine the effects of exposure to BCES on the kinetics of cell production in the monolayer, cell counts of monolayer cells exposed to BCES or solvent were carried out at 24-hr intervals up to 7 days using a hemocytometer. The monolayer cells were collected by disaggregation with 0.05% crude trypsin/0.02% EDTA and light scraping with a rubber policeman.

Autoradiography. Monolayer cultures were exposed to 1.0 μ M BCES or solvent at Day 1. At 24-hr intervals postexposure up to 7 days, cultures were pulse-labeled with [³H]TdR (2 μ Ci/ml \times 2 hr, 35°C). Cultures were then rinsed with PBS, fixed in neutral buffered formalin (10%), and coated with Kodak NTB-2 emulsion. The autoradiograms were developed after 5 days of exposure. One to two thousand cells were scored in both control and BCES-exposed cultures.

Mitotic counts. Monolayer cultures were exposed to BCES (0.25, 0.5, and 1.0 μ M) or solvent at Day 1. Medium was replaced, and cultures were harvested at 44, 48, and 53 hr in culture for the quantitative analysis of mitotic figures. A selective staining procedure developed by Fraser (1982) was utilized to identify mitotic figures. The highly selective method stains mitotic figures an intense blue (crystal violet) against a background of red-staining resting cells (counterstain with nuclear fast red). Mitotic figures at various stages (metaphase, anaphase, telophase) were quantified in BCES-exposed and solvent control cultures and expressed as the total number of mitotic figures/1000 cells. A minimum of 20,000 cells were evaluated for each condition.

Nature of BCES-induced large cells. Monolayer cultures were exposed to 5.0 μ M BCES or solvent. At this BCES concentration, virtually all of the cells in the monolayer at Days 6-7 were enlarged, with diameters as

much as fourfold greater than solvent controls. These large cells were examined at Day 7 for (a) staining characteristics by FITC-labeled GS I-B₄ and rhodamine-labeled UEA by methods previously described (Ku and Bernstein, 1988) and (b) their ability to incorporate [³H]TdR into nuclei as revealed by autoradiography. Results were recorded by photomicrography and compared to solvent controls.

Statistics. Two sample *t* tests were used to compare differences in mean values between control and BCES-exposed monolayer cultures. One-way and two-way analyses of variance were used to assess BCES concentration effects for various parameters.

RESULTS

Effect of BCES on the UEA/B₄ ratio. The UEA/B₄ ratio was utilized to determine changes in the relative proportion of early differentiated to germinative (basal) cells in BCES-exposed monolayer cultures. The results of previous studies showed that monolayer cultures exposed to 1.0 μ M BCES at Day 1 exhibited a significantly higher UEA/B₄ ratio when compared to controls at 5-6 days postexposure (Day 6-Day 7) (Ku and Bernstein, 1986) (Fig. 1) (two-sample *t* test, α = 0.05 level of significance). The results presented in Fig. 2 demonstrated a significant concentration effect with respect to the BCES-associated increase in the UEA/B₄ ratio (one-way analysis of variance, multiple comparison, α = 0.05 level). Monolayer cultures exposed to 0.5, 1.0, 2.0, and 4.0 μ M BCES at Day 1 showed a 5, 38, 39, and 49% increase in the UEA/B₄ ratio at Day 7 over the appropriate solvent controls, respectively. This increase was the result of a dose-related decrease in the absolute amount of GS I-B₄ bound with little or no change in the amount of UEA bound compared to controls. Scatchard analysis (Scatchard, 1949) of UEA and GS I-B₄ binding sites at Day 7 in monolayer cultures exposed to 5.0 μ M BCES showed significantly fewer (48%) GS I-B₄ binding sites with no significant change in UEA binding sites compared to solvent controls (Table 1, two-sample *t* test, α = 0.05 level). The results also showed that the affinity constants (K_a)

