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THE EFFECT OF 2,2′-DICHLORODIETHYL SULFIDE ON DNA SYNTHESIS OF A MURINE STRATIFIED KERATINOCYTE CULTURE SYSTEM

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SUMMARY

A primary stratified keratinocyte culture resembling the epidermis in situ was used as a model for studying the effects of exposure to 2,2′-dichlorodiethyl sulfide, or sulfur mustard (SM), on DNA synthesis. A method that distinguishes between semi-conservative (s.c.) DNA synthesis and repair synthesis was used to determine if the former was inhibited following treatment with SM. In this method the density of the newly synthesized DNA was increased by incorporation of 5-bromo-2-deoxyuridine. Density gradient centrifugation was then used to isolate the heavy DNA for quantification. It was demonstrated that topically applied SM in the dose range of 1 – 10 nmole/cm^2 inhibited s.c. DNA synthesis (replication) in a dose and time related manner. Inhibition of DNA replication by SM would result in inhibition of cell division which must be preceded by s.c. DNA synthesis. This failure to replace damaged germinative cells may lead to the destruction of the basal layer which is observed in vivo and in our epidermal culture following exposure to SM. This may also be related to development of vesication observed in exposed intact human skin.

Key words: 2,2′-Dichlorodiethyl sulfide — Sulfur mustard — Keratinocytes — DNA synthesis — In vitro — Epidermal culture

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Abbreviations: SM, sulfur mustard; SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide; EDTA, ethylene-diamine tetraacedic acid; EBSS, Earle's balanced salt solution; PBS, phosphate buffered saline; MEM, Eagle's minimum essential medium; FBS, fetal bovine serum; 5-BUdR, 5-bromo-2-deoxyuridine; 5-FUdR, flurodeoxyuridine; s.c., semi-conservative.

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INTRODUCTION

This paper reports the use of a primary stratified keratinocyte culture grown at the air-liquid interface as an in vitro model of the epidermis to investigate the effect of SM on DNA synthesis in the culture. This keratinocyte culture represented an integrated tissue having most of the components of the parent tissue in terms of organization, morphological features, biochemistry and homeostasis [1–3]. By virtue of these properties, the culture system was amenable to topical exposure, which is the major route of exposure in cutaneous toxicology. Therefore this system offered the advantages of a simple and convenient in vitro system permitting more focused experimentation compared to using in vivo models. Another merit of this system was that it did not suffer from the usual pitfall of most cell cultivation systems, i.e. not adequately resembling the parent tissue.

SM is a potent vesicant for human skin. It is also a powerful alkylating agent capable of alkylating DNA, RNA and protein [4,5]. The mechanism of vesication of this agent is unknown. However, it selectively affects the basal layer, the compartment to which DNA replication and cell division are normally restricted [6,7].

In a previous study using the same culture system, it was demonstrated that the synthesis of DNA was more sensitive to SM than the syntheses of RNA or protein, suggesting that DNA was the primary target for SM [3]. Since the synthesis of DNA was studied simply by observing the incorporation of [^{3}H]thymidine into DNA, no distinction could be made between s.c. DNA synthesis (i.e. DNA replication) and repair synthesis. There is ample evidence in the literature to indicate that DNA alkylated by SM is subject to repair [8–10].

This study was undertaken to determine if the previously observed inhibition of DNA synthesis in the culture following exposure to SM resulted specifically from the inhibition of DNA replication. Both DNA replication and repair synthesis occur when DNA polymerase uses one strand of the double stranded DNA as a template to synthesize a new strand. However, s.c. DNA synthesis involves the synthesis of an entire new strand of DNA using one old strand as the template and results in two new DNA strands. Repair synthesis occurs only in small patches in the loci of damage. Synthesis of a new strand of DNA in the presence of 5-BUdR would result in the incorporation of enough 5-BUdR to increase the mass of this DNA to allow it to be separated from parental DNA in a neutral gradient of cesium chloride. In the case of repair, incorporation of 5-BUdR would be insufficient to render the resulting DNA heavy enough to be separated in a neutral gradient.

