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Chemical Blistering: Cellular and Macromolecular Components (U)

Annual Report

I. A. Bernstein, Ph.D.

December 31, 1989

Supported by

U. S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701-5012

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The University of Michigan
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The overall purpose of this investigation is to elucidate the molecular mechanisms by which bis(beta-chloroethyl)sulfide (BCES) exerts its vesicant action when applied topically to human skin. The technical objectives of the project are to establish morphological, cytochemical, and/or biochemical indicators of epidermal mustard toxicity and to examine and evaluate the relevance of these indicators to vesication. The following three biological systems have been used in this study: a) stratified, cornified primary cultures of keratinocytes grown on nylon microporous membranes positioned at the air-medium interface; b) submerged monolayer cultures of primary keratinocytes that contain early differentiated as well as germinating cells and are grown in a medium having a low level (0.08-0.10 uM) of Ca^{2+}; and c) cultures of the BSC 40 line of monkey kidney cells grown as a submerged monolayer.

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Ca2+, (calcium ions)

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The following specific aims guide the project: a) to identify the most sensitive biochemical and/or morphological parameter(s) of toxicity in keratinocyte cultures exposed to BCES by determining dose-responsive changes in DNA structure and metabolism, mitosis, differentiation and morphology, and b) to ascertain the relevance of the abnormality seen at the lowest dose of BCES in such cultures to the cellular necrosis which is requisite for vesication.

Earlier studies in this investigation showed that a) DNA synthesis was inhibitied by a lower concentration of BCES than were RNA or protein synthesis, b) the integrity of DNA structure was compromised by exposure of keratinocytes to this low level of BCES but that gross structural damage could be repaired by the cells, c) such low levels of exposure decreased the viability of the cultures, and d) exposed cultures developed abnormalities in cell surface glycoconjugates, in keratins and in morphology and physiology. The morphological changes included the appearance of abnormally large cells that had the cell-surface characteristics of differentiated cells and did not exhibit mitotic activity. Physiological changes included extended inhibition of DNA replication and mitosis. These residual effects of exposure to BCES suggest that the mustard imposes an informational error in the DNA and that this misinformation is responsible for the ultimate demise of the culture and, possibly, to vesication in intact skin exposed to BCES in vivo.

During the period covered by the present report, it has been shown that a) alkylation of DNA is dose-responsive to the concentration of BCES, b) basal cells are more susceptible to alkylation from topically applied BCES than are differentiated cells, c) BCES applied topically penetrates the tissue in affecting the basal layer, d) exposure of monkey kidney cells to a low level of BCES interfers with the repair of DNA containing mismatched base pairs, and e) HPLC with a reverse phase C18 column and an elution program of an increasing concentration of methanol and decreasing concentration of tetrabutyl ammonium hydrogen sulfate, can be used to collect BCES-mediated adducts from the DNA of exposed cells.

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The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

I. SUMMARY

The overall objective of this investigation is to elucidate the molecular mechanisms by which bis(beta-chloroethyl)sulfide (BCES) exerts its vesicant action when applied topically to human skin. The study is being done using cultures of cutaneous keratinocytes in order to focus on the direct interactions between the mustard and its cellular targets. The technical objectives of the project are to establish morphological, cytochemical, and/or biochemical indicators of epidermal mustard toxicity and to examine and evaluate the relevance of selected parameters to vesication. This information should help to develop an understanding of the molecular etiology of the cutaneous injury that results from exposure to BCES and to provide a rational basis for proposing and testing prophylactic and/or therapeutic measures.

The following specific aims guide the study: a) to identify the most sensitive biochemical and/or morphological parameter(s) of toxicity in keratinocyte cultures exposed to BCES by determining dose-responsive changes in DNA structure and metabolism, in mitosis, and in differentiation and morphology; and b) to ascertain the relevance of the abnormality (or abnormalities) seen at the lowest dose of BCES in such cultures to the cellular necrosis which is requisite for vesication.

Earlier studies, using both submerged monolayer cultures and stratified, cornified cultures grown at the air-liquid interface, showed that the incorporation of ^{3}H-thymidine into DNA was inhibited by exposure to a lower concentration of BCES than was the incorporation of ^{3}H-uridine into RNA or ^{3}H-leucine into protein. In the monolayer culture, exposure to 20 uM BCES inhibited the incorporation of ^{3}H-thymidine by about 80%, whereas an inhibition of only 18% in the incorporation of ^{3}H-uridine and no significant inhibition in the incorporation of ^{3}H-leucine was observed. In the stratified, cornified culture, application of 1 nmol BCES/cm^2 of culture surface area resulted in an inhibition of 20% as compared to a solvent control. At this level of exposure, neither the incorporation of ^{3}H-uridine nor the incorporation of ^{3}H-leucine was significantly affected. By the criterion of the incorporation of precursor, it appears that DNA is the most sensitive macromolecular target for BCES.

Studies carried out in the monolayer culture by the nucleoid sedimentation assay showed that gross structural damage to the DNA occurred as a result of exposure to 1 uM BCES. The nucleoid sedimentation rate was equal to control by 22 hr after exposure, which suggested that repair of the DNA occurred by that time after exposure. However, when the incorporation of ^{3}H-thymidine was measured by autoradiography to evaluate replication of DNA, no replication occurred until 24 to 48 hr after exposure. After exposure to 1 uM BCES, mitosis did not resume until 72 hr. No gross morphological effects were observed during the first 48 hr after exposure to 5 uM BCES. Thereafter, abnormally large cells appeared in the culture. These cells had the characteristics of the differentiated state. The number of these cells increased and the time after exposure for their appearance decreased as the dose of BCES was increased.

The inhibition of mitosis for several days after exposure to 1 uM BCES and the appearance of the abnormally large cells, as well as changes in the

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cell surface glycoconjugates and the maturation of keratin which occurred after exposure to this level of BCES, suggest that the mustard imposes a lesion upon the cells which the cells cannot reverse and which leads to loss of cellular viability. An informational change in the DNA could account for these abnormalities.

By lightmicroscopic observation of gross morphology in stratified, cornified cultures exposed to mustard, necrosis of the basal layer - an effect seen in cutaneous vesication after exposure in vivo - was complete by 48 hr after a topical exposure to 50 nmol/cm^2 of culture surface. After application of 10 nmol/cm^2 only focal necrosis of this layer was seen. Because the toxic effect was seen only some time after exposure, it appears unlikely that the toxic response was a result of dissolution of the cell by the mustard. As a working hypothesis, it is proposed that the cell suffers a lesion in the DNA - as a result of either a failure in the repair process or repair that occurs with low fidelity - such that the cell's normal biochemical and physiological activities cannot be carried out without resultant cell death.

During the period covered by this report, the following four goals were sought: a) completion of an alkylation study in the stratified, cornified culture; b) demonstration that BCES applied topically permeated down through the tissue; c) evaluation of the hypothesis that errors in the repair system are responsible for the failure of exposed cells to recover completely; and d) identification of the nature of the lesion in the DNA caused by BCES.

It was shown that alkylation of DNA by ^{14}C-BCES is dose-responsive in the stratified, cornified culture and that DNA in the basal cells is more highly alkylated than is the nucleic acid in the differentiated population. From the level of incorporation of the labeled mustard, it appears that this technique will be useful in future studies designed to isolate and identify adducts.

Autoradiography demonstrated that ^{14}C-BCES applied topically reached the basal layer by permeation through the culture rather than by spillage into the medium and diffusion into the culture from the medium. By the end of the first 3 min of exposure, ^{14}C was localized in the cornified and granular layers. After 6 min, a few silver grains were seen over the basal layer. By 15 min, silver grains were present evenin the nylon membrane.

The methodology required to evaluate the effects of BCES on mismatch repair of DNA was developed in monkey kidney cells and is now ready for application to keratinocytes. Preliminary experiments with the monkey kidney cells indicated that exposure to BCES results in a dose-responsive decrease in the rate at which mismatch repair is carried out.

The development of an HPLC technology for identifying DNA adducts has been initiated using a C18 reverse phase column eluted with a gradient of increasing concentrations of methanol and decreasing concentrations of tetrabutyl ammonium hydrogen sulfate.

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II. FOREWORD

The source of animal tissue for the primary cultures described in this report was skin from neonatal rats derived from the CFN strain by random mating and reared in The University of Michigan School of Public Health animal f facility is under the supervision of the University Unit for Laboratory Animal Medicine. The University of Michigan is accredited by the American Accreditation Association for Laboratory Animal Care (AAALAC). In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW Publication No. (NIH) 78-23, Revised 1978].