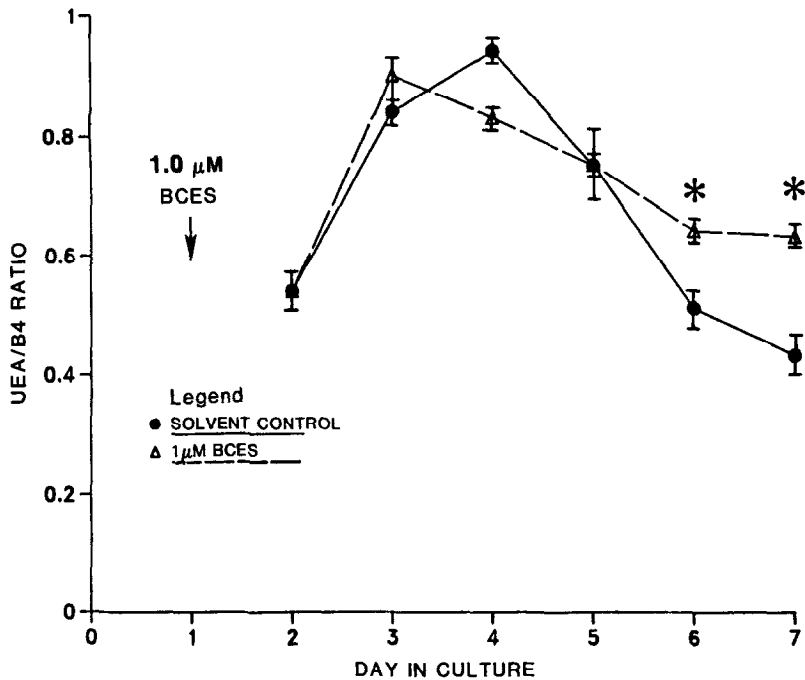


FIG. 1. Effect of a single exposure to 1.0 μM BCES at Day 1 on the UEA/B₄ ratio. Monolayer cultures were exposed to 1.0 μM BCES or solvent at Day 1 as described under Materials and Methods. The UEA/B₄ ratio was determined 1–6 days postexposure (Days 2–7 in culture). The results represent the mean ratio \pm standard deviation (SD) from eight independent determinations from two separate experiments. Asterisk denotes significant difference compared to control values, two-sample *t* test, $\alpha = 0.05$ level.

for GS I-B₄ and UEA binding were not significantly different for BCES-exposed and solvent control cultures. Thus, the Scatchard analysis supported the interpretation that the BCES-associated increase in the UEA/B₄ ratio was due to a loss of GS I-B₄ binding with no change in UEA binding.

The effect of BCES on cell number in the monolayer was measured by determination of DNA content. In addition to the change in the UEA/B₄ ratio, there was a significant concentration effect with respect to the content of DNA in BCES-exposed monolayers (Fig. 3) (one-way analysis of variance, multiple comparison, $\alpha = 0.05$). The results showed 7, 18, 32, and 45% decreases in monolayer DNA content (μg DNA/16-mm well) at Day 7 in cultures exposed to 0.5, 1.0, 2.0, and 4.0 μM BCES, respectively. BCES-exposed monolayers also revealed a dose-related decrease in monolayer cell density with

the presence of uniform enlarged flat cells throughout the monolayer (Fig. 4). These large flat cells were most notable at 5–6 days postexposure (Day 7). In monolayer cultures exposed to 4.0–5.0 μM BCES, these enlarged flattened cells occupied nearly 100% of the monolayer culture surface at Day 7. These cultures also exhibited the loss of the characteristic morphology of the low Ca^{2+} monolayer (Hennings and Holbrook, 1983) (i.e., wide intercellular spaces, dark nuclei, “cobblestone” appearance). Although there was a decrease in monolayer cell density, confluency was maintained in BCES-exposed cultures as a result of an increased average surface area per cell.

Effect of BCES on replicative DNA synthesis. The effect of BCES exposure on replicative DNA synthesis in monolayer cultures was determined by estimation of the incorporation of [³H]TdR into DNA by autoradiog-

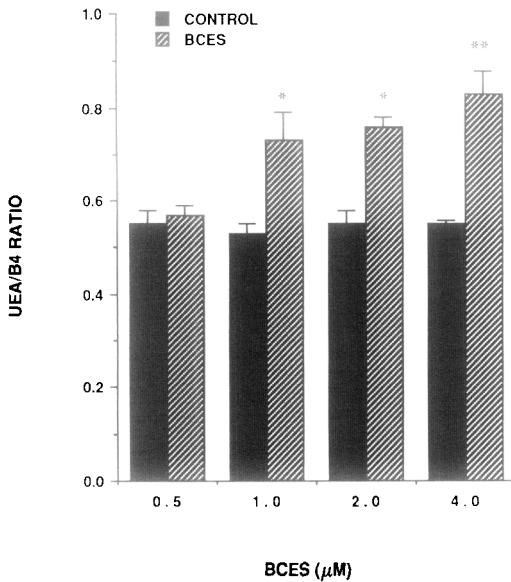


FIG. 2. BCES dose-response relationship: The UEA/B₄ ratio. Monolayer cultures were exposed to 0.5, 1.0, 2.0, and 4.0 μM BCES or solvent at Day 1. The UEA/B₄ ratio was determined at Day 7. The results represent the mean ratio ± SD from 12 independent determinations from three separate experiments. *Significance compared to 0.5 μM BCES; **Significance compared to 0.5, 1.0, and 2.0 μM BCES, one-way analysis of variance, multiple comparison, $\alpha = 0.05$ level.

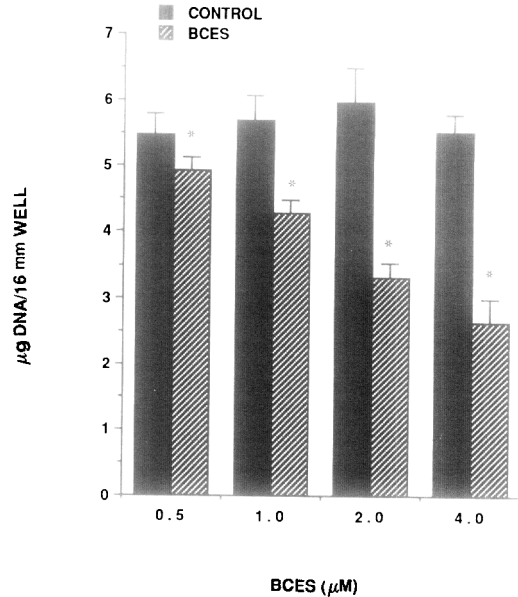


FIG. 3. BCES dose-response relationship: Monolayer DNA content. Monolayer cultures were exposed to 0.5, 1.0, 2.0, and 4.0 μM BCES or solvent at Day 1. The content of DNA in BCES-exposed and control monolayers was determined at Day 7 following determination of the UEA/B₄ ratio. The results represent the mean μg DNA/16-mm well ± SD from eight independent determinations from three separate experiments. Asterisk denotes significant difference upon multiple comparisons, one-way analysis of variance, $\alpha = 0.05$ level.

