MOLECULAR AND CELLULAR MECHANISMS OF SULFUR MUSTARD-INDUCED LESIONS OF HUMAN SKIN

PROEFSCHRIFT

ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden,
op gezag van de Rector Magnificus
Dr. L. Leertouwer,
hoogleraar in de faculteit der
Godgeleerdheid,
volgens het besluit van het College van Dekanen
te verdedigen op woensdag 29 januari 1992
te klokke 15.15 uur

door

Maria Anna Elisabeth Mol

geboren te Rijswijk in 1955

NKB Offset BV, Bleiswijk

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STELLINGEN

1. *In vitro* screeningstesten zijn pas dan op zinvolle wijze in de risicoanalyse voor humane toxiciteit toe te passen als door fundamenteel onderzoek de betekenis van cellulaire toxische mechanismen voor de mens *in toto* duidelijk is.

2. Zweet levert een bijdrage aan het volume van blaarvocht.

3. Bij het sluiten van grote huiddefecten, zoals brandwonden, verdient het aanbrengen van een dermaal substituut dat is samengesteld op basis van niet-gedenatureerd collageen de voorkeur boven synthetische materialen, vanwege de natuurlijke eigenschap van collageen om cellen en groeifactoren te binden.

4. Resultaten van onderzoek naar de percutane absorptie van stoffen uitgevoerd in "isolated perfused porcine skin flaps" moeten kritisch beschouwd worden, omdat men door het gebruik van Krebs-Ringer buffer als perfusievloeistof geen rekening houdt met het verschil in oplosbaarheid van een stof in waterige oplossing en in bloed.
Riviere, J.E., Bowman, K.F., Monteiro-Riviere, N.A., Dix, L.P., and Carver, M.P. (1986). Fund. Appl. Pharmacol. ***7****, 444 - 453.*

5. Het feit dat bis-2-chloroethyl-sulfide betiteld wordt als "nitrogen mustard" betekent een blamage voor zowel de auteurs als het tijdschrift dat het betreffende artikel publiceert.
Requena, L., Requena, C., Sanchez, M., Jaqueti, G., Aguilar, A., Sanchez - Yus, E., and Hernandez - Moro, B. (1988). J. Am. Acad. Dermatol. ***19****, 529 - 536.*

6. Een wettelijke verplichting om vrachtwagens te voorzien van "dode hoek" spiegels zal het aantal verkeersslachtoffers meer reduceren dan de invoering van snelheidsbegrenzers.

7. Met het bevorderen van de uitstroom van oudere werknemers via VUT-regelingen bereikt men bij bedrijfsreorganisaties slechts een schijnoplossing, vooral vanwege de financiële consequenties voor de komende jaren.

8. De procedure die de Landbouwuniversiteit Wageningen volgt bij de werving en selectie van gekwalificeerde vrouwen in hogere functies zal in kwalitatieve zin eerder leiden tot een geringere dan tot een grotere respons, aangezien vrouwen die zichzelf respecteren een functie ambiëren die zij op grond van hun capaciteiten in een eerlijke competitie met mannelijke kandidaten kunnen verwerven.
Intermediair, 15 november 1991, p.30

9. Een mol bevat méér dan 6,02 x 10^{23} moleculen.

Rijswijk, 29 januari 1992

Marijke Mol

In dank opgedragen aan allen die op welke manier ook aan de verwezenlijking van dit proefschrift hebben bijgedragen.

CONTENTS

1 INTRODUCTION

Skin is the largest organ of man and its main function is to provide a protective envelope for the maintenance of body homeostasis. The conventional view that skin is a rather passive and metabolically inactive barrier had to be reconsidered, when research showed that skin is a complex organ, well equiped to carry out its protective function. This may be illustrated by the fact that skin, like the liver, is an active site of biotransformation, a process aimed at detoxifying many hazardous chemicals. Or, by the fact that skin is very immunocompetent, due to the immunoreactivity of the epidermal cells and the presence of Langerhans cells and mast cells, which form part of the immunologic system of the body. In addition to these few examples, the complexity of skin structure and function is further underlined by the occurrence of numerous skin diseases, indicating that there are many ways in which skin function can deviate.
In the past decades, there has been an enormous increase in the knowledge on biochemical processes in the human skin under physiological or pathological conditions, stimulated by the availability of new models for human skin and of advanced biochemical techniques. Until then, human and animal skin had been studied predominantly in a descriptive way, based on clinical signs and histopathology, and although this approach revealed much about underlying mechanisms, understanding of many phenomena of the skin was incomplete. A complicating factor has been the difference between the skin of man and that of other mammals, which greatly interfered with extrapolation to man of data obtained in animals. The development of *in vitro* techniques such as the serial culture of human epidermal cells, provided the basis for a more scientific approach in skin research on damage due to exposure of skin to chemical and physical factors. Whereas in the past the study of skin toxicity was mainly confined to clinical observations, it is now possible to investigate the mechanisms that cause the observed signs.