MATERIALS AND METHODS

Obtaining stratified keratinocyte cultures

The two stage cultivation procedure used to obtain the mature keratinocyte cultures has been described elsewhere [1,3]. Briefly, the epidermis of rat skin was mechanically separated from the dermis after enzymatic treatment of full

thickness skin with trypsin. The under side of the epidermis was then brushed into growth medium (90% MEM:10% FBS) to obtain a suspension of basal and supra-basal cells. The basal cell population was then purified in a density gradient of percoll and resuspended in fresh growth medium to obtain approximately 5×10^5 cells/ml of predominantly basal cell keratinocytes, which were then seeded on Puropor nylon membranes (Gelman Sciences catalogue 66–470) (47 cm in diameter) used as the growth substratum. Once attached, the cells were cultivated in growth medium in a 5% CO_2-95% air incubator at 35°C and 95% humidity. When a confluent monolayer was formed, the nylon membranes with the monolayer were lifted to the air-medium interface by placing them on glass fiber pads soaked in growth medium. This enhanced differentiation and stratification of the cultures. They were maintained in this state for approximately 2 weeks. At this cultivation stage, they formed a multilayered epidermal-like tissue with cornified layers [1]. Epidermal cultures were always incubated under these standard conditions unless otherwise noted.

Materials for measuring s.c. DNA synthesis of the keratinocyte cultures following exposure to SM

SM was provided by the United States Army Medical Research Institute of Chemical Defense, Aberdeen Proving Grounds, MD. DMSO was obtained from Fisher Scientific, Fairlawn, NJ. Tritiated thymidine ([^{3}H]thymidine) (specific activity 80 Ci/mmol) was purchased from New England Nuclear, Boston, MA. EDTA, tris base, 5-BUdR, 5-FUdR, SDS and protease K were all obtained from Sigma Chemicals, St. Louis, MO. Cesium chloride (CsCl) was purchased from US Biochemicals Corp., Cleveland, OH. Aqueous counting solution (ACS) was purchased from Amersham, Arlington Heights, IL.

Methodology

Topical exposure of the keratinocyte cultures to SM. Prior to treatment with SM, keratinocyte cultures were incubated for 2 h in growth medium containing BUdR:FUdR (100 μM:10 μM). This was determined by previous investigators to ensure that no artefact would result from the incorporation of the heavy base into the parental strand during the labeling period [10]. The cultures were then transferred to fresh growth medium and exposed topically to 0.8 ml of various doses of SM (diluted in 70% DMSO) by placing the solution gently on the surface of the cultures. The doses used in this study were 0.01, 1.0 and 10 nmol SM/cm^2 of culture surface. The fact that exposure was indeed topical and not happening via spillage into the underlying medium was confirmed in another study by autoradiographic observation of cultures treated with [^{14}C]SM [11]. The SM was then removed after 30 min by washing with EBSS, following which the cultures were transferred to another petri dish containing fresh growth medium and incubated for the selected post-exposure periods.

Procedures for radiolabeling and density labeling the DNA of the cultures and for obtaining a DNA solution. The DNA of the cultures were radiolabeled with 5 μCi/ml of [^{3}H]thymidine and density labeled with a mixture of 5-BUdR:FUdR (100 μM:10 μM) by transferring the cultures to a petri dish with medium contain-

ing the above stated quantities of labels and incubating the cultures under standard conditions for 4 h. It was established by previous investigators that use of a combination of [^{3}H]thymidine and cold 5-BUdR was more efficient than [^{3}H]BUdR, which is labile and more expensive [12]. The small quantity of 5-FUdR was included in the labeling medium to prevent de novo synthesis to ensure maximum incorporation of thymidine [12]. After the labeling period was completed, the cells from the cultures were harvested by exposure to trypsin. Prior to trypsin treatment, the cultures were incubated for 10 min at 35°C in PBS containing 0.02% EDTA, following which they were incubated for 15 min with 0.05% trypsin dissolved in the PBS/EDTA solution. The cells from the culture were then collected by brushing them from the membrane into growth medium. The cell suspension was centrifuged and the cell pellet obtained was stored at 4°C until the cells were lysed.