raphy during the 7-day culture period. In control monolayer cultures, there was a nearly synchronous pattern of DNA synthesis during the 7-day culture period, with

peaks at or around 34–48 hr, 82 hr, and 130 hr (Fig. 5). A synchronous pattern of growth has also been reported for primary cultures of

TABLE I
SCATCHARD ANALYSIS OF UEA AND GS I-B₄ BINDING SITES IN SOLVENT CONTROL AND 5.0 μM BCES-EXPOSED MONOLAYER CULTURES

	Solvent control		5.0 μM BCES	
	GS I-B ₄	UEA	GS I-B ₄	UEA
$K_a (\times 10^{-11})^a$	1.14 ± 0.03	3.18 ± 0.16	1.00 ± 0.06	3.44 ± 0.37
$N (\times 10^{12})^b$	8.67 ± 0.33	5.28 ± 0.66	3.85 ± 1.12*	6.27 ± 0.03
R^2	-0.94	-0.96	-0.95	-0.97

^a Apparent association constant for the major lectin-binding sites (M^{-1}).

^b Total number of lectin binding sites in the monolayer culture (16-mm well) adjusted by a surface area correction factor.

* Significant difference, solvent control vs. 5.0 μM BCES-exposed cultures, two-sample *t* test, $\alpha = 0.05$ level.

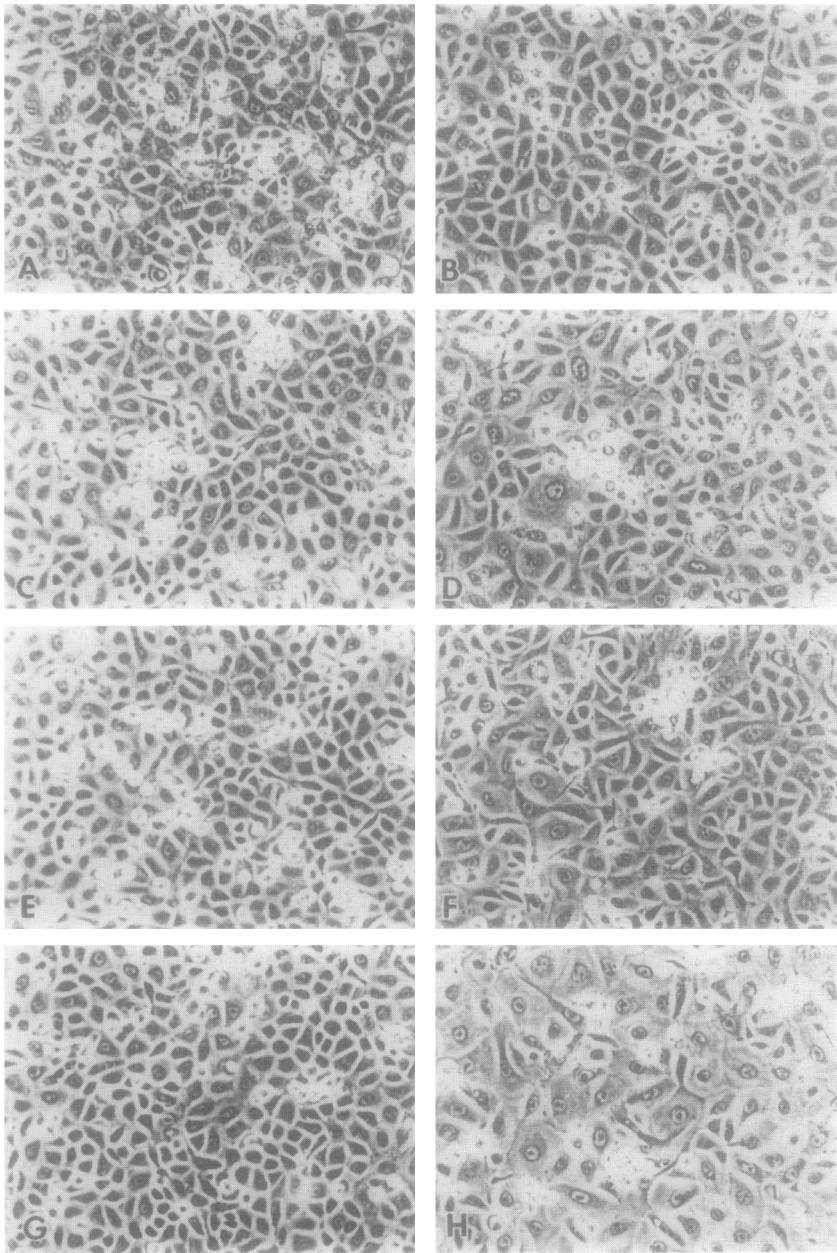


FIG. 4. BCES dose-response relationship: Morphology of monolayer cultures. Monolayer cultures were exposed to 0.5, 1.0, 2.0, and 4.0 μM BCES or solvent at Day 1. Phase contrast photomicrographs were taken at Day 7. (A, C, E, G) 0.5, 1.0, 2.0, and 4.0 μM solvent controls, respectively; (B, D, F, H) cultures exposed to 0.5, 1.0, 2.0, and 4.0 μM BCES, respectively.