It has been known for more than hunderd years that, upon contact with human skin, sulfur mustard causes blisters. Blistering as a response to exogeneous stimuli is a unique aspect of human skin. Formation of full, fluid-filled blisters due to heat, cold or chemical vesicants like mustards usually does not occur in animal skin. The

biochemical process underlying the blister formation on human skin by these stimuli, is not clear. The blisters caused by sulfur mustard, a warfare agent, are extremely large and slowly healing. Even today, preventive or therapeutic measures to these disabling lesions are ineffective. The development of a rational intervention of vesication depends on an understanding of the mechanism of action of sulfur mustard. In chapter 2 the literature on the mechanism of action of sulfur mustard at the molecular and cellular level has been reviewed. For an exhaustive review of the current state of the toxicology of sulfur mustard and its mechanisms of action, the reader is referred to Papirmeister and coworkers (1991). A brief description of sulfur mustard-induced injuries in man is given in chapter 3, and the specific effects of sulfur mustard on the skin are discussed in chapter 4. In that chapter also sections are included on skin structure and function (4.1), on skin reactions upon contact with irritants, in particular blistering (4.2), and on skin models and their application in dermatotoxic research (4.3).

From the literature review it could be concluded that the sulfur mustard concentration that reaches the basal epidermal cells of human skin after topical exposure to concentrations inducing vesication, is unknown. If an estimate could be made, this would aid the choice for concentrations of sulfur mustard in future *in vitro* studies, and give an indication of the biochemical sequela primary responsible for the onset of blistering. Experimental studies aimed to obtain an answer to these questions have been performed in two *in vitro* models for human skin. Early studies had provided evidence that DNA is an important cellular target of the bifunctional alkylating agent sulfur mustard. Based on the wide-spread opinion that sensitivity of cells to sulfur mustard is dependent on their capacity to overcome DNA interstrand cross-links, the formation and repair of these cross-links in cultured human epidermal keratinocytes have been studied (chapter 5). In addition to DNA lesions, sulfur mustard causes disturbance of metabolic processes in the cell. The various toxic effects induced by sulfur mustard and the cell's capacity to recover from these damage has been examined in cultured human epidermal keratinocytes by assays on several cell functions (chapter 6). Investigations on the possible use of nicotinamide as a therapeutic agent have been performed in both sulfur mustard-exposed cultured human epidermal cells (chapter 7) and organ cultured pieces of human skin (chapter 8). These experiments were based on a hypothesis postulated by Papirmeister et al. (1985), suggesting that cellular NAD^+ depletion might play a crucial role in the formation of blisters in sulfur mustard-exposed human skin. Finally, in chapter 10 some alternative mechanisms of sulfur mustard-induced blister

formation in human skin are proposed.

This thesis also contains a chapter on the application of a cultured skin equivalent to promote the healing of extended or slowly improving skin defects. Experiments with this cultured skin equivalent can be considered as a "spin off" of the development of *in vitro* models of human skin for dermatotoxic research. In chapter 9 the successful treatment of venous leg ulcers with these skin grafts, composed of a dermal and an epidermal component, is reported.

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2 MOLECULAR AND CELLULAR TOXICITY OF SULFUR MUSTARD

2.1 Chemistry of alkylation

Sulfur mustard (*bis*-(β-chloroethyl)sulfide; HD) is a representative of the mustards, a group of bifunctional alkylating agents containing at least two β-chloroethyl groups attached to a sulfur or nitrogen atom (Fig. 2.1). Monofunctional analogues, containing one β-chloroethyl group, are called semi-mustards.

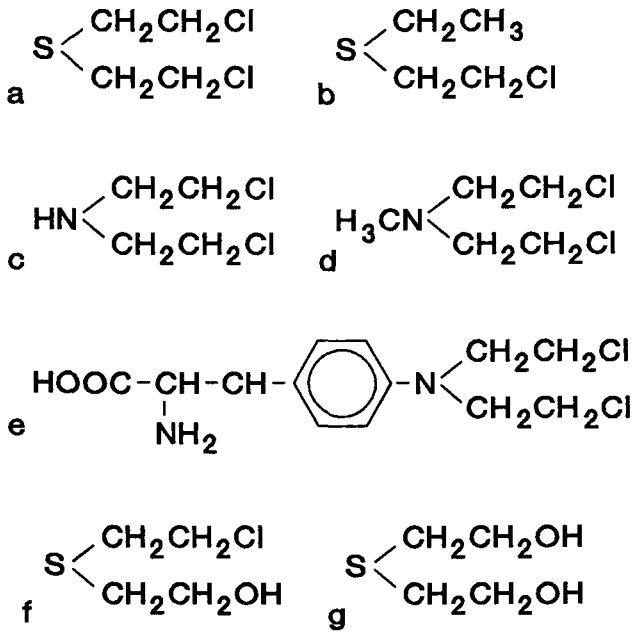

Figure 2.1. Structural formulas of several mustards and some of their hydrolysis products. a) bis(β-chloroethyl)sulfide; sulfur mustard. b) β-chloroethyl-ethylsulfide. c) bis(β-chloroethyl)-amine; nornitrogen mustard. d) bis(β-chloroethyl)methylamine; nitrogen mustard. e) bis(β-chloroethyl)phenylalanineamine; melphalan. f) β-chloroethyl-β-hydroxyethylsulfide;semi sulfur mustard. g) bis(β-hydroxyethyl)sulfide; thiodiglycol.