The source of human tissue was foreskin obtained at routine circumcision done at Women's Hospital, The University of Michigan Medical Center, and provided without identification of the donor. The form utilized to obtain "informed consent" was the one in use by the hospital for routine circumcision. Signature on this form allows experimental use of tissues. The use of this tussue for the present project has been approved by a University Human Subjects Review Committee and, for the protection of human subjects, the investigators have adhered to policies of applicable Federal Law 45CFR46.

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III. NARRATIVE REPORT OF PROJECT PROGRESS

A. Statement of the Problem and Experimental Rationale for the Study

The overall objective of this investigation is to elucidate the molecular mechanisms by which bis(beta-chloroethyl)sulfide (BCES) exerts its vesicant action when applied topically to human skin. The theoretical and technical advances which have occurred in cutaneous biology over the past several decades encourage the view that this objective can be achieved. It appears likely that the most productive application of the new knowledge is in experiments involving cultures of epidermal keratinocytes. Studies _in vitro_ should allow better-controlled experiments and should yield more reproducible data than will studies _in vivo_.

A tissue culture of cutaneous keratinocytes provides a biological system in which the direct interaction of BCES with molecular and cellular elements can be studied without the systemic influences that secondarily affect the toxic manifestations _in vivo_. Since the culture has the morphological and biochemical characteristics of the epidermis _in vivo_, the mode of human exposure, topical application, can be mimicked experimentally and the results can be credible in terms of application to the situation _in vivo_. Furthermore, use of human keratinocytes allows the study to be as close to the "human condition" as one can come without trauma to a human volunteer.

The technical objectives of this project are to establish morphological, cytochemical, and/or biochemical indicators of epidermal mustard toxicity and to examine and evaluate the relevance of selected parameters to vesication. Molecular parameters of toxicity are to be tested in the submerged monolayer culture to establish a range of effective exposures to BCES, to determine which indicators of toxicity appear at the lowest exposure, and then to determine whether these parameters can be monitored in stratified cultures after topical application of BCES. The information derived from this study should aid in elucidating the molecular etiology of the cutaneous injury from exposure to BCES _in vivo_ and in providing a rational basis for proposing and testing prophylactic and therapeutic measures.

The specific aims which guide the study are:

1. To identify the most sensitive biochemical and/or morphological parameter(s) of toxicity in stratified, cornified keratinocyte cultures exposed topically to BCES by determining dose-responsive changes in DNA structure and metabolism, in mitosis, and in differentiation and morphology.

2. To ascertain the relevance of the abnormality (or abnormalities) seen at the lowest dose of BCES in such cultures to the cellular necrosis which is requisite for vesication.

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B. Background and Literature Review

1. Anatomy of blistering

Application of BCES to human skin results in an initial erythema
followed by blistering. Stoughton (1971) has noted that in vesication,
"fluid accumulation is almost always secondary to fundamental damage to the
cellular structures," and has defined a blister as an "abnormal
accumulation of fluid, completely replacing the pre-existing tissue
structure, capped by a part or all of the epidermis." The blister seen
after exposure to BCES fits this description. After exposure to this agent,
the progression to ultimate blistering proceeds irrevocably unless the
action of the chemical is neutralized within the first several minutes of
exposure. Warthin and Weller (1919) and Sinclair (1949) noted that the
process initially involves destruction of the basal and lower spinous
layers of the epidermis. Presumably, the cellular destruction that
produces a cavity in the tissue precedes the actual accumulation of fluid.
At later stages in the destructive process, the necrosis may spread to the
upper spinous and granular layers as well as into the dermis. Blistering
is rare in animals and appears to be a reaction seen primarily in man, in
whom it may arise as a component of various cutaneous diseases and as a
result of exposure to some biological (e.g., herpes virus), physical (e.g.,
ultraviolet radiation), or chemical (e.g., mustards) stressors. The blister
may develop at different levels in the skin, depending upon the etiologic
agent involved.

2. Biochemistry of blistering

Since different vesicants produce blisters at different levels in the
tissue, it would not be unexpected if the biochemistry involved were
different, depending on the location of the blister.

Exposure to a mustard _in vivo_ will cause separation of the dermal-
epidermal junction; i.e., the blister appears at that level in the tissue.
From a description of the time course of dermatopathologic development and
the time after exposure within which the process can be reversed, it seems
clear that the molecular course of the pathologic process is set within
the first 3 min of exposure _in vivo_. Warthin and Weller (1919) found that
erythema, inflammation, and vesication were not reversed by therapy (i.e.,
application of chlorinated lime) initiated after 3 min of exposure to BCES.

Although the molecular mechanism of the effect of BCES is unknown, it
is known that substances in this class are powerful alkylating agents of
DNA; it could be reasoned then, that alkylated DNA could lead to inhibition
(or at least delay) of replication; to generalized breakdown of damaged DNA
-- leading to cell death (cf. Wheeler, 1962) and to low-fidelity repair --
resulting in mutations, with consequent disruption of normal metabolic
function (Kirner, 1946; Wheeler, 1962). Mustards can also alkylate RNA,
with consequent interference in the translation of genetic information and
protein (Ross, 1962), resulting in metabolic disruption (Wheeler, 1962).
BCES, being a bifunctional mustard, can also produce inter- and intra-
strand DNA cross-links and cross-links between DNA and RNA or DNA and
protein. The most important molecular target of BCES appears to be DNA (Fox
and Scott, 1980). BCES alkylates and cross-links at the purine bases.

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Alkylation of the phosphate groups in DNA can also occur. On the basis that damaged proteins can be replaced, whereas damaged DNA may result in irrevocable changes, the DNA seems to be the most important target of toxicity by BCES.

Exposure of isolated skin to a vesicant for as little as 5 min can result in the inhibition of glycolysis and respiration (Barron et al., 1948). Glycolysis is inhibited by a lower concentration of toxicant than is necessary to obtain inhibition of respiration. In the case of exposure to mustards, this effect seems to be a consequence of a reduced level of pyridine nucleotides in the cell (Holzer and Kroger, 1958; Frazer, 1960). Recent evidence suggests that BCES causes a lowered level of pyridine nucleotides by virtue of the stimulation of poly-ADP-polymerase (Gross et al., 1983).

3. Repair of alkylated DNA

In bacteria, the ability to excise BCES-alkylated products from DNA is associated with increased resistance to the mustard (Lawley and Brookes, 1965). Apparently, both mono- and bifunctional adducts can be removed from DNA, since comparable amounts of induced repair synthesis have been observed in HeLa cells exposed to doses of BCES and to the analogous half-mustard both of which compounds are equally toxic (Roberts et al., 1971). However, there appears to be disagreement as to the relative rates at which repair of the two types of lesion occurs (Reid and Walker, 1969; Roberts et al., 1971). Given the mechanisms that are probably involved in the two repair processes, it seems unlikely that removal of the cross-links could occur as quickly as repair of monofunctional adducts (cf. Fox and Scott, 1980). It appears more likely that the cross-link would be removed one arm at a time rather than by both arms being removed simultaneously, since the latter process results in a double-strand break, which could be lethal. Of course, this hypothesis is based upon the assumption that the mechanism of repair is not simple base replacement.

Detailed information on the molecular mechanisms by which BCES-mediated lesions in DNA are repaired does not seem to be available. However, some possible mechanisms can be formulated based upon repair of DNA that has been damaged by other alkylating agents. BCES has been shown to alkylate guanine at N7 and adenine at N3 in DNA in the ratio of 60% to 15%, while other sites in guanine and adenine are alkylated to lesser extents (Fox and Scott, 1980). The pyrimidines can also be alkylated. Cross-linking between interstrand guanines and alkylation at O6 of guanine may indeed be the most damaging mechanism from a physiological point of view (cf. Ludlum et al, 1984). Alkylated bases may depurinate spontaneously at $37^{\circ}C$ and neutral pH (Lindahl, 1979). Alkylated guanine appears to be released faster from its glycosidic linkage at acid and neutral -- but not at alkaline -- pH than is alkylated adenine, and both are released faster than alkylated pyrimidines (Lindahl and Nyberg, 1972). Alkylated purines and pyrimidines may also be hydrolyzed at the glycosidic linkage by specific glycosylases; a specific glycosylase, though, has yet to be unequivocally demonstrated for alkylated guanine (Lindahl, 1982). Some evidence exists for an enzyme which inserts the appropriate base at an apurinic site (Deutsch and Linn, 1979). Another mechanism for the specific repair of an apurinic or apyrimidinic site is via excision of the

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deoxyribose phosphate moiety and replacement of the entire nucleotide. The apurinic or apyrimidinic site is excised by the sequential action of an endonuclease specific for the apurinic or apyrimidinic deoxyribose phosphate, which inserts a nick in the DNA chain on one side of the sugar phosphate to be removed, and a non-specific exonuclease, which hydrolyzes the phosphate sugar linkage on the other side of the unit to be excised. According to this mechanism, replacement of the new nucleotide would occur by DNA polymerase and DNA ligase. Another mechanism for the excision of the damaged nucleotide is dark excision, by which some of the nucleotides on each side of the lesion as well as the damaged nucleotide are excised and replaced with new nucleotides complementary to the nucleotides in the undamaged chain.