Cells were lysed with SDS and the cell lysate was digested with protease K [12]. To 3.5 ml of DNA solution in Tris buffer at pH 8.0, 4.3 g of CsCl was added to obtain a refractive index of 1.400, which was equivalent to about 1.7 g of CsCl/ml. This solution was centrifuged at 27 500 rotations per min for 48 h in a SW50.1 rotor in a L8 Beckman ultracentrifuge. These conditions were determined to be optimal for separating the heavy DNA from the normal density DNA.

Fractions of equal volume were collected from the bottom of the tube with a needle. From each fraction, a 50 μl aliquot was dissolved in ACS and used to quantify radioactivity by liquid scintillation spectrometry. Another 50 μl from each sample was used to quantify DNA by a fluorescence method using diaminobenzoic acid [13].

Measuring s.c. DNA synthesis. When the DNA labeled with [^{3}H]thymidine and 5-BUdR was subjected to ultracentrifugation in CsCl, a density gradient was formed. The 'heavy' DNA sedimented to the lower region of the gradient compared to the normal density DNA. For each dose, a profile of counts/min and quantity of DNA in μg versus the fraction numbers of each gradient had two peaks, i.e. the [^{3}H]thymidine labeled heavy DNA peak and the normal density DNA peak [12].

s.c. DNA Synthesis in counts/min/μg DNA was determined for each curve. The percent inhibition of s.c. DNA synthesis for each dose was calculated by comparing the quantity of [^{3}H] in the heavy band obtained for the treated cultures with that of the untreated control cultures. The means of at least two samples for each observation were statistically evaluated and the null hypothesis tested by comparing two means using the Student's *t*-test.

RESULTS

These results describe the data obtained when the cultures were exposed to three concentrations of SM, i.e. 0.01, 1 and 10 nmol/cm^2 of culture surface. Figure 1 was taken from experiment number 1 and shows the density gradient profiles of DNA obtained for untreated cultures (Fig. 1a), cultures exposed to solvent alone (Fig. 1b) and cultures 4 h after an initial 30 min exposure to 0.01,