An alkylating agent is an electrophile and may combine with a variety of nucleophilic centers. In general, a nucleophilic substitution reaction may be defined as the conversion

$$R—X + Y^- \longrightarrow R—Y + X^-$$

where R-X represents the alkylating agent, Y^- the nucleophile, R-Y the alkylated nucleophile and X^- the leaving group. Theoretically, nucleophilic substitution reactions may follow first order or second order kinetics, but in practice a strict discrimination can not be made. In a nucleophilic substitution with first order kinetics (S_N1) the formation of a reactive intermediate (R^+) is the rate limiting step and is followed by a rapid reaction with a nucleophile (Y^-) to produce the alkylated product (R-Y).

$$R—X \longrightarrow R^+ + X^- \xrightarrow{Y^-} R—Y + X^-$$

The reaction rate is essentially independent of the concentration of the target nucleophile. In contrast, the rate of a nucleophilic substitution with second order kinetics (S_N2) is dependent on the collision frequency between the alkylating agent (R-X) and the target nucleophile (Y^-), and thus on the concentrations of both reactants.

$$R—X + Y^- \longrightarrow [\, Y \ldots R \ldots X \,] \longrightarrow R—Y + X^-$$

Mustards are one of the earliest known alkylating agents. The mechanism by which they alkylate is rather unusual, as mustards undergo two subsequent nucleophilic substitutions. The first is an internal S_N2 reaction by the sulfur or nitrogen atom on the β-carbon atom with replacement of chlorine, resulting in a cyclic intermediate. The second nucleophilic substitution is the reaction of the intermediate with external nucleophilic centers (Fig. 2.2). In the case of nitrogen mustard (bis-(β-chloroethyl)methylamine; mechlorethamine; HN2), the cyclization in aqueous solution results in an aziridinium ion. Because of the stability of the aziridinium intermediate the consecutive reaction with an external nucleophilic group occurs relatively slowly. Overall, alkylation by nitrogen mustard exhibits second order kinetics (S_N2). In contrast, the intermediate episulfonium ion in sulfur mustard alkylation is highly reactive and will react rapidly with a nucleophilic center, presumably due to the ring strain in the episulfonium ion. This may also explain why the episulfonium ion can only be isolated in the presence of counter-ions with a very low nucleophilic reactivity

(Vorob'eva et al., 1975). Alkylation by sulfur mustard approaches S_N1 mechanism, as has been shown for hydrolysis, on condition that the agent is dissolved in an organic solvent prior to addition to water and that the concentration is kept below 1 mM (Yang et al., 1988).

Recently, it has been established that episulfonium ion intermediates are also involved in the reaction of 1,2-dibromoethane and other *vic*-dihaloalkanes with DNA. The compounds are first transferred enzymatically to glutathione by a GSH-S-transferase mediated step to produce S-(2-haloethyl)conjugates. Then, these metabolites can react with DNA as monofunctional alkylatings agents through the formation of episulfonium ion intermediates (Peterson et al., 1988).

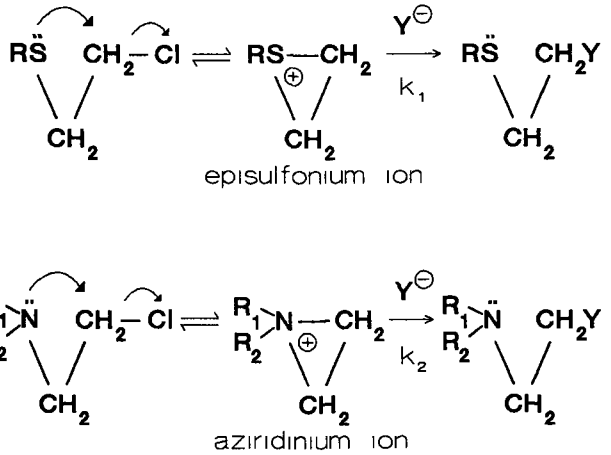

Figure 2.2. Assumed mechanism of alkylation of sulfur and nitrogen mustard, where Y represents an external nucleophilic group. The rate constant for reaction with an external nucleophilic group is higher for sulfur mustard (k_1) than for nitrogen mustard (k_2).