4. Cultivation of keratinocytes

The two main types of cells in the skin are fibroblasts (dermal) and keratinocytes (epidermal). In the epidermis, only the keratinocytes existing at the dermal-epidermal junction can double their DNA and undergo mitosis, although all nucleated keratinocytes can repair their DNA (Karasek and Moore, 1970; Vaughan and Bernstein, 1971). Fibroblasts are easily cultivated in basal medium supplemented with serum (Earle, 1958); Cultivation of keratinocytes requires more stringent attention. Several systems are available for cultivating keratinocytes. The best systems involve use of a substratum of collagen (Karasek and Charleton, 1971; Freeman et al., 1976) or a feeder layer of irradiated fibroblasts (Rheinwald and Green, 1975). Conditioned medium is useful (Ham, 1982) -- but not necessary (Peehl and Ham, 1980; Eisinger et al., 1980). Good growth can be obtained by supplementing the medium with growth factors, such as epidermal growth factor (Cohen and Savage, 1974; Rheinwald and Green, 1975), and hormones (Hayashi et al., 1978). Vaughan et al. (1981) reported increased plating efficiency and successful cultivation and passage of murine keratinocytes after supplementing basal medium with hydrocortisone and insulin.

Most of the cultures of keratinocytes referred to above form monolayers, with some multilayering and production of cornified layers. However, they do not reproduce structural characteristics typical of their counterparts _in situ_. There have been previous reports of stratification of rabbit, human, and rat keratinocytes with some cellular characteristics similar to those of intact epidermis (Karasek and Moore, 1970; Vaughan and Bernstein, 1971; Kitano, 1979). Lillie et al. (1980) cultured a line of rat lingual epithelial cells at the air-liquid interface by lifting collagen-supported cultures on organ culture grids. This resulted in stratification and terminal differentiation, with organellar components similar to the parent tissue. Modification of this technique led to the development of protocols for the successful production of an "epidermis" from primary isolates of cutaneous keratinocytes (Vaughan et al., 1986; Rernstam et al., 1986).

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C. Progress Report

1. Status of the project as of January 1, 1988

Progress toward the achievement of the specific aims of this project as of the beginning of the current reporting period is as follows:

a. To identify the most sensitive biochemical and/or morphological parameters of toxicity in cultures exposed to BCES

By the criterion of incorporation of radiolabeled precursor, DNA synthesis is the most sensitive metabolic target of BCES (cf., Bernstein, 1987). Whereas at an exposure to 20 uM BCES for 1 hr, the incorporation of ^{3}H-leucine into protein in a submerged monolayer culture of rat keratinocytes was not significantly inhibited, and the incorporation of ^{3}H-uridine into RNA was inhibited only to the extent of about 18%, the incorporation of ^{3}H-thymidine into DNA was inhibited to nearly 80% under these conditions. An exposure to even 1 uM BCES for 1 hr was sufficient to prevent the initiation of new DNA replication (Ku and Bernstein, 1988a). In fact, the appearance of heavily labeled nuclei (which is indicative of DNA replication) was not seen in the culture during the first 48 hr following exposure (Ku and Bernstein, 1988a).

The primacy of DNA synthesis as the target of BCES was also seen in stratified, cornified cultures of keratinocytes derived from newborn rat skin (Vaughan et al., 1988). These cultures were exposed for 30 min to BCES in 70% DMSO by topical application, and synthesis of protein, RNA, and DNA was monitored by incorporation of the respective tritiated precursors. The solvent alone had an inhibitory effect with all three precursors although much less than was true with BCES in the solvent. Exposure to 1 nmol BCES/cm^2 of surface for 30 min resulted in approximately 20% less incorporation of ^{3}H-thymidine than was true in the cultures exposed to solvent alone. Significant inhibition of ^{3}H-uridine incorporation into RNA was seen only at an exposure of 10 nmol/cm^2 and ^{3}H-leucine into protein, at 50 nmol/cm^2.

Further evidence for the sensitivity of DNA to low levels of BCES was obtained using the nucleoid sedimentation assay (Cook and Brazell, 1976; Romagna et al., 1985). Nucleoids consist of supercoiled DNA associated with a small amount of non-histone protein obtained from cells by gentle lysis at neutral pH in the presence of a non-ionic detergent. The assay involves sedimentation of the nucleoids in a 15-30% linear gradient of sucrose containing 2M NaCl, 10 mM EDTA, 10 mM Tris buffer at pH 8, and a dye that interacts with DNA and causes the nucleoid band to fluoresce when illuminated with light at 366 nm. Nucleoids made from relaxed DNA (i.e., DNA containing single-strand breaks) sediment more slowly in the sucrose gradient than native DNA does so the decrease in the rate of sedimentation is proportional to the number of single-strand breaks in the DNA. Because one break is sufficient to relax an entire loop of the DNA, the assay can only be used for a small amount of single-strand breakage.

Exposure of submerged monolayer cultures of rat keratinocytes to 1.0 uM BCES caused a major decrease in the sedimentation rate of

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the nucleoids isolated from such cells within 15 min after the exposure (cf., Bernstein, 1987, 1988); and exposure to a level of as low as 0.1 uM seemed to have some effect. The damage inflicted by exposure to 1 uM BCES was sufficient to produce a maximal response in this highly sensitive assay. Increasing the level of BCES beyond 1 uM resulted in only a minor increase in this effect. Exposure to levels of BCES higher than 1 uM undoubtedly produces greater damage -- even though the greater effect was not detectable by this assay. The nucleoid sedimentation assay was shown to return to control values even after an exposure to 5 uM BCES if submerged cultures were allowed to grow for a period of 22 hr subsequent to the exposure. Apparently, gross damage mediated by BCES could be repaired when exposure was limited to 5 uM BCES.

In the stratified, cornified culture of rat cells, a reduction in the sedimentation rate of the nucleoids was also observed (cf., Bernstein, 1988); application of 10 nmol BCES/cm^2 was required to obtain a positive response. Only the DNA of the germinative cells exhibited a response at this level of exposure. Differentiated cells exhibited the response after exposure to 50 nmol/cm^2.

Although the nucleoid sedimentation assay indicated that the gross structural integrity of the DNA from submerged cultures of rodent cells exposed to 1 uM BCES was completely restored during 22 hr of incubation subsequent to exposure, replication of DNA did not resume until 24 to 48 hr later in this system. After exposure to 5 uM BCES, no resumption of DNA replication occurred at all (Ku and Bernstein, 1988a) even though, as noted above, the nucleoid sedimentation assay indicated that gross structural repair could occur. Of course, in the absence of DNA replication, mitosis did not occur. The number of mitotic figures per 1,000 cells decreased as BCES was increased from 0.2 to 1 uM for an exposure of 1 hr. Observations were made at 44, 48, and 53 hr after exposure -- the time when control cultures showed the highest number of mitotic figures. After exposure to 1 uM BCES, mitosis resumed by 72 hr after exposure - but in a smaller percentage of cells than was the case in control cultures.

These mitotic effects correspond to the data on inhibition of DNA replication described above and identify mitosis as a target for BCES, but one that is secondary to the effect on DNA per se.

No gross morphological change was observed during the first 48 hr after exposure of submerged monolayer cultures of rat cells to solvent alone or to 1 or 5 uM BCES (cf., Bernstein, 1988). Three to four days after exposure to these levels of BCES, very large cells were observed in the exposed cultures (Ku and Bernstein, 1988a). Relatively few enlarged cells were seen in cultures exposed to 1 uM BCES or to lower concentrations. A greater number of such cells was seen in the cultures exposed to the higher level of BCES. In cultures treated with 20 uM BCES, enlarged cells could be seen as early as 18 hr after exposure. By 4 days after exposure most cells were large. These large cells carried the lectin binding sites for differentiated cells and did not divide (Ku and Bernstein, 1988b).

Exposed cells seemed to have more vacuoles and many more mitochondria than control cells. The mitochondria in exposed cells seemed

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to be abnormally small and to exhibit different degrees of degeneration. The large cells had large, pale-staining nuclei that contained very little heterochromatin but had large nucleoli. Also eexposed cells appeared to be deficient in ribosomes as compared with control cells. In some cells from cultures exposed to 5, 10, and 20 uM BCES, tonofilaments formed unusally thick bundles. The ultrastructural changes could be detected at about 24 hr before the large cells were seen in an exposed culture but the damage to DNA preceded any obvious morphological change.