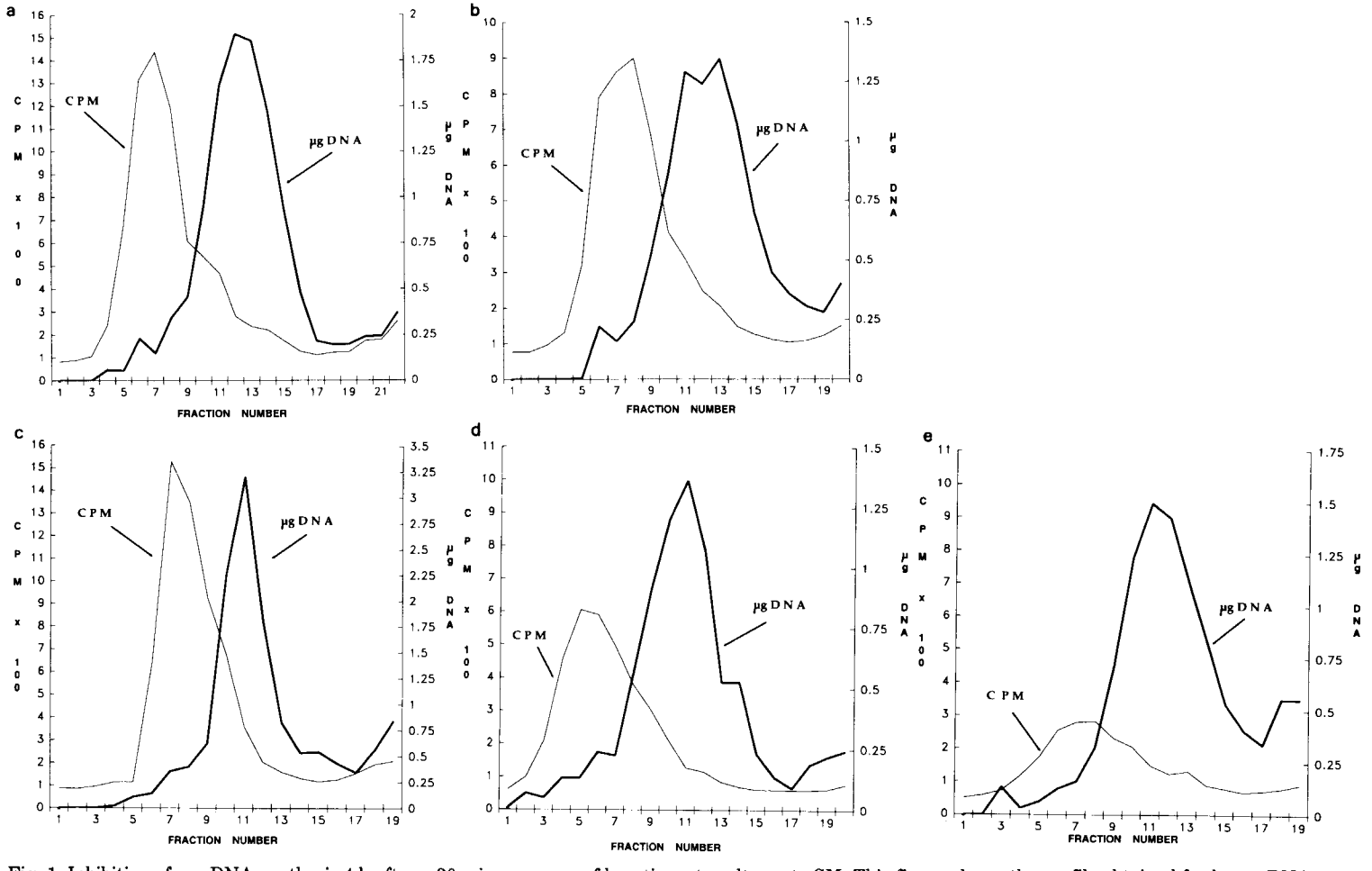

Fig. 1. Inhibition of s.c. DNA synthesis 4 h after a 30-min exposure of keratinocyte cultures to SM. This figure shows the profile obtained for heavy DNA (counts/min) and normal density DNA (μg DNA) versus fraction number in each gradient. The figure contains the untreated control (a), DMSO only (b), and each dose of SM in nmoles/cm^2, i.e. 0.01 (c), 1.0 (d) and 10.0 (e). Epidermal cultures which were pre-treated for 2 h in 5BUdR:FUdR (100 μM:10 μM) were exposed to various doses of SM. They were then labeled for 4 h in a solution containing [^{3}H]thymidine:5-BUdR:5-FUdR (5 μCi/ml:100 μM:10 μM) and then lysed to obtain DNA. The DNA was then mixed with CsCl and ultracentrifugation performed to obtain a density gradient. After centrifugation, the centrifuge tubes were pierced at the apex and fractions of equal volume obtained. For each fraction, the amount of heavy DNA in counts/min and normal density DNA in μg were determined. All of the above procedures are described in the text. Note that the scales for counts/min and μg DNA (y 1 and y 2) were adjusted for each curve resulting from each treatment because the individual epidermal cultures used did not contain identical amounts of DNA.

1.0 and 10 nmol/cm^2 SM (Fig. 1c, Fig. 1d and Fig. 1e, respectively). This figure represents the data from one of two sets of experiments performed to measure the level of inhibition of s.c. DNA synthesis 4 h after exposure to concentrations of SM. Similar curves were generated to determine the level of inhibition 6 h after exposure to SM. The first (left) peak in each profile represents the newly synthesized heavy DNA measured from the [^{3}H]thymidine labeling of DNA, whereas the peak in the right of each profile represents the parental DNA determined by the fluorescence method. It is apparent that the first peak representing heavy DNA diminishes in size when the cultures were exposed to concentrations of SM at 1.0 nmole/cm^2 and above. However, no comparisons between treatments can be made until s.c. DNA synthesis is calculated as a specific activity, i.e. counts/min/μg DNA. Table I from experiment number 1 shows the data used to determine the amount of s.c. DNA synthesis in counts/min/μg DNA that occurred during the 4-h labeling period. These data were derived from the profiles generated in Fig. 1.