The structure and physicochemical properties of the alkylating agent are of fundamental importance in determining the selectivity for a nucleophilic target. Additionally, the selectivity is influenced by the nucleophilicity of the nucleophilic groups. The selectivity of sulfur mustard for a number of nucleophilic groups in aqueous solution was determined by Ogston (1948) as a competition factor. This factor

$$F_y = K_y/K_{H2O}$$

describes the relative affinities of various substances in competition with water for

sulfur mustard. K_{H2O} and K_y are rate constants for reactions of an alkylating agent with water and added nucleophilic agents, respectively. Later, Swain and Scott (1953) developed a two parameter equation to correlate the relative rates of reactions of various nucleophilic reagents with a series of substrates in aqueous solution

$$\log (K_y/K_{H2O}) = s.n$$

in which s is a constant charactistic of the alkylating agent that measures the ability to select between nucleophiles with different strenghts, expressed as n. Usually, the s-value of an alkylating agent is indicated for reaction with nucleophilic centers with strength around n=2, representing the average nucleophilicity of DNA (Table 2.1). The high s-values (>0.9) of sulfur and nitrogen mustards indicate that they are rather selective, with a preference to react with soft (easily polarizable) nucleophilic centers such as thiol groups of proteins and nitrogen atoms of purines. The affinity of sulfur mustard to soft nucleophiles is further illustrated by the high competition factors manifested by sulfur compounds such as thiophosphates and thiols (Stein, 1946). In this section a very brief description of the chemical processes of alkylation was given. More information on the chemistry of alkylation can be found in textbooks on organic chemistry and review articles (Sykes, 1981; Coles, 1984/1985).

2.2 Alkylation of cellular macromolecules

2.2.1 *Introduction*

Cellular constituents, such as nucleic acids and proteins contain a variety of nucleophilic sites, to which considerable binding by alkylating agents will take place. In Table 2.2 the macromolecular nucleophiles are classified in order of their chemical "hardness", which is an inversed function of the polarizability of the nucleophilic center. Similarly, alkylating agents can be classified according to polarizability of the electrophilic centers. It is expected that the softest electrophiles will preferentially attack the softest nucleophilic centers. However, the biological alkylation reaction is not only determined by the selectivity, represented by n and s. Firstly, the specificity of an alkylating agent is determined by its uptake into the cell and the passage to the nucleus. The lipophilicity of a compound influences strongly its penetration rate and cellular distribution. Lipid soluble compounds will easily penetrate by means of passive diffusion. Nitrogen mustard enters the cell via active transport over the choline carrier (Goldenberg et al., 1971; Doppler et al., 1988). The aromatic nitrogen

Table 2.1. S-values of some alkylating agents

COMPOUND	S-VALUE
N-Ethyl-N-nitrosourea	0.26[a]
N-methyl-N-nitro-N-nitrosoguanidine	0.42[a]
Diethyl sulfate	0.64[a]
Ethyl methanesulfonate	0.67[b]
Methyl methanesulfonate	0.83[a]
Dimethyl sulfate	0.86[a]
Bis-chloroethylnitrosurea	0.94[a]
Episulfonium ion of sulfur mustard	0.95[c]
Ethylene oxide	0.96[a]
Methyl bromide	1.00[a]
Nitrogen mustard	1.18[a]
2-Chloroacetaldehyde	1.30[b]
Melphalan	1.39[a]

Adopted from a) Barbin and Bartsch, 1989; b) Vogel, 1989; c) Whitfield, 1987.

mustard derivative, phenylalanine mustard or melphalan (see Figure 2.1), is taken up by a transport system that normally reacts with leucine and other neutral amino acids. Knowledge on the mechanism of cellular uptake of sulfur mustard is lacking, but due to its small size and rather high lipophilicity (the solubility of sulfur mustard in water is 0.7 mg/ml) it might be a passive process. Secondly, the rate of hydrolysis of the alkylating agent may determine the biological activity since alkylation of water is a principal route of degradation of alkylating agents. Hydrolysis of mustards causes the formation of β-hydroxyethyl groups, resulting in monofunctional semi-mustards and subsequently in non-toxic bis-(β-hydroxyethyl) compounds (see Figure 2.1). Finally, the accessibility of nucleophilic sites in macromolecules may be limited by steric hindrance.

2.2.2 *DNA alkylation*

The nitrogen and oxygen atoms of the DNA molecule present many potential sites for alkylation. The primary sites of attack of the purine and pyrimidine bases are shown in Fig. 2.3. Many electrophilic species react predominantly at the N-7 position of guanine, because this position is rather electronegative and also exposed to the major groove of the DNA molecule. Sulfur mustard and nitrogen mustard were amongst the first compounds for which the alkylated DNA adducts were identified. *In vitro* reactions of mustards with single-stranded DNA yielded derivatives resulting

Table 2.2. Nucleophiles and electrophiles of biological importance, arranged in order of increasing hardness. (From Coles, 1984/85)