The inhibition of DNA replication and cell division for several days after exposure to 1 uM BCES and the appearance of abnormally large differentiated cells in such cultures several days after exposure -- even though the structural integrity of DNA had been grossly restored -- support the hypothesis that BCES imposes a subtle lesion upon the cells which the cells cannot repair. The lesion could consist of unrepaired adducts and/or cross links.

It is also possible that adducts including interstrand crosslinks are repaired but that the wrong bases are inserted into the DNA because BCES decreases the fidelity of the repair process itself. Insertion of an erroneous base could lead to a cell's death or to an abnormality in its structure or function if the cell survives.

Further support for the concept of such a BCES-mediated informational change in the DNA is the observation (Ku and Bernstein, 1988a) that there is a dose-responsive change in the carbohydrate moiety of the glycoconjugates on the cell surface in cultures of rat keratinocytes exposed to low levels of sulfur mustard. This alteration was visualized as a decrease in the number of binding sites per unit cell surface for the alpha-D-galactoside-specific *Griffonia simplicifolia*, $I-B_4$ isolectin, with no change in the number of binding sites for the L-fucoside-specific *Ulex europeus*, Agglutinin I lectin (cf., Bernstein, 1988). This conclusion was based on Scatchard (1949) analysis after correction for the large size of many of the cells. In fact, the large cells did not bind the $I-B_4$ isolectin at all, indicating that they were terminally differentiated in contrast to the cells in control cultures. In the latter condition, all cells bind the $I-B_4$ isolectin, whether or not they bind the Agglutinin I lectin.

Additional support for the concept of an informational lesion in the DNA is the appearance of an abnormality in keratin maturation in submerged monolayer cultures of rat cells exposed to BCES (cf., Bernstein, 1988). Exposure of a culture to 1 uM BCES resulted in a 15% increase over control in the percentage of cells which bound 2D6 monoclonal anti-keratin. The increase reached 42% after exposure to 10 uM BCES. This anti-keratin binds only to cells in the epidermal basal layer of newborn rat skin, although the 55Kd antigen for this antibody has been shown to be present throughout the epidermis. Apparently, the antigen has its binding site for the anti-body blocked in the differentiated cells of the epidermis. Since the ratio of differentiated to basal cells in the exposed culture is no different than it is in the control culture (Ku and Bernstein, 1988b), it is surprising that the percentage of cells that bind 2D6 becomes greater when cultures are exposed to the mustard. Although the explanation for this anomaly is not known, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, applied to the 2D6-positive protein

from cells exposed to 10 uM BCES showed the presence of an abnormal band which migrated slightly faster than the normal 2D6-staining protein. One possible explanation would be that masking of the 2D6-positive keratin peptide involves the addition of a small-molecular-weight unit and that this process is inhibited in differentiating cells which have been exposed to low levels of BCES. This abnormality could be a function of an informational error in the DNA.

In summary, DNA synthesis was found to be more sensitive to BCES than was protein or RNA synthesis in both the monolayer culture and the stratified, cornified culture. Similarly, the DNA in the two types of culture exhibited single-strand breakage upon exposure to BCES. Basal cells were found to be more susceptible than differentiated cells with regard to this parameter. Cells exposed to low levels of BCES can restore the gross structural integrity of their DNA, but appear to be left with a lesion which they cannot obviate. This lesion is expressed in inhibition of mitosis, enlargement of cells in the culture, change in the carbohydrate moiety of the glycoconjugates on the cell surface, and aberration in the maturation of a particular keratin. The lesion is likely to be located in the DNA. If this hypothesis is true, the lesion may be a function of the failure to repair a BCES-alkylation in the DNA or may be a result of an error inserted in the DNA by the repair process itself.

b. To ascertain the relevance of the BCES-mediated toxic responses seen in cultures to the cellular necrosis that is requisite for vesication

By light-microscopic observation of gross morphology, exposure of a stratified, cornified culture of rat cells for 30 min to 1 nmol of BCES/cm^2 of surface had no observable effect (cf., Bernstein, 1987). Application of even 10 nmol/cm^2 resulted in only focal necrosis. However, after application of 50 nmol/cm^2, the basal layer and lower spinous layers completely disappeared by 48 hr after exposure. The upper layers remained essentially intact. Thus an exposure to between 10 and 50 nmol BCES/cm^2 is necessary to achieve the destruction of the germinative layer of the epidermis - a major concomitant of vesication _in vivo_. It should be noted that, as is true with BCES-mediated vesication _in vivo_, this necrotic response is not instantaneous in the culture; rather, it requires time (i.e., between 24 and 48 hr) after exposure to develop.

The destruction of the epidermal basal layer by topical application of BCES, either _in vivo_ or _in vitro_, is obviously not a result of a direct dissolution of the cell by the mustard; rather it must be a consequence of the interaction between BCES and a cellular component that is critical to the cell's survival. It is reasonable to assume, from all the available data, that damage to the DNA is central to the necrotic response. Since the cultured germinative cell can repair the gross structural damage to its DNA (cf., Bernstein, 1987) resulting from exposure to a level of BCES that essentially destroys the cell's viability, it is possible that the degree of fidelity in the repair process is inconsistent with cellular viability; i.e., the cell's normal biochemical and physiological activities cannot be carried out and as a result, the cell dies.

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Initial studies of the repair process in submerged cultures of rat keratinocytes exposed to BCES failed to yield evidence for the incorporation of ^{3}H-thymidine in the repair mode (cf., Bernstein, 1988). The small amount of labeled thymidine that was incorporated was found essentially only in replicated strands of DNA.

Further investigation of the repair process produced the observation (cf., Bernstein, 1988) that, in contrast to ^{3}H-thymidine, ^{3}H-deoxyguanosine did label the DNA in the repair mode. This was shown by using the density gradient technique. Monolayer cultures were exposed to BCES for 1 hr, washed, and incubated for 10 hr in the presence of bromodeoxyuridine, fluorodeoxyuridine and ^{3}H-thymidine or ^{3}H-deoxyguanosine. Both tracers were used at the same level of radioactivity. The DNA was isolated and submitted to centrifugation in a gradient of CsCl at a pH of 8.0. The peak of material corresponding to the parental DNA in each case was then sedimented (i.e., "rebanded") in a gradient of alkaline CsCl (pH 12.5). This rebanding of the original light band demonstrated that ^{3}H-thymidine did not label the parental DNA peak, but ^{3}H-guanosine did. This observation supports the claims of Hennings et al. (1974) and Hennings and Michael (1976) that there is a repair pathway which is distinct from the one in which thymidine is incorporated and that this new pathway involves the replacement of damaged guanine residues. It appears reasonable to propose, therefore, that the repair of single-strand breaks from exposure to BCES is associated with a specific replacement of the damaged guanine moieties of DNA. The possibility that apurinic sites might also have been repaired by the incorporation of adenine moieties has not yet been tested.

No information is available as yet on the degree of fidelity that is inherent in this repair system nor on whether it is present only in either the germinative or differentiated cells.

2. Progress report for the period, January 1, 1988 - December 31, 1989

   a. Plans for the period:

      1) Complete the study of alkylation in stratified, cornified cultures exposed to BCES by topical application

      2) Demonstrate that the toxic effects on basal cells in stratified, cornified cultures exposed topically are a result of the penetration of mustard through the tissue

      3) Investigate the effect of BCES on the fidelity of repair in order to evaluate the hypothesis that errors in the repair process are responsible for the failure of exposed cells to recover completely after exposure to mustard

      4) Identify the molecular nature of the lesion in the DNA caused by BCES

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b. Progress on the individual items of the plan

    1) Complete the study of alkylation in stratified, cornified cultures exposed to BCES by topical application

    Alkylation was studied by exposing submerged monolayer cultures and stratified, cornified cultures of keratinocytes from newborn rats to ^{14}C-BCES and determining the amount of ^{14}C which became associated with the DNA isolated from the exposed cells.

a) Methods

    Monolayer cultures were grown submerged in a medium low in Ca^{2+}, as described by Ku and Bernstein (1988b). Stratified, cornified cultures were grown on nylon microporous membranes for 7 days submerged and 14 days lifted, as described by Vaughan et al. (1986). Submerged monolayer cultures, grown for 24 hr, were exposed for 1 hr to 1, 10, and 100 uM BCES (final concentration in the medium) containing 2 mCi of ^{14}C per 10 mg of the mustard. Stratified, cornified cultures were exposed for 30 min to 5, 10, and 50 nmol BCES/cm^2 of culture surface by application of 800 ul of BCES in 70% DMSO to the surface of each culture (on a 47 mm membrane). The BCES had a specific activity of 32 mCi/mmol.