Figure 2 shows the combined results of the effect of SM on the inhibition of s.c. DNA synthesis 4 and 6 h after exposure. The inhibition of s.c. DNA synthesis was expressed as percent of control, as described above and each observation is an average of two experiments. As can be seen from this figure, the inhibitory effect of SM increased with dose and period of exposure. The solvent caused some inhibition of synthesis both 4 and 6 h after exposure. The inhibitory effect of 0.01 nmol/cm^2 of SM was not significantly different from that of the solvent at either 4 or 6 h following exposure. However, 1 nmol/cm^2 caused a significant amount of inhibition of s.c. DNA synthesis 4 h after exposure and this inhibition

TABLE I

S.C. DNA SYNTHESIS 4 h AFTER EXPOSURE OF EPIDERMAL CULTURES TO SM: EXPERIMENT NUMBER 1

	Treatment				
Control	SM (nmol/cm^2)				
		Solvent	0.01	1.0	10.0
Radioactivity (counts/min) in heavy DNA peak	5167	3252	5004	2514	1622
Total DNA in the gradient (μg)	11.96	9.48	13.0	8.14	10.07
s.c. DNA synthesis (counts/min/μg DNA)	432	343	384.9	308.8	161.1

This table contains the data generated in Experiment number 1 to measure s.c. DNA synthesis 4 h after a 30-min exposure to SM. Similar data were generated for the 6 h post-exposure period and both exposure periods duplicated in experiment number 2. The table shows (1) the amount of heavy DNA (in counts/min) which was obtained by pooling all counts in the heavy DNA peak in the region that did not overlap with the normal density DNA peak and (2) the total amount (in μg) of normal density DNA. These data were obtained from the curves in Fig. 1. With these data, s.c. DNA synthesis was calculated in counts/min/μg DNA, i.e. as a specific activity of the total quantity of DNA in each culture. Only when this is computed can a comparison be made between treatments.

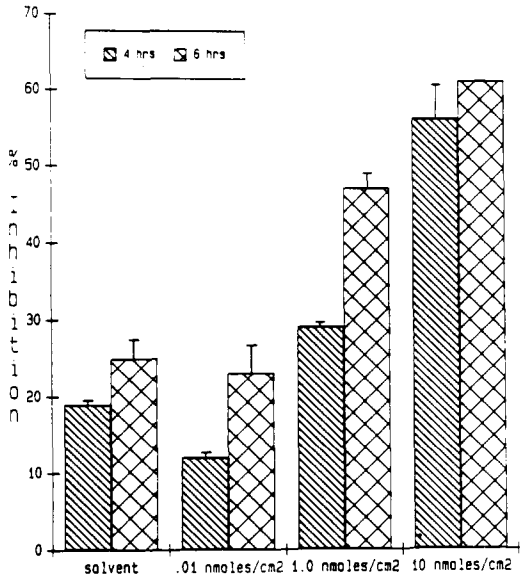

Fig. 2. The inhibitory effect of 0 – 10 nmole/cm^2 of SM on s.c. DNA synthesis by epidermal cultures 4 and 6 h following a 30-min exposure. The data are expressed as percent inhibition of s.c. DNA synthesis as compared to untreated control cultures. Bar values represent means ± S.D. for two experiments.

increased to 38% by 6 h after exposure. With the highest dose of 10 nmol/cm^2 SM, a much greater amount of inhibition occurred at both time periods examined.

DISCUSSION

The murine primary culture used in this study represents a system deviating minimally from the parent tissue hence permitting the investigation of the effect of SM on the epidermis when applied topically. Application of SM to the surface of this stratified, cornified culture of keratinocytes derived from newborn rat skin has previously been shown to result in a dose-responsive inhibition of DNA, RNA and protein synthesis [3]. The inhibitory effect on DNA synthesis, presumably a result of alkylation of the nucleic acid by SM [5,15,17], was seen at a lower concentration and sooner than on RNA or protein synthesis. However, each of these synthetic processes was affected at much lower exposure than was necessary to obtain irreversible necrosis of the germinative cell layer in the culture [11] suggesting that inhibition of s.c. DNA synthesis is a specific effect of SM and not a result of general cytotoxicity. Destruction of the epidermal basal layer of cells is a major concomitant of the vesicant response in skin exposed to SM [6,20]. Since replication of cellular DNA is a prerequisite for mitosis in the germinative population and, therefore, for the survival of a stratified, cornified culture of keratinocytes, the inhibitory effect of SM on s.c. DNA synthesis has been evaluated in terms of the level of exposure to SM.