NUCLEOPHILES
Thiol groups of cysteinyl residues in protein and glutathione
Sulfur atoms of methionyl residues in protein
Primary amino groups in protein (arginine and lysine)
Secondary amino groups in protein (histidine)
Amino groups of purine bases in RNA and DNA
Oxygen atoms of purines and pyrimidines
Phosphate oxygen of RNA and DNA
ELECTROPHILES
Aldehydes, polarized double bonds
Epoxides, strained-ring lactones, aryl sulfates, alkyl halides
Arylcarbonium ions
Benzylic carbonium ions, nitrenium ions

from reaction at the N-7 atom of guanine, the N-1 and N-3 atom of adenine, the N-3 atom of cytosine and the O^6 of guanine (Lawley and Brookes, 1967; Price et al., 1968; Lawley et al., 1971/72; Ludlum et al., 1986). However, probably due to steric and electrostatic factors, only monoadducts of N-7 guanine and N-3 adenine, representing approximately 65% and 17% of total alkylations, respectively, could be identified from reaction with native DNA (Brookes and Lawley, 1963; Lawley and Brookes, 1967; Lawley et al., 1969). Recently, it was shown that nitrogen mustards react preferentially at guanines flanked by other guanines in DNA, probably due to the favorable electrostatic charge resulting from interactions with the neighbouring guanines (Mattes et al., 1986; Kohn et al., 1988). Besides monoadducts, diadducts involving the N-7 atoms of two guanines accounted for approximately 17% of all alkylations by mustards in native DNA. Based on the isolation of these di(guanin-7-yl) products from DNA of cells treated with sulfur mustard or nitrogen mustard, Brookes and Lawley (1961, 1963) postulated that the bifunctional mustards produced DNA cross-links. From a subsequent study, Walker (1971) estimated that interstrand cross-links (covalent bindings that occur between guanine moieties in opposite DNA strands) represent one-third of the total diguaninyladducts and that intrastrand cross-links (diadducts occurring between adjacent guanines in the same DNA strand) form

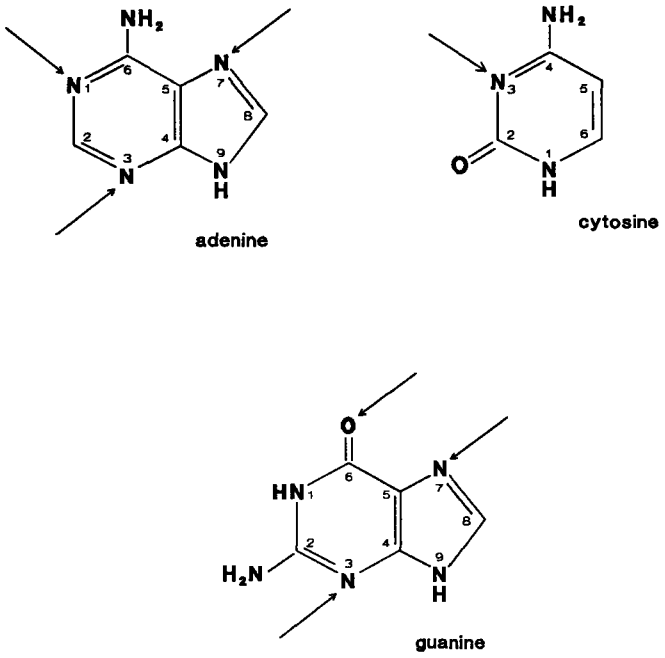

Figure 2.3. Base positions that are preferentially attacked by electrophilic agents: the N-7, N-3, and O-6 atoms of guanine; the N-1, N-3 and N-7 atoms of adenine; the N-3 atom of cytosine

the major part of the mustard-induced diadducts. Ball and Roberts (1970) showed that the percentage of interstrand cross-links relative to total alkylations is independent of the dose of sulfur mustard. Direct evidence for theformation of interstrand cross-links in DNA of mammalian cells resulting from treatment with sulfur mustard or nitrogen mustard was provided by various methods (Kohn et al., 1966; Ball and Roberts, 1971/1972; Jolley and Ormerod, 1973). However, these techniques were rather insensitive. The introduction of the alkaline elution technique by Ewig and Kohn (1977) allowed detection of interstrand crosslink formation after exposure of human melanoma cells to concentrations as low as 0.1-0.2 μM nitrogen mustard, which are subtoxic concentrations that cause >50% survival of the cells (Hansson et al., 1987). The maximum number of interstrand cross-links induced by sulfur mustard or nitrogen mustard could be measured between 0 and 1.5 hr after a 30 min exposure, whereas the levels of crosslinking decreased during further post-exposure incubation (Ewig and Kohn, 1977; Ross et al., 1978; Murnane and Byfield,

1981; Hansson et al., 1987; this thesis, chapter 5). In contrast, the maximum level of cross-links induced by melphalan, an aromatic derivative of nitrogen mustard, was reached 12 hr after exposure (Ross et al., 1978).