    After exposure the cultures were washed with phosphate buffered saline having less than 0.1 mM Ca^{2+} (submerged) or with Eagle's balanced salt solution (stratified).

    The cells of the submerged culture were harvested using a rubber policeman and collected by centrifugation. The pellets were resuspended in 2.4 ml of a mixture of 10 mM Tris, pH 8, and 1 mM EDTA. SDS (0.6 ml of a 5% solution (w:v) was added to lyse the cells. The cellular material was treated with 150 ug/ml of Proteinase K for 16 hr at 37° C. The DNA was then extracted with an equal volume of phenol saturated with a mixture of 10 mM Tris, pH 8, and 1% hydroxyquinone. The aqueous layer was separated and extracted with an equal volume of 1:0.5:0.5 (v:v:v) saturated phenol:chloroform:isoamyl alcohol and then ether. The DNA was precipitated by making the aqueous solution 0.1 N with respect to NaCl and adding 2 volumes of cold absolute alcohol. After recovery by centrifugation, the DNA was solubilized in 0.1 N NaOH and incubated at room temperature overnight to hydrolyze any RNA present. The DNA was again precipitated by neutralizing the solution with 0.1 N HCl and adding alcohol as before. The recovered DNA was solubilized in 0.1 N NaOH and counted for associated ^{14}C using a Packard Liquid Scintillation Spectrometer.

    The washed, stratified, cornified cultures were incubated with phosphate-buffered saline containing 0.02% EDTA for 10 min and then with 0.05% trypsin for 15 min at 35°C. The action of the trypsin was inhibited by addition of growth medium (containing serum). The membranes were brushed gently to release the cells into the medium and the cells were recovered by centrifugation. The cell pellet was resuspended in 38% isotonic Percoll and centrifuged at 30,000 xg for 20 min in order to separate the basal and differentiated cells in the density gradient of Percoll. The basal cells banded at a density >1.074 and the differentiated

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cells accumulated in a band at a density of 1.049.

The cells were collected from each band, recovered by centrifugation, and resuspended in 1.5 ml of 10 mM Tris, pH 8. One and a half ml of 1% SDS in the same buffer were added to lyse the cells during 30 min of incubation at room temperature. The mixture was then incubated with Proteinase K overnight at 37°C. The volume of the mixture was adjusted to 3.5 ml with the buffer, and 4.3 g of CsCl were added with gentle stirring. This solution was submitted to centrifugation at 27,500 rpm at 20°C for 48 hr in a 50.1 SW rotor in a Beckman L8 ultracentrifuge to separate RNA, DNA and protein. The DNA band was identified by the "DABA" procedure (Setaro et al., 1976) and the associated ^{14}C was determined by liquid scintillation counting.

b) Results

Alkylation of DNA was dose-responsive, doubling from 10 to 20 dpm/ug of DNA when the exposure of monolayer cultures was increased from 1 to 10 uM BCES (Figure 1). The radioactivity associated with the DNA increased fivefold when the level of exposure was increased to 100 uM BCES. The reason for the non-linearity of the results is unknown.

When the level of alkylation was determined in stratified, cornified cultures of rat cells in two separate experiments, the association of labeled BCES with DNA was also found to be dose-responsive (Table 1). The associated ^{14}C was higher in basal than in differentiated cells. At an exposure of 5 nmol of ^{14}C-BCES/cm^2, the incorporated tracer was not different in the two types of cell, but after exposure to 10 nmol of ^{14}C-BCES/cm^2, the tracer was 50% higher in the DNA of the basal cells as compared with that in the differentiated cells. After an exposure to 50 nmol/cm^2, the DNA from the basal cells contained about 35% more tracer than the DNA from the upper cells.

d) Discussion

An exposure of 10 nmol of ^{14}C-BCES/cm^2 in the stratified culture resulted in a specific activity (i.e., dpm/ug DNA) of tracer associated with DNA which was about four times higher in the basal cells and nearly three times higher in the differentiated cells than the specific activity found in the DNA of the submerged culture exposed to 10 uM ^{14}C-BCES.

It is tempting to compare the level of ^{14}C-BCES observed to be associated with DNA isolated from monolayer cultures exposed to a particular dose of BCES with that found in the DNA isolated from stratified, cornified cultures exposed to what is assumed to be a similar dose of the mustard and to make conclusions about the similarity or dissimilarity of effects in the two culture systems. Since the amounts of BCES which actually reach any cell in the two culture systems is unknown, such comparisons must be considered tenuous at best and might better be avoided.

Although, because of their wide variance, the data generated in these experiments have only qualitative credibility, they are important in that they encourage the view that the labeled BCES can be used

Figure 1. Alkylation of DNA in a submerged monolayer culture exposed to different concentrations of ^{14}C-BCES added to the medium. For technical details of the procedure, see the text. The data represent mean values derived from analysis of three cultures for each of the different concentrations. Values for a particular concentration varied from the mean by ±10%.
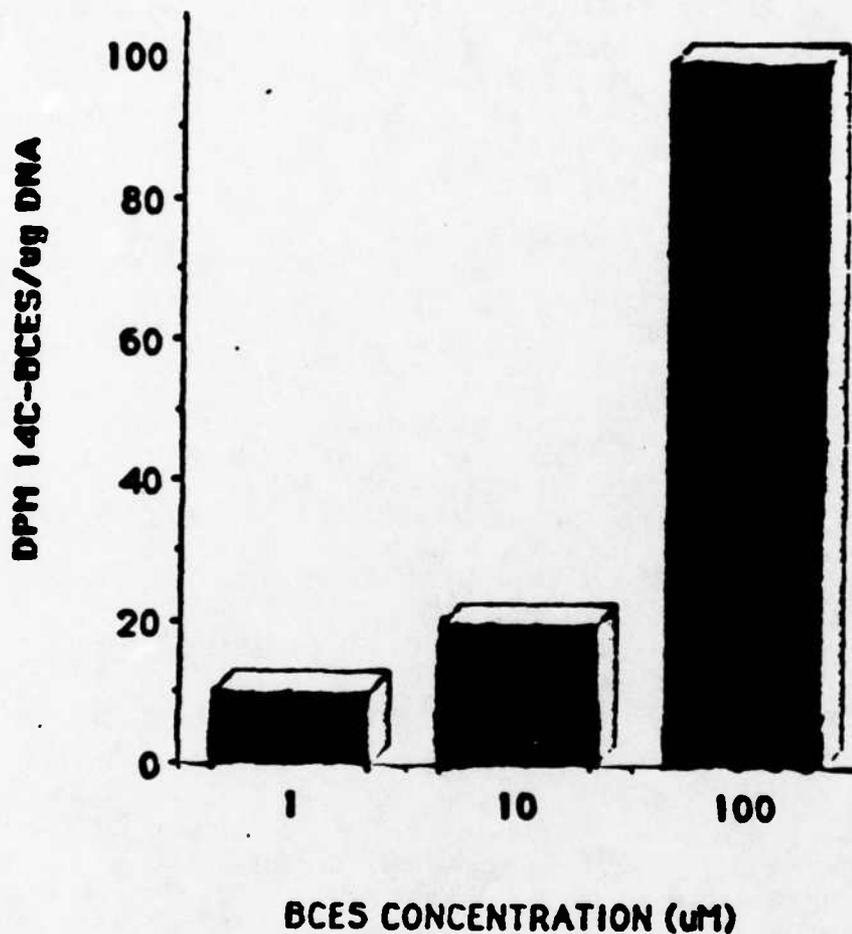
Table 1. Alkylation of DNA in a stratified, cornified culture exposed to ^{14}C-BCES by topical application of the mustard in 70% DMSO. For technical details of the procedure, see the text. Data represent the average of results from two experiments ± the deviation from the average.

Concentration of BCES (nmol/cm^2)	5	10	50
		dpm/ug DNA	
DNA from basal cells	5.4±1.2	84±31	418±90
DNA from differentiated cells	4.6±1.0	54±28	310±110

in later efforts to isolate and identify DNA adducts.