It was clearly demonstrated in the present study that SM inhibits s.c. DNA synthesis in the epidermal culture in a dose and time related manner. However, the solvent, i.e. DMSO, had some effect on s.c. synthesis as well. DMSO is a widely used solvent for topical exposure by virtue of its ability to penetrate the epidermal barrier. For topical exposure of a chemical such as SM which is very unstable in an aqueous environment, selection of a completely innocuous organic solvent is difficult and in a search for an appropriate solvent, we found that 70% DMSO was the least toxic [3]. The dose level of 0.01 nmol/cm^2 SM did not result in a significant inhibition when compared to the data obtained from solvent controls following both exposure periods. In fact, this dose of SM caused an apparent lower inhibition of s.c. DNA synthesis than the solvent control. In a previous study [3] exposure of the epidermal culture to 0.01 nmol/cm^2 SM resulted in an increase in [^{3}H]thymidine uptake as compared to solvent controls. Observations from that study and the present one suggests that SM may stimulate DNA synthesis at low concentrations. Nevertheless, a dose of 1.0 nmol/cm^2 caused a significant amount of inhibition after a 4-h exposure. This inhibition had increased by 38% at 6 h after exposure. Ten nmol/cm^2 caused 56% inhibition of s.c. DNA synthesis which persisted up to 6 h after exposure. In contrast, protein synthesis was unaffected by an exposure of 10 nmol/cm^2 but was significantly inhibited by 50 nmol/cm^2 [3]. Similarly, cells in the germinative basal layer showed slight pathology at 10 nmol/cm^2 and nearly complete destruction after an exposure to 50 nmol/cm^2 [11].

Roberts et al. [10] studied effects of SM on s.c. DNA synthesis indirectly utilizing synchronous cultures of HeLa cells. There appears to be no publication of a prior study of the effects of this agent on DNA synthesis in cultures of keratinocytes by the technique utilized in this investigation which allows one to evaluate the degree of s.c. DNA synthesis in a culture that is not synchronized. More importantly, since this stratified, differentiated epidermal culture system more closely mimics in vivo characteristics of the epidermis both structurally and biochemically, it serves as a better model for studying the effects of SM following topical exposure. By allowing the culture to carry out new DNA synthesis in the presence of [^{3}H]thymidine, 5-BUdR and 5-FUdR, sedimenting the isolated DNA in a CsCl gradient and determining the [^{3}H] in the heavy (i.e. more dense) band in the gradient, one can determine the amount of s.c. DNA synthesis (relative to total synthesis) which has occurred. The amount of s.c. DNA synthesis is proportional to the amount of ^{3}H found. The amount of repair synthesis is proportional to the amount of ^{3}H in the less dense or parental band.

SM has been shown to react directly with DNA in bacteriophage and mammalian cells [4,15]. Inhibition of DNA synthesis in an exposed cell is probably a result of alkylation of the nucleic acid by SM [4,9,14,17]. The sites of attack appear to be mainly at the N^7 position of guanine and to a lesser extent at other positions on guanine and on the other bases [5,15,17]. Inter- and intrastrand diguanyl cross links have been detected (compare with Ref. 17) and may be responsible for the cytotoxic action of SM [9,18]. Further studies directed towards identifying and quantifying the different alkylation products in a similar time frame in this culture exposed to SM are needed to establish the underlying

cause for the inhibition of s.c. DNA synthesis. Eukaryotic cells seem to be able to remove cross links [21] as well as mono-functional adducts [10] and resume synthesis of DNA. In fact, the appearance of single strand breaks in the DNA of this epidermal culture exposed to SM [19], is consistent with the cell's attempt to repair its damaged DNA.

Since s.c. DNA synthesis is inhibited following exposure to SM, it can be presumed that mitosis in the germinative population is also inhibited (compare with Ref. 22). The absence of cell division in an exposed epidermis would seriously compromise the integrity of the tissue since the epidermis relies on a constant supply of new cells to replace the cells normally sloughed from the surface of the skin. If the level of exposure is sufficiently great that repair of the DNA does not occur, s.c. DNA synthesis is irreversibly inhibited and mitosis will not resume in the epidermal germinative cells. Necrosis of the basal layer is an absolute consequence leading to vesication at the site of exposure.

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