Only a few studies have been conducted on mustard-induced cross-linking between DNA and proteins. DNA-protein cross-links are thought to be important lesions because they might impede replication, transcription or repair of DNA. Tew et al. (1983) reported that interference of DNA-matrix association by DNA alkylation could contribute to the cytotoxicity of alkylating agents. A study of Moy and Tew (1986) indicated that the difference between mustard-resistant and -sensitive cells might reside within the nuclear matrix phosphoproteins. Both reports suggest the importance of the interaction of mustards and nuclear proteins. Both histones as well as non-histone proteins can be involved in DNA-protein cross-linking. Recently, nitrogen mustard-induced cross-linking of DNA to the DNA matrix protein nucleophosmin has been shown (Chan, 1989). Nucleophosmin is essential for ribosome assembly. The availability of a new immunochemical method for detection of DNA-protein cross-linking might enhance the number of investigations on this subject (Miller and Costa, 1990). Data obtained by Ewig and Kohn (1978) suggested that a small part of the measured cross-links induced by nitrogen mustard in L1210 cells consist of DNA-protein cross-links. However, Murray and Meyn (1986) reported that 26% of total cross-links induced in CHO cells by nitrogen mustard were DNA-protein cross-links and Hansson et al. (1987) even mentioned that 60-70% of the total amount of cross-links induced by nitrogen mustard in RPMI 8322 cells occurred between DNA and protein.
The alkylation of RNA by sulfur and nitrogen mustard has only been assessed in studies in bacteria and yeast (Venitt, 1968; Shooter et al., 1971).

2.2.3 *Protein alkylation*

Amino acid residues on protein represent nucleophilic sites of varying strength. The most easily alkylated nucleophilic centers are the sulfur containing groups. Studies on the binding of alkylating agents with hemoglobin have shown that, in addition to the sulfur atom of cysteine residues, other sites of attack may be the terminal nitrogen of valine, the two nitrogen atoms of histidine and several carboxylic groups (Osterman-Golkar et al., 1976; Segerbäck, 1990). The pattern of protein adducts following alkylation is dependent on the selectivity of the compound, just as with DNA base alkylation. Since protein adducts, in contrast to DNA adducts, are

generally stable, they are increasingly used in dosimetry studies in which the biologically effective dose is determined by measurement of the reaction adducts following alkylation. Especially electrophiles with s-values higher than 0.9, such as sulfur and nitrogen mustard, easily alkylate proteins (Coles, 1984/1985). This may indicate that the reaction of mustards with proteins is favored compared to reaction with DNA. Studies on the proportional alkylation between DNA and protein have not been performed, but numerous investigations have assessed the reaction of sulfur mustard with a variety of proteins, such as serum proteins and nuclear proteins, keratin, ovalbumine, fibrinogen and collagen (Levy, 1946; Pirie, 1947; Peters and Wakelin, 1947; Boursnell, 1948). Furthermore, studies on *in vitro* systems using cell homogenates or purified extracts showed that several enzymes could be inhibited by sulfur mustard (Bailey and Webb, 1948; Wheeler, 1962). Although it should be noted that in these experiments very high concentrations of sulfur mustard were used, these data suggest a high affinity of sulfur mustard for proteins. It was remarkable that the alkylation reactions of sulfur mustard with proteins at pH 6.0 - 8.0 predominantly involved carboxyl groups, instead of the more nucleophilic sulfhydryl groups (Banks et al., 1946; Herriot et al., 1946). Probably, the accessibility of sulfhydryl groups of proteins is limited, whereas sulfhydryl groups which form part of small molecules (e.g. glutathione) are easily alkylated.

2.3 DNA alkylation repair

2.3.1 *Mechanism of removal of monofunctional adducts*

Most monofunctional adducts of alkylating agents with DNA are eliminated relatively rapidly, due to chemical instability or to biochemical repair. The repair of N-7 guanine, N-3 adenine and O^6 guanine adducts in bacterial and mammalian cells has been a major focus of study (for reviews see Roberts, 1978; Singer and Grunberger, 1983; Bohr et al., 1989). There is increasing evidence that DNA adducts formed in active genes are repaired preferentially to those formed in inactive genes. (Bohr and Wasserman, 1988). Usually, monofunctional guanine-adducts are removed through nucleotide excision repair, an enzyme-mediated process, whereby the damaged bases are excised together with a number of undamaged nucleotides and replaced by a new DNA strand. This mechanism has been extensively described by Ross and Carter (1984). Both the enzymatic repair as well as the spontaneous loss of the alkylated purines 3-alkyladenine and 7-alkylguanine by chemical instability lead to