2) Demonstrate that the toxic effects on basal cells in stratified, cornified cultures exposed topically are a result of the penetration of the mustard through the tissue

In order to verify that BCES applied topically to stratified, cornified cultures reached the viable layers by penetrating the culture from above rather than by diffusing into the culture after first spilling into the medium, an autoradiographic time study was carried out. ^{14}C-BCES was applied topically to stratified, cornified cultures of rat cells. A level of 50 nmol/cm^2 was used. After 3, 6, and 15 min, cultures were processed for histology and autoradiography in order to show the progressive movement of the tracer into the deeper layers from the surface.

a) Methods

^{14}C-BCES (32 mCi/mmol) was applied topically as described above to cultures of rat cells grown on nylon membranes (Vaughan et al., 1986). Thirteen-mm membranes were used and 40 ul of solution were applied. After exposure for the desired times, the cultures were washed with Eagle's balanced salt solution, and fixed with Cornoy's solution for 1 hr. After fixation the cultures were dehydrated with absolute alcohol, washed with toluene and embedded in paraffin. Sections of 5u were prepared. Standard autoradiographic techniques were then applied using Kodak NTB-3 emulsion. Exposure was allowed to proceed for 24 hr at 4°C in the dark, after which the autoradiographs were developed for 3 min with D-19 and treated with Kodak fixative. The slides were then stained with hematoxylin and eosin and observed by light microscopy.

b) Results

Three min after the tracer was applied, the autoradiograph (Figure 2) showed that most of the silver grains were present in the upper layers, mainly in the granular and cornified layers. In the autoradiograph taken after 6 min of exposure (Figure 3), some grains are seen in the basal layer, although most of the radioactivity is still localized in the upper layers. After 15 min of exposure (Figure 4), silver grains are seen to have penetrated into the basal layer and to have migrated even into the nylon membrane.

c) Discussion

The autoradiographic data are consistent with the conclusion that the tracer reached the basal layer by penetrating into the tissue from above. This kind of experiment cannot exclude the possibility that some BCES entered the tissue from the medium, clearly, though the main route of advance appears to be through the tissue. In applying BCES to the surface of a stratified, cornified culture, care is always taken to carefully add the BCES solution at the middle of the culture and to use the amount of liquid which had previously been determined to barely spread to the edge of the culture.

3) Investigate the effect of BCES on the fidelity of repair in order to evaluate the hypothesis that errors in the repair process are responsible for the failure of exposed cells to recover completely after exposure to mustard

A technique is being developed to measure the degree of fidelity with which keratinocytes exposed to BCES can repair lesions in their DNA. The technique is a modification of the "host cell reactivation" phenomenon (cf. Defais et al. 1983) and can be applied only to human cells. In principle, this technique involves transfection of a culture with a heteroduplex of SV40 DNA in which one mismatched base has been inserted in each strand at the gene for the large T antigen. The two lesions have also made the DNA replicative process temperature-sensitive. It will occur at $37^{\circ}C$ but not at 41° C. In order for new viral DNA to be made at the non-permissive temperature, at least one of the two mismatched bases must be repaired. If exposure to BCES results in a decreased effectiveness of this repair system, the appearance of new viral DNA will be delayed in a dose-responsive manner.

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Figure 2. Autoradiograph of a stratified, cornified culture exposed topically to ^{14}C-BCES for 3 min. For technical details, see the text.
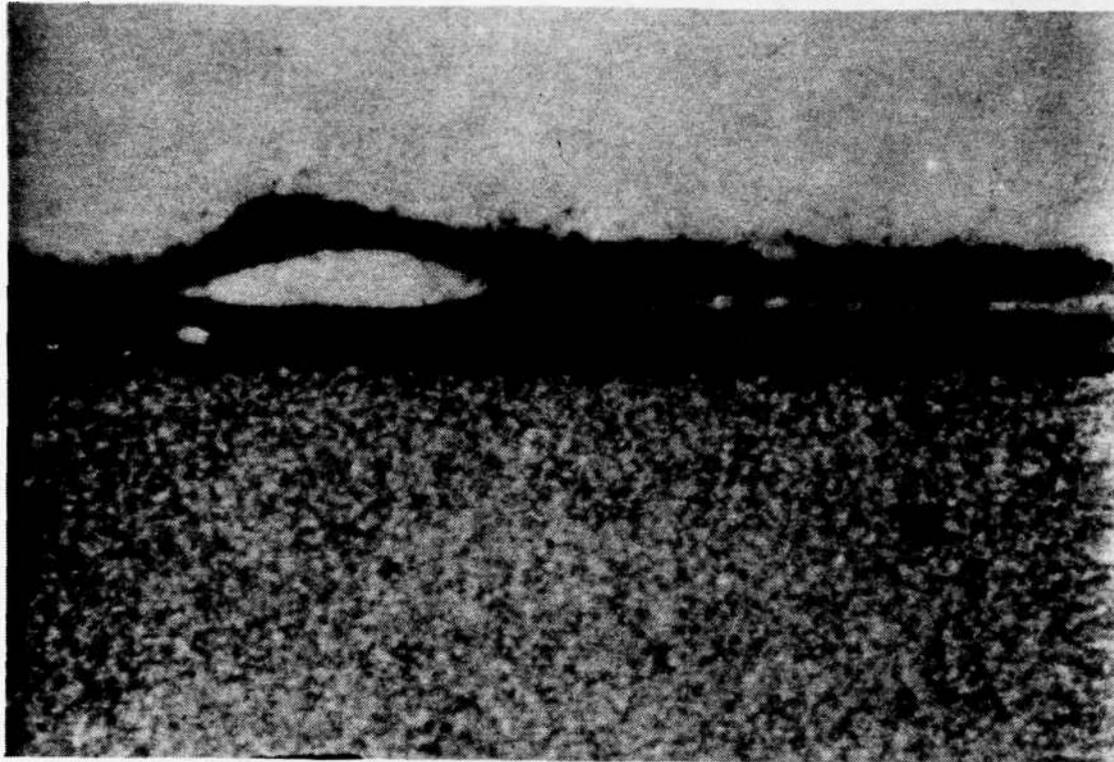

Figure 3. Autoradiograph of a stratified, cornified culture exposed topically to ^{14}C-BCES for 6 min. For technical details, see the text.
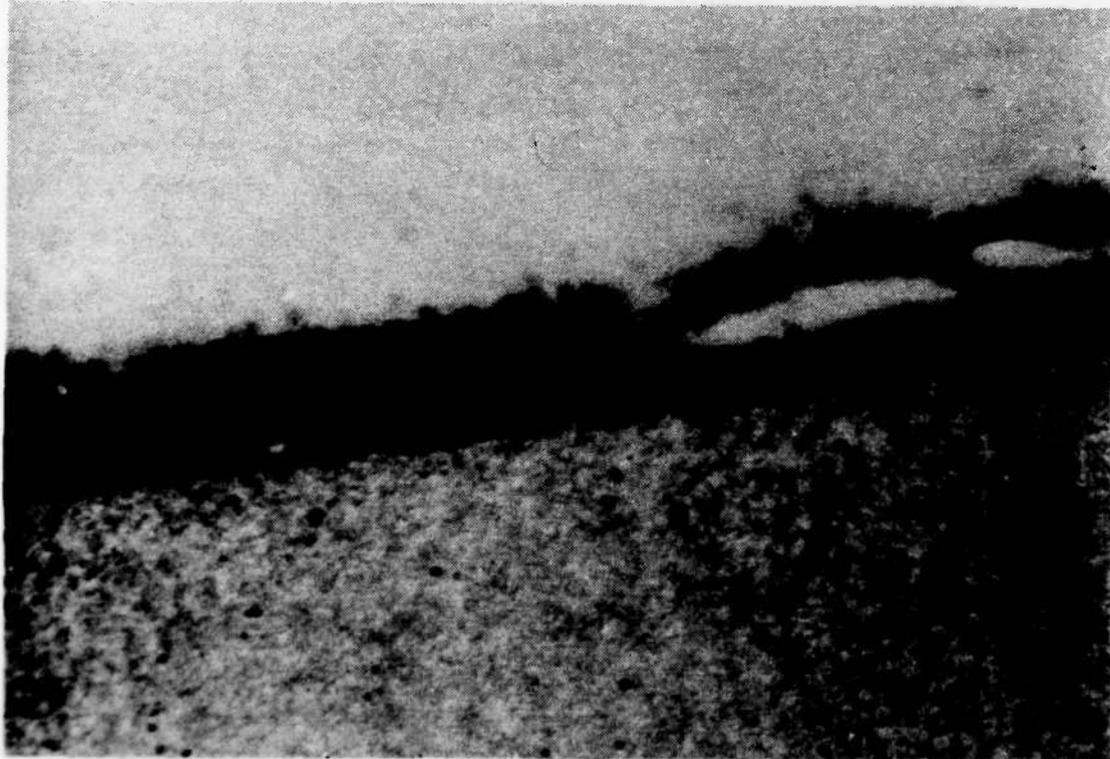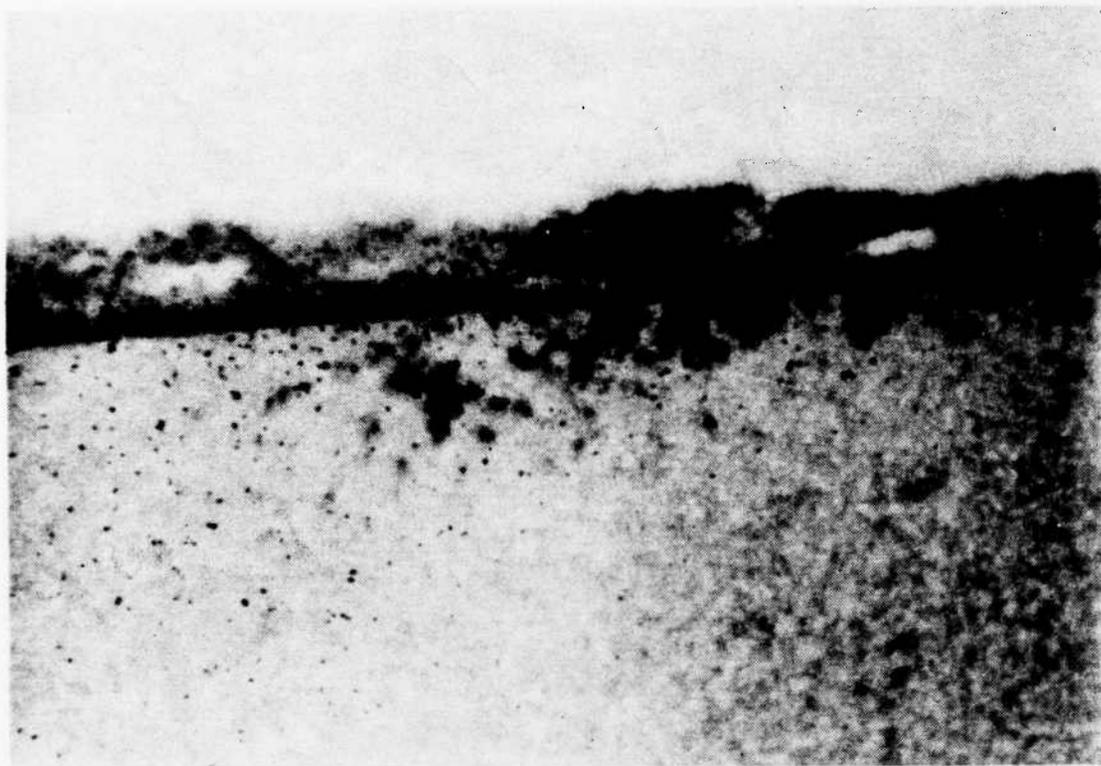
Figure 4. Autoradiograph of a stratified, cornified culture exposed topically to ^{14}C-BCES for 15 min