newborn mouse epidermal cells (Elgjo *et al.*, 1976). The results from the autoradiographic analysis of monolayer cultures exposed to 1.0

μM BCES showed that there was a reduced intensity of [^3H]TdR labeling per cell rather than a reduction in the fraction of densely la-

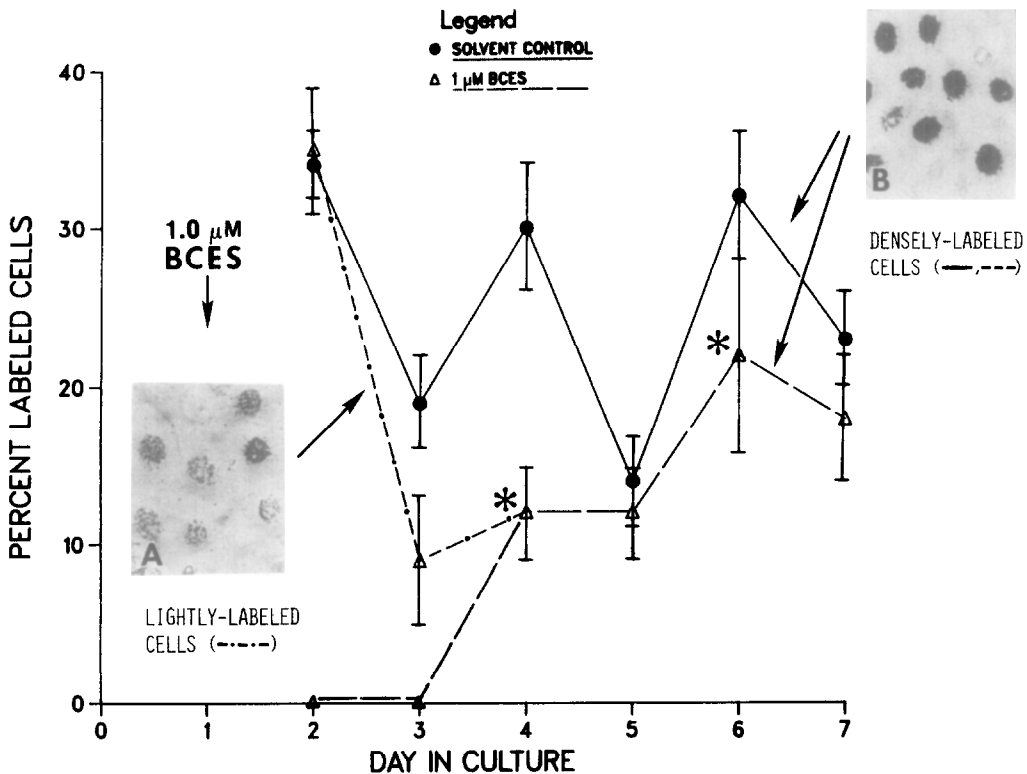


FIG. 5. Autoradiographic analysis of monolayer cultures exposed to 1.0 μM BCES. Monolayer cultures were exposed to 1.0 μM BCES or solvent at Day 1. At 24-hr intervals postexposure to 7 days, cultures were processed for autoradiography as described under Materials and Methods. There were 1000–2000 cells scored for both control and BCES-exposed cultures. The results represent the mean percentage labeling \pm SD from four independent determinations from two separate experiments. Asterisk denotes significant difference compared to control values, two-sample *t* test, $\alpha = 0.05$ level. (—) densely labeled cells in solvent controls; (---) densely labeled cells in BCES-exposed cultures; (- - -) lightly labeled cells in BCES-exposed cultures.

beled cells characteristic of control cultures noted at 1–2 days postexposure (Day 2–Day 3). The presence of lightly labeled nuclei in BCES-exposed monolayers as revealed by autoradiography was believed to reflect repair synthesis as a result of gross DNA damage (Cleaver and Thomas, 1981). Thus, the actual fraction of densely labeled cells, interpreted to be cells which were undergoing replicative DNA synthesis, was almost zero for cultures exposed to 1.0 μM BCES during this initial period in culture. Beginning at Day 4 (3 days postexposure), densely labeled cells were observed in BCES-exposed cultures, however, at a significantly reduced percent-

age of labeling compared to controls (12% vs. 30% for controls) (two-sample *t* test, $\alpha = 0.05$ level). Beginning at Day 5 (4 days postexposure), BCES-exposed monolayer cultures appeared to exhibit a recovery, however, at still a slightly reduced level of labeling compared to controls (Day 5: 12% vs. 14% for controls, not significant; Day 6: 22% vs. 32% for controls, significant, $\alpha = 0.05$; Day 7: 18% vs. 27% for controls, not significant).