apurinic sites or breaks in the DNA strands. In recent years it has become clear that DNA strand breaks activate the nuclear enzyme poly(ADP-ribose)polymerase (Zahradka and Ebisuzaki,1982; Ferro and Olivera,1982; Berger, 1985; for review see Althaus and Richter, 1987). This enzyme cleaves the β-N- glycosidic links of NAD^+ molecules to obtain the ADP-ribose groups, which are subsequently added to various proteins, forming large, negatively charged polymers (Ueda and Hayashi, 1985; Cleaver and Morgan, 1991). In addition to histones and other chromatin proteins, the enzyme itself is the major substrate of poly(ADP-ribose)polymerase. The poly(ADP-ribose) chains have a short lifetime and cause a transient structural modification of proteins. It has been postulated that ADP-ribosylation of chromatin might promote a transitory decondensation of the chromatin which makes the access of DNA repair enzymes sterically possible (Niedergang et al., 1985; De Murcia et al., 1986, 1988). Thus, low levels of damage activate the polymerase, causing an increased rate of DNA repair and ligation. However, when DNA is extensively damaged, activation of the polymerase might deplete the cellular NAD^+ pool to such an extent, that its levels become reduced to a point at which the cell cannot survive (Berger, 1985). In this way, activation of poly(ADP-ribose)polymerase may play an important role in the regulation of the cell response to high levels of DNA damage.

2.3.2 *Mechanism of removal of DNA-DNA and DNA-protein cross-links*

The ability of mammalian cells to overcome DNA interstrand cross-links has been unequivocally established. Most of DNA interstrand cross-links induced by sulfur mustard (Lawley and Brookes, 1965; Crathorn and Roberts, 1966; Roberts et al., 1968, 1971a; Reid and Walker, 1969), by nitrogen mustard and its derivatives (Yin et al., 1973; Hansson et al. 1987, 1988) or by cis-platin (Plooy et al., 1984) disappeared within 24-36 hours. Although the precise mechanism by which cross-linking agents cause cell death is still subject to question, the ability to circumvent DNA cross-links is generally thought to determine the sensitivity of cells to cross-linking agents. It is assumed that even one cross-link may inhibit cell replication (Lawley et al., 1969).

Only a few studies exist on the mechanism of repair of DNA cross-links. Papirmeister and Davison (1964) and Lawley and Brookes (1965) suggested that excision repair of DNA cross-links occurs in *E. coli*. Similarly, Cole et al. (1976) proposed that in *E. coli* psoralen-induced cross-links might be removed by two strand cuts on each side of one arm of the crosslink and subsequent repair of the DNA strand. The actual

mechanism that removes DNA cross-links in mammalian cells is not clear. It is supposed that one arm of the cross-link is detached, followed by nucleotide excision repair of the remaining bulky diadduct on the second DNA strand (Reid and Walker, 1969; Walker and Reid, 1971).

The removal of DNA-protein cross-links has been scarcely investigated and some controversial results have been reported (Fornace and Kohn, 1976; Cress and Bowden, 1983; Sugiyama et al., 1986). Generally, the disappearance of DNA-protein cross-links is relatively slow. Mechanisms by which DNA-protein cross-links are removed have not been examined, although the involvement of proteases has been supposed.

2.3.3 *Repair of mustard-induced DNA damage*

Removal of mustard-induced damage of DNA in *E. coli* and mammalian cells has frequently been demonstrated (Papirmeister and Davison, 1964; Kohn et al., 1965; Lawley and Brookes, 1965, 1968; Crathorn and Roberts, 1966; Roberts et al., 1968, 1971b; Venitt, 1968; Chun et al., 1969; Reid and Walker, 1969; Walker and Reid, 1971; Yin et al., 1973; Ewig and Kohn, 1977; Ross et al., 1978; Murnane and Byfield 1981; Murray and Meyn, 1986; Hansson et al., 1987, 1988; Sorscher and Conolly, 1989). Some contradictionary reports exist about the relative rates of removal of the mono- and bifunctional adducts. Random excision of both adducts was observed in HeLa cells, but preferential loss of the bifunctional adducts was seen in mouse L cells and Lettre-Ehrlich cells (Roberts et al., 1971a; Reid and Walker, 1969; Yin et al., 1973). There is also some disagreement on the question whether differences in sensitivity between sensitive and resistant cell lines to sulfur mustard or nitrogen mustard could be explained on the basis of overall differences in repair of alkylated DNA (Yin et al., 1973; Chun et al., 1969; Helliger et al., 1988).

Removal of a part of the DNA-protein cross-links at 24 hr following treatment with nitrogen mustard has been demonstrated by Fornace and Kohn (1976), Ewig and Kohn (1978) and Murray and Meyn (1986).