To date, the work on the assay has involved mostly establishing satisfactory protocols for transfection and assay of the newly synthesized viral DNA. The work was started using monolayer cultures of human keratinocytes. A major problem that surfaced early in the work was the low order of transfection which was achieved in the keratinocyte cultures and, later, in monolayers of human fibroblasts. If only a few cells are transfected, the amount of newly formed DNA will be too small to measure accurately. To obviate this difficulty, several different transfection techniques were applied and compared with the efficiency of the transfection process in monkey kidney cells, the natural host of the virus. In addition, a better technique for making the heteroduplex DNA was tried, in an effort to increase the yield of the DNA probe. When the required techniques became satisfactory when applied to monkey kidney cells, cultures of these cells were exposed to BCES and the effect of the mustard on mismatch repair in this system was evaluated.

a) Methods

Heteroduplex DNA with two mismatched base pairs within the large T antigen gene were constructed from ts239 and ts255 SV40 virus DNA mutants, as described by Peden and Pipas (1985). DNA from ts239 was linearized with Eco RI restriction endonuclease and DNA from ts255 with BamHI endonuclease, as described by Maniatis et al. (1982). The two digests were mixed, denatured by making the solution 0.1 N NaOH and incubating the mixture for 10 min at 37°C, and renatured by neutralizing the solution to pH 7.2 with HCl and Tris buffer and incubating at 61°C. The mixture was slowly cooled to 4°C, and the DNA was precipitated by addition of 24 volumes of ethanol. The steps in the process were monitored by agarose gel electrophoresis. A parallel preparation was made of wild-type SV40 DNA to be used in control experiments.

Green monkey kidney (BSC-40) cells were transfected with heteroduplex or homoduplex (duplex from wild-type DNA only or duplex from each of the mutant DNAs separately) by a cationic liposome-mediated procedure (lipofection), as described by Felgner et al. (1987). Three ug of DNA and 30 ul of lipofectin were used per 60-mm dish of culture. After lipofection at 37°C, 3 ml of minimal essential medium supplemented with 20% fetal bovine serum were added and the cultures were transferred to an incubator set at 41°C.

At the desired times for measuring synthesis of new viral DNA, the cultures were labeled with ^{3}H-thymidine, harvested, and lysed (Hirt, 1967). Viral DNA was isolated by salt precipitation of total DNA in the cell and purified by digestion with RNase, phenol extraction, and ethanol precipitation (Maniatis et al., 1982). The viral DNA was separated from mitochondrial and chromosomal DNA by agarose gel electrophoresis. The gel was sliced, and the slices were melted and digested in TS-2 digestion fluid; the ^{3}H present was determined by liquid scintillation counting.

b) Results

Figures 5a, b, c, and d are plots of the appearance of tritiated viral DNA in cultures exposed to 0.1, 0.5, 1, and 5 uM BCES, respectively, and then transfected with heteroduplex DNA. In each case, a solvent control was run using the same amount of methylene chloride as was present in the medium for the BCES-exposed cells. This was done to normalize for any possible effect which the solvent might have on the system. The figures show that as the concentration of BCES increased, there was an increase in the delay, when compared with the control, before synthesis of new DNA became apparent. The curves were generated using a computer statistical program. The experiments which provided the data for Figures 5a and d have been repeated once with similar results.

Figures 6a, b, c, and d are plots of the appearance of new viral DNA in cultures exposed to 0.1, 0.5, 1, and 5 uM BCES and then transfected with homoduplex DNA made from wild-type DNA. In each case, the solvent control contained the same amount of methylene chloride as was present in the medium of the exposed culture. Although the exposed cultures exhibited a greater delay with respect to control before

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Figure 5. The effect of exposure to different concentrations of BCES on the repair of mismatched bases in heteroduplex SV40 ts mutant DNA previously introduced into monkey kidney cells. Repair was observed at the nonpermissive temperature of 41°C. Concentrations of BCES used: a - 0.1 uM; b - 0.5 uM; c - 1 uM, and d- 5 uM. For technical details see the text. Curves were generated by a computer statistical program.