Effect of BCES on cell production. The results of studies estimating the kinetics of cell production showed that, in monolayer cultures exposed to 1.0 μM BCES at Day 1, there appeared to be no cell production in the

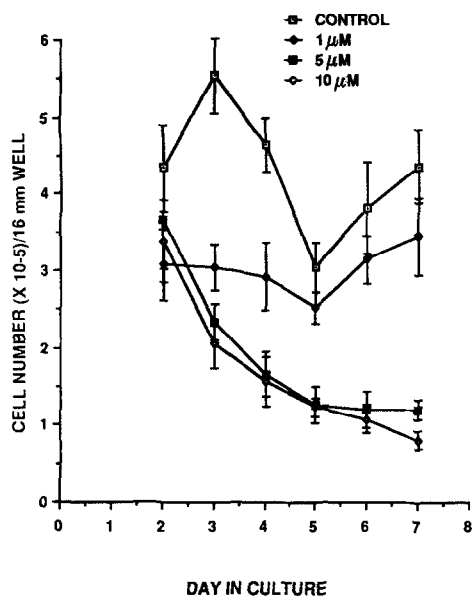


FIG. 6. Effect of BCES exposure on the kinetics of cell production in the monolayer. Monolayer cultures were exposed to 1.0, 5.0, and 10.0 μM BCES or solvent at Day 1. At 24-hr intervals postexposure to 7 days, the cells in the monolayer were harvested and counted as described under Materials and Methods. The results represent the mean cell number ($\times 10^{-5}$)/16-mm well \pm SD from four independent determinations from two separate experiments.

monolayer subsequent to the major 34- to 48-hr peak of DNA synthesis noted for control cultures between Day 2 and Day 3 (Ku and Bernstein, 1988) (Fig. 6). This was the same period of time in which lightly labeled nuclei were detected by [^3H]TdR autoradiography (Fig. 5). Monolayer cultures exposed to 5.0 and 10.0 μM BCES actually showed a loss of cells (38% loss for both concentrations between Day 2 and Day 3) from the monolayer during this early time period postexposure. Cultures exposed to both of these high BCES concentrations continued to exhibit a loss of cells from the monolayer to a low of 20–30% of controls at 3–6 days postexposure. However, cultures exposed to 1.0 μM BCES showed a recovery of cell production in the monolayer starting at 4 days postexposure (Day 5), although at a slightly reduced level as compared with controls (80, 84, and 79%

of control cell numbers at Days 5, 6, and 7, respectively). This period was also associated with the restoration of the characteristic darkly labeled nuclei in exposed cultures and a recovery to slightly below control levels in the [^3H]TdR labeling index as revealed by autoradiography (Fig. 5). Cultures exposed to 5.0 and 10.0 μM BCES failed to show this recovery in [^3H]TdR labeling.

Effect of BCES on mitosis. The results from studies estimating the kinetics of cell production suggested that BCES affected the ability of cells to undergo mitosis. Studies were designed to determine whether there was a block or delay in the mitotic count as a result of BCES exposure. Monolayer cultures were assayed for mitotic figures at short intervals post-BCES exposure following the 34-hr peak of DNA synthesis noted for controls (20, 24, and 29 hr postexposure). Therefore, during this 9-hr interval, a subsequent delay in mitosis, if any, would be noted. The results revealed that BCES-exposed monolayer cultures showed a significant concentration effect with respect to the total mitotic count at 20, 24, and 29 hr postexposure (Fig. 7) (two-way analysis of variance, multiple comparison, $\alpha = 0.05$ level). Monolayers exposed to as low as 0.25 μM BCES showed a 38, 34, and 48% decrease in total mitotic counts at 20, 24, and 29 hr postexposure, respectively, compared to controls. However, when monolayer cultures were exposed to 1.0 μM BCES, the results showed a 98, 88, and 60% decrease in total mitotic counts at these time points postexposure. Monolayer cultures exposed to 5.0 and 10.0 μM BCES showed the complete absence of mitotic figures at these time points (data not shown). Overall, the results suggested that, in addition to an inhibition in replicative DNA synthesis, there was a subsequent block in the cell cycle which resulted in the partial absence of mitotic figures without a delay in their appearance.

Nature of large cells. The large flat cells produced as a result of BCES exposure were studied with respect to (a) their ability to incorporate [^3H]TdR into their nuclei as re-

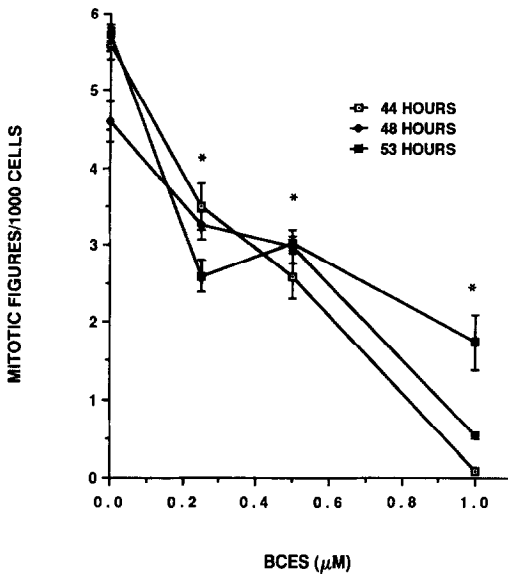


FIG. 7. Effect of BCES exposure on the mitotic counts in monolayer cultures. Monolayer cultures were exposed to solvent ($0 \mu\text{M}$) or to 0.25 , 0.5 , and $1.0 \mu\text{M}$ BCES at Day 1. Cultures were harvested at 44, 48, and 53 hr in culture for the quantitative analysis of mitotic figures. The results represent the mean total number of mitotic figures (in metaphase, anaphase, and telophase) per 1000 cells counted \pm SD from four independent determinations from two separate experiments. A minimum of 20,000 cells were evaluated for each condition. Asterisk denotes significant difference upon multiple comparisons within each time point, two-way analysis of variance, $\alpha = 0.05$ level.

vealed by autoradiography and (b) their staining characteristics by FITC-labeled GS I-B₄ and rhodamine-labeled UEA.

The results from autoradiographic studies showed that the large flat cells failed to incorporate [³H]TdR into their nuclei (Fig. 8). On the other hand, the small cells in solvent control monolayers appeared with the characteristic densely labeled nuclei (Figs. 8A, 8B). Monolayer cultures exposed to $1.0 \mu\text{M}$ BCES (Figs. 8C, 8D) showed significantly more of the smaller nuclei which became labeled than cultures exposed to $5.0 \mu\text{M}$ BCES (Figs. 8E, 8F) (18% labeling vs. 3% for $5.0 \mu\text{M}$ BCES, two-sample *t* test, $\alpha = 0.05$ level). However, under both exposure conditions, the large flat cells failed to show evidence of [³H]TdR incorporation into their nuclei.

Lectin staining was determined in monolayer cultures exposed to $5.0 \mu\text{M}$ BCES. The results of staining with GS I-B₄ and UEA revealed that some of the large flat cells exhibited granular staining of both GS I-B₄ and UEA over the entire cell surface (Figs. 9a, 9b). However, there were also areas in the monolayer (30–40% of total monolayer surface) in which these large cells showed intense UEA staining with only faint GS I-B₄ staining (Figs. 9c, 9d, highlighted by arrows). The presence of these groups of cells probably contributes to the increase in the UEA/B₄ ratio noted in BCES-exposed monolayer cultures.

DISCUSSION

The results from other efforts in this laboratory (Ribeiro, 1987) have shown that low concentrations of BCES react rapidly with and damage cellular DNA through alkylation and/or crosslinking in the monolayer cultures. Exposure of monolayer cultures to $1.0 \mu\text{M}$ BCES for 1 hr produced a maximal effect in such damage. Further studies showed that such gross damage was repaired within 22 hr postexposure (Ribeiro *et al.*, 1986).

The results of the present investigation suggested that, although gross DNA damage was repaired, certain lesions remained in the genome with consequent compromise in the ability of germinative (basal) cells to undergo replicative DNA synthesis. Coincident with this early disruption in replicative DNA synthesis was a subsequent failure of BCES-exposed cultures to produce cells through mitosis into the monolayer. This inhibition in replicative DNA synthesis and reduced level of mitosis appeared to persist until around 3 days postexposure.

At Day 4 in culture, replicative DNA synthesis appeared to resume in cultures exposed to $1.0 \mu\text{M}$ BCES, but with the fraction of nuclei replicating their DNA being only about 40% of controls. Monolayer cultures exposed to BCES concentrations greater than $1.0 \mu\text{M}$