Figure 6. The effect of different concentrations of BCES on the production of new viral DNA at 41° C in monkey kidney cells previously transfected with homoduplex DNA made from wild type SV40. Concentration of BCES used: a - 0.1 uM; b - 0.5 uM; c - 1 uM, and d - 5 uM. For technical details see the text. Curves were generated with a computer statistical program.
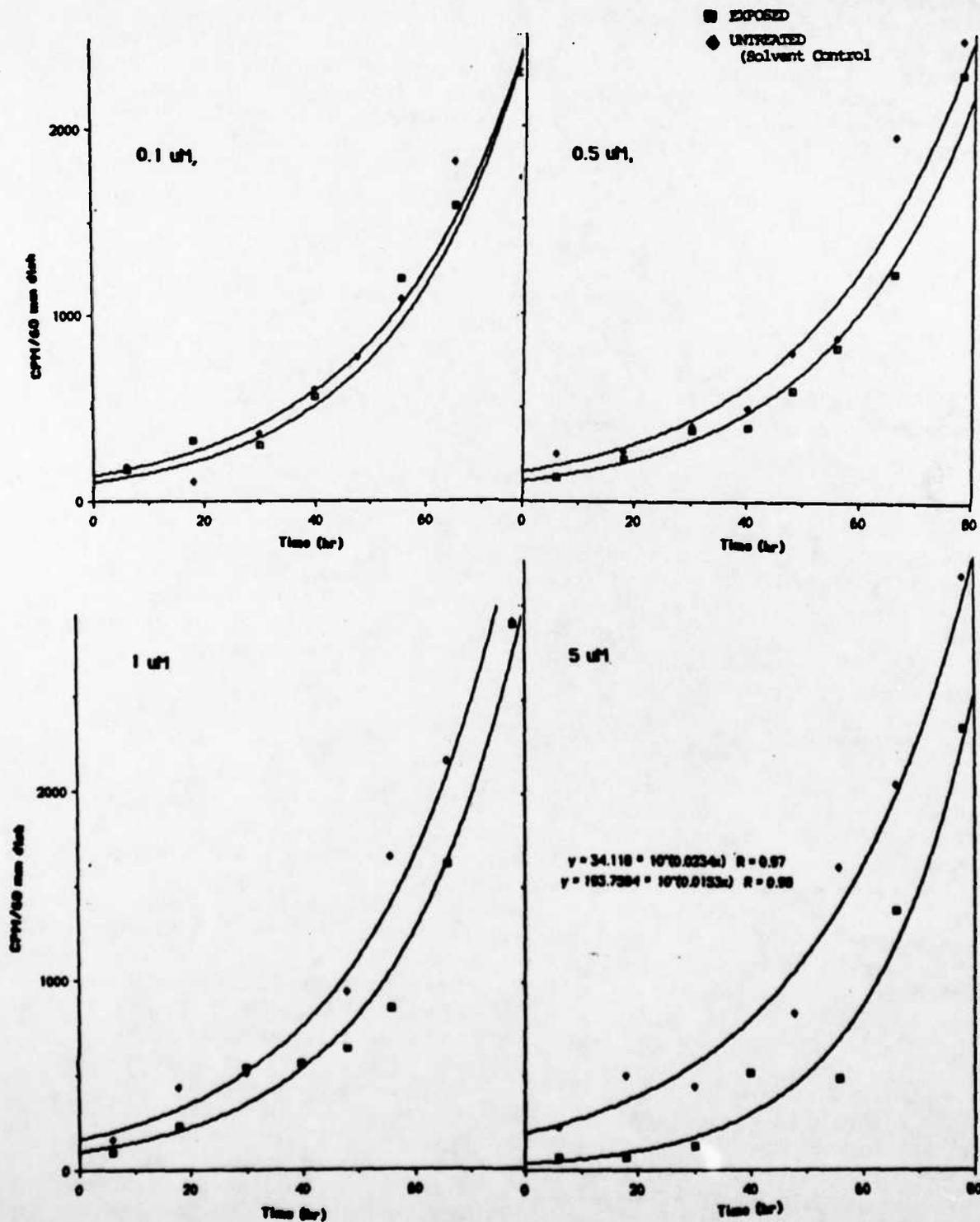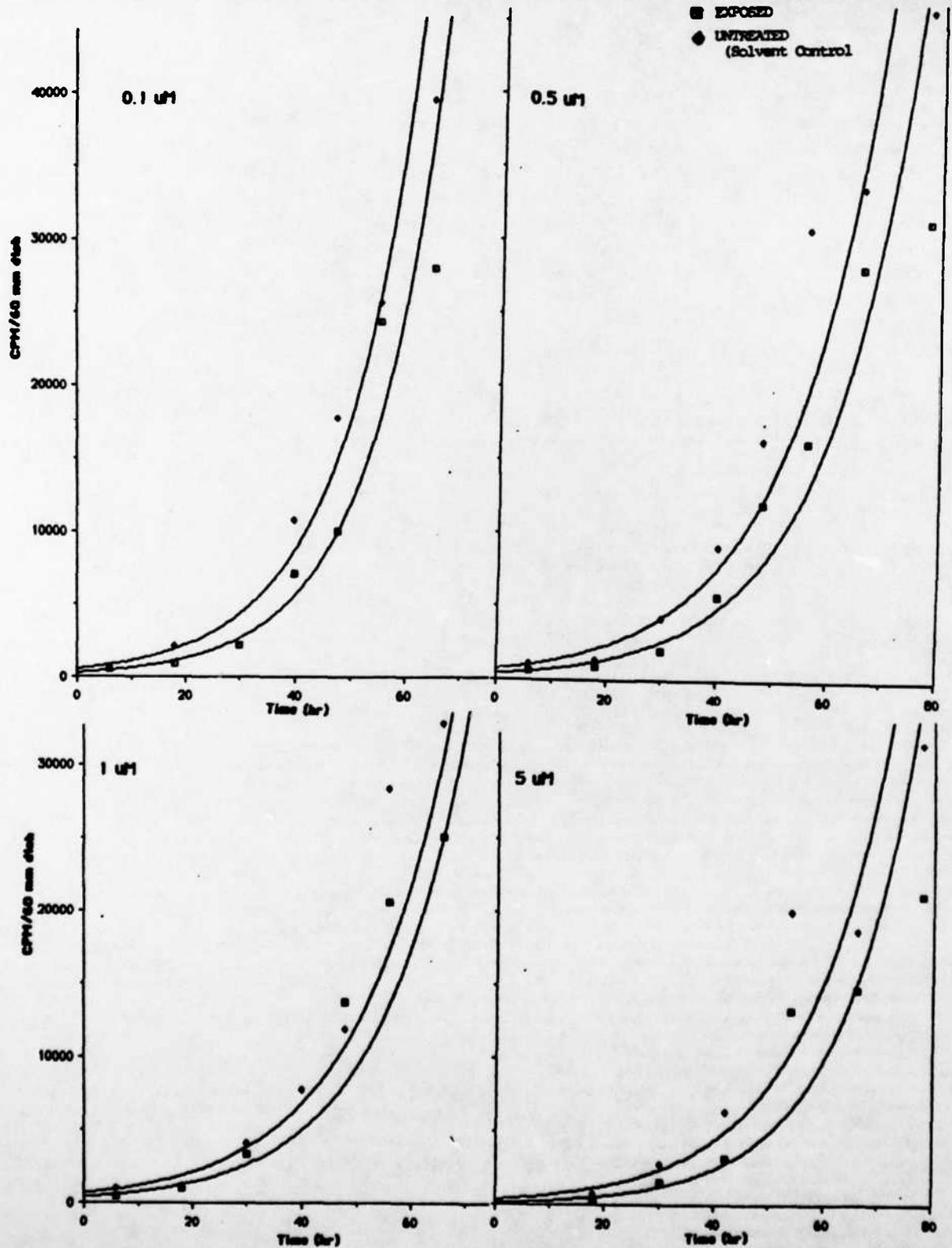
new viral DNA became detectable, the length of the delay was approximately the same at all concentrations of BCES used.

When homoduplex DNA made from either mutant virus was transfected into the cells, no newly synthesized viral DNA was detected.

c) Discussion

The plots in Figure 5 clearly indicate that exposure to BCES resulted in a dose-responsive increase in the delay before new viral DNA was detected. This type of experiment alone cannot determine whether the effect of BCES is on the repair system alone or on one or more of the steps in the entire transfection process. The fact that cells transfected with wild-type homoduplex DNA showed a small delay which was not dose-responsive whereas the cells transfected with heteroduplex DNA showed a dose-responsive delay does suggest that the effect of BCES was primarily on the repair system and not on other aspects of the transfection process.

The absence of any new viral DNA in cells transfected with homoduplex DNA made from either mutant virus indicates that the mutants were not leaky and that the new viral DNA made by cells transfected with the heteroduplex DNA involved repair of at least one of the mismatched bases.

The technology is now satisfactory and should be applicable to human keratinocytes which are permissive for SV40 (Banks-Schlegel and Howley, 1983).

4) Identify the molecular nature of the lesion in the DNA caused by BCES.

An effort has been initiated to identify the lesion in DNA caused by BCES by first establishing the nature of the adducts present in the DNA of cultured cells exposed to low levels of BCES. A protocol using HPLC is being developed with which to identify bases and other components of DNA which are alkylated by BCES. Alkylation will be followed by exposing cells to ^{14}C-BCES and then looking for DNA components that are labeled.

a) Methods

The instrumentation in use includes the following: a Varian Model 5000 HPLC with Waters Model 440 absorbance detector operating at 254 nm and a Hewlett-Packard Model 3390A integrator. The column currently in use is a Spherisorb C-18 reverse-phase column which is 25 x 4.6 cm in size having 5 um particles.

The solvent system in use consists of (a) 5 mM tetrabutyl ammonium hydrogen sulfate and 3.1 mM potassium phosphate adjusted to pH 7 with 4 N potassium hydroxide and (b) absolute methanol. The flow rate is 1 ml/hr. The solvent program is as follows: isocratic elution for 5 min using solvent a followed by a linear gradient with b

starting at 0% and running to 25% over a period of 35 min. Temperature: 24°C.

b) Results

To date, no BCES adducts have been generated. The column procedure seems adequate to at least initiate the search for adducts of guanine and adenine. Table 2 shows that alkylation on adenine, guanine, and cytosine results in retardation of these bases in their elution from the column. It appears likely that this column procedure can be used to at least separate the adducts from the unmodified bases.

Table 2. Elution times of standard compounds run on a reverse phase HPLC column. For technical details, see the text.

Compound	Elution time (Min)
Uracil	3.78
Thymine	5.82
Cytosine	3.30
5-Methyl cytosine	4.06
Adenine	9.73
6-Methyl adenine	19.79
Guanine	5.35
8-Azoguanine	11.73

Note: Uracil and 5-methyl cytosine do not separate when run together. All other combinations separate sufficiently for identification.

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