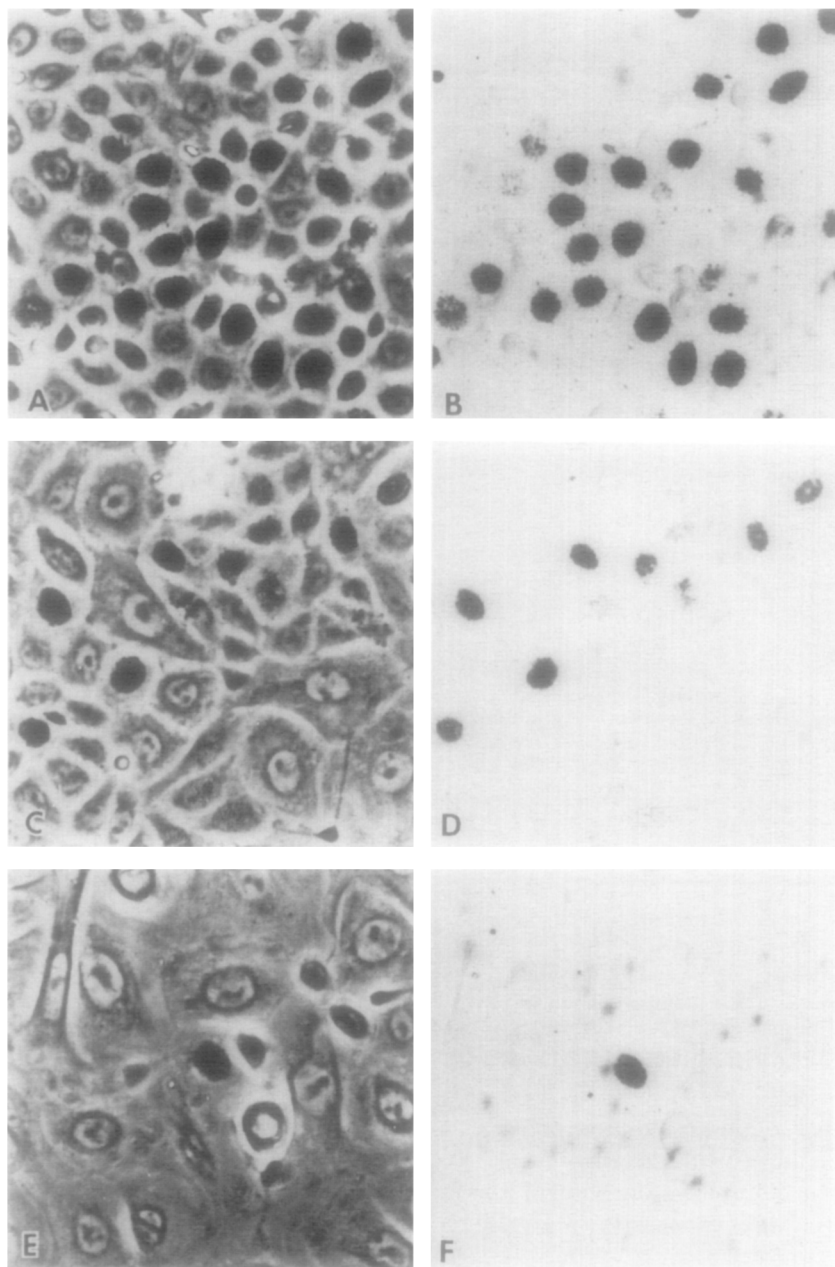


FIG. 8. Nature of large cells produced as a result of BCES exposure: Autoradiography. Monolayer cultures were exposed to 1.0 and 5.0 μM BCES or solvent at Day 1. At Day 7 (6 days postexposure), monolayer cultures were processed for autoradiography. (A, C, E) Solvent controls and 1.0 and 5.0 μM BCES-exposed monolayer cultures, respectively; (B, D, F) minus phase ring to highlight labeled nuclei.

failed to show this recovery. These findings suggested that there was an early loss of potentially proliferative (basal) cells from the

monolayer which were unable to recover from the lesions introduced as a result of BCES exposure and which would normally

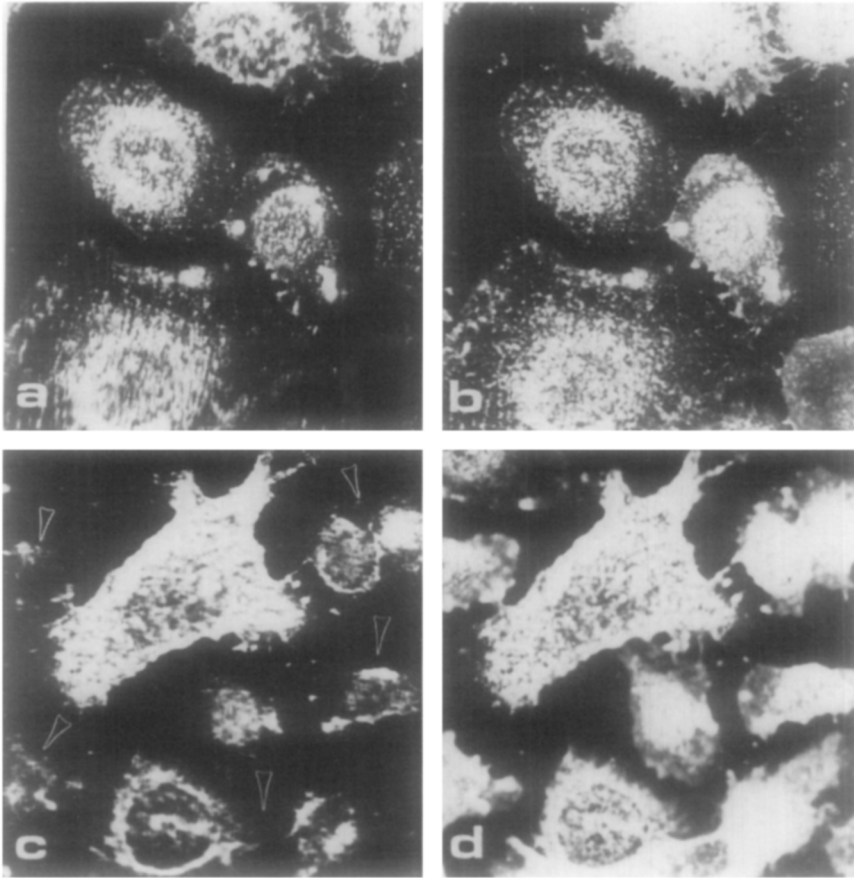


FIG. 9. Nature of large cells produced as a result of BCES exposure: UEA and GS I-B₄ staining. Monolayer cultures were exposed to 5.0 μM BCES at Day 1. At Day 7, monolayer cultures were stained with rhodamine-labeled UEA and FITC-labeled GS I-B₄ and examined fluoromicroscopically. (a, b) Large flat cells which exhibited granular staining of both UEA and GS I-B₄; (c, d) group of large flat cells which stained intensely with UEA and showed less GS I-B₄ staining (indicated by arrows). (a, c) Photographed with FITC-filters; (b, d) Same fields as (a, c) photographed with rhodamine filters.

have been part of the germinative pool (i.e., a reduction in the growth fraction).

At Day 5 in culture, a proportion of cells in monolayer cultures exposed to 1.0 μM BCES appeared to make a recovery to control levels. This proportion of cells regained their ability to undergo DNA replication and produce cells in the monolayer through mitosis albeit at a decreased level compared to controls. Monolayer cultures exposed to BCES concentrations greater than 1.0 μM failed to show this recovery and continued to lose cells from the monolayer into the medium as a result of cell death.

From the lectin binding data, the following conclusions can be drawn. First, BCES-exposed monolayer cultures appear to exhibit a gradual and selective loss of germinative (basal) cells as indicated by a loss of GS I-B₄ binding compared to controls when determined at the later stages in culture (Day 7). Second, the absence of a change in the amount of UEA bound to BCES-exposed monolayers suggests that there is a retention of some early differentiated cells which would normally slough into the medium between Days 4 and 6 in culture (Ku and Bernstein, 1988).

As mentioned previously, BCES-exposed monolayers revealed a dose-related decrease in the cell density of the monolayer with the presence of uniform enlarged flat cells throughout the monolayer. Although there was a decrease in cell density, confluency in the monolayer was maintained in BCES-exposed cultures as a result of an increased average surface area per cell.

Upon further examination, these large flat cells failed to show evidence of replicative DNA synthesis. The results from lectin staining showed that some of the large flat cells exhibited granular staining of both UEA and GS I-B₄, while other areas in the monolayer revealed groups of these cells which exhibited intense UEA staining with only faint GS I-B₄ staining. It is likely that the presence of these groups of intense UEA-staining cells contributed to the increase in the UEA/B₄ ratio noted in BCES-exposed monolayers during this time period.

The enlargement of cells after treatment with mustards has been widely reported *in vitro* (Brewer *et al.*, 1961; Crathorn and Roberts, 1965, 1966; Roberts *et al.*, 1971). Studies have demonstrated that these enlarged cells did not undergo replicative DNA synthesis, or did so at a reduced rate, and failed to undergo mitosis. To the contrary, RNA and protein synthesis continued at a normal rate in these cells. This resulting delay in cell division with continued RNA and protein synthesis led to cell enlargement (Roberts *et al.*, 1971). In these studies, rapidly proliferating and nondifferentiating mammalian cell systems were utilized.

In the present study, a proliferating and differentiating cutaneous keratinocyte culture system was employed. It is proposed that, in this cell culture system, two classes of large flat cells arise as a result of exposure to BCES: (a) basal (germinative) cells which would have normally been in the proliferative pool but being unable to replicate their DNA and/or undergo mitosis as a result of BCES exposure, continue to enlarge in size, eventually die and disintegrate (Crathorn and

Roberts, 1965; Roberts *et al.*, 1971), thus leading to a reduction in the growth fraction and (b) early differentiated cells (early spinous or spinous cells) which would normally detach and slough into the medium between Day 4 and Day 6 in culture, but which were retained in the monolayer. This may be a reflection of the failure of BCES-exposed cultures to produce new cells into the monolayer compartment through mitosis and thus to produce the proliferative pressure required to force early differentiated cells to detach and slough.

The concept of epidermal cell homeostasis was introduced to explain how the various cell compartments of the epidermis maintain a steady state in response to environmental stimuli. According to this concept, a negative feedback type mechanism exists which assures, in normal circumstances, that cells lost through desquamation are replaced by cell proliferation (Weiss and Kavanau, 1957).

The ability of the epidermis to undergo cell replacement in response to cell loss is highly dependent on an intact proliferating (basal) cell compartment. Exposure of the skin to a cytotoxic chemical such as BCES, which exerts preferential damage to the lowermost nucleated cell layers (i.e., germinative (basal) layer), could alter the regulation of cell proliferation and the commitment to differentiation and thus disrupt this steady state. The replacement of differentiated cells lost through desquamation is dependent on the capacity of germinative (basal) cells to repair gross DNA damage induced by BCES and to continue through the cell cycle and undergo cell division. It is proposed that in the absence of an intact proliferative component which exerts spatial pressure by the introduction of new progeny, the retention of some differentiated cells with a failure to desquamate results as a mechanism to preserve structural integrity.

This proposal is supported by studies on the effects of the cytostatic agent bleomycin on cell proliferation and maturation time in hairless mouse epidermis (Iversen *et al.*,

1976). The results of these studies showed that, in addition to a long-lasting inhibitory effect on epidermal cell proliferation, bleomycin prolonged the turnover time of the differentiating cells.

Overall, from the data generated in this study, it is proposed that low concentrations of the vesicant and alkylating agent BCES disrupts the normal homeostasis of cell proliferation and differentiation in this monolayer culture system. This disruption is primarily through a reduction in the growth fraction (fraction of germinative (basal) cells) consequent with a retention of some early differentiated cells, presumably early spinous or spinous cells. The failure of germinative cells to replicate their DNA and divide as a result of exposure to BCES is a responsible factor in initiating this disruption in homeostasis. The retention of some early differentiated cells may be a result of a homeostatic response mechanism designed to preserve the integrity of the monolayer under conditions in which cell replacement through proliferation is compromised by an environmental influence (i.e., BCES exposure). This response may be analogous to the situation *in vivo* for maintaining structural integrity and preserving the skin's function as a protective barrier from the external environment.

ACKNOWLEDGMENT

The authors thank Kenneth Guire, Department of Biostatistics, School of Public Health, The University of Michigan, for his assistance in the statistical evaluation of the data.

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