2.4 NAD^+ depletion and its consequences

Conditions that cause DNA damage, including ionizing irradiation or exposure to alkylating agents, including sulfur mustard, specifically induce a depletion of NAD^+ (Roitt, 1956; Hilz et al., 1961; Scaife, 1963; Goodwin et al., 1978; Jacobson et al., 1980; Rankin et al., 1980; Berger et al., 1986; Oleinick and Evans, 1985; Meier et al.,

1987; Mol et al., 1989). Initially, this has been interpreted as an inhibition of NAD^+ synthesis and subsequently by an activation of NAD^+ase, since NAD^+ depletion could be prevented by nictotinamide (Hilz et al., 1961). The detection of poly(ADP-ribose)polymerase activation by DNA fragmentation (Miller, 1975; Whish et al., 1975) and of poly(ADP-ribose) formation and metabolism (Hilz and Stone, 1976; Hayaishi and Ueda, 1977; Purnell et al., 1980) provided a new interpretation. Since then, a link between the drop in NAD^+ and elevated poly(ADP-ribose) synthesis due to DNA damage caused by radiation or alkylation has been frequently demonstrated (Davies et al., 1977; Skidmore et al., 1979; Benjamin and Gill, 1980; James and Lehmann, 1982; Wielckens et al., 1982; reviewed by Cleaver and Morgan, 1991).

In mammalian cells NAD^+ is involved in at least two different types of metabolism. In addition to its involvement in the above mentioned cellular ADP-ribosylation reactions, it is utilized as a coenzyme in many enzymatic oxidation-reduction reactions in which it is rapidly conversed from the oxidized into the reduced form and vice versa without loss of NAD^+ molecules from the NAD^+ pool (Kaplan, 1985). The decrease in the cellular NAD^+ concentration due to extensive DNA damage inhibits the NAD^+-dependent enzymes of the oxidation-reduction reactions, which may eventually lead to ATP depletion (Skidmore et al., 1979; Seto et al., 1985; Tanizawa et al., 1987). In turn, ATP depletion may cause cell death. This cell death initiated by extensive DNA damage is termed "the suicide response" of mammalian cells (Berger, 1985; Berger et al., 1983). The rationale may be that the genetic fixation of an excess of aberrant DNA is prevented (Gaal et al., 1987).

Conflicting reports exist on the question whether depletion of cellular NAD^+ and ATP levels following treatment of cells with monofunctional alkylating agents or irradiation could be prevented by inhibitors of poly(ADP-ribose)polymerase, such as 3-aminobenzamide, thymidine, theophylline and nicotinamide (Durkacz et al., 1980, 1981; Berger and Sikorski, 1980; James and Lehmann, 1982; Sims et al., 1982, 1983; Cleaver et al., 1985; Schraufstatter et al., 1986; Tanizawa et al., 1987; Stubberfield and Cohen, 1988). In addition to the question of their efficacy, evidence was shown that inhibitors of poly(ADP-ribose)polymerase might enhance cytotoxicity and mutagenicity of cells treated with alkylating agents (Jacobson et al., 1984; Lunec et al., 1984; Schwartz et al., 1985; Ben Hur et al., 1985; Cleaver and Morgan, 1987) and disturb other metabolic processes (Milam and Cleaver, 1984; Grunfeld and Shigenaga, 1984; Nadeau and Lane, 1989).

Early studies had clearly demonstrated that sulfur and nitrogen mustards generally inhibited glycolysis and respiration (Berenblum et al., 1936; reviewed by Wheeler,

1962). Fraser (1960) suggested that the inhibition of glycolysis following exposure to nitrogen mustard might be due to the decrease of NAD^+ levels. Later, depletion of NAD+ levels due to sulfur mustard-exposure has been demonstrated in several cell types (Gross et al., 1985; Meier et al., 1987; Mol et al., 1989).

2.5 Genotoxicity and cytotoxicity

As a result of the alkylation of DNA, alkylating agents cause genotoxic and cytotoxic damage. Genotoxic damage involves the formation of potential miscoding DNA adducts, which lead to mutagenic and carcinogenic alterations in next cell generations. In the case of cytotoxic damage no progeny can be formed since cell death occurs by inhibition of replication due to DNA template inactivation.
There is an increasing need for risk evaluation of genotoxic agents. The chemical structure of an alkylating agent, which is closely related to its nucleophilic selectivity, is an important determinant for the mutagenicity and carcinogenicity of a potential genotoxic agent. Ashby and Tennant (1989) proposed 18 structural groups that may be considered conspicious with respect to genotoxicity. The nucleophilic selectivity can be expressed either as the Swain-Scott constant s (see section 2.1) or as the N-7/O^6 ratio, indicating the preference of the alkylating agent for alkylation of the N-7 position of guanine or the $O^{6\ \text{position of}}$ guanine. Alkylating agents with preference to alkylate O^6 guanine will potentiate mutagenicity by miscoding of the alkylated guanine with thymine instead of cytosine, resulting in GC-AT transitions. If an alkylating agent shows preference to alkylate N-7 guanine, this may promote the incidence of base-pair substitutions at the apurinic sites resulting from spontaneous depurination or enzymatic repair of the N-7 guanine adduct (see section 2.3).
For a small number of carcinogens Barbin and Bartsch (1989) reported a linear relationship between the nucleophilic selectivity and the median TD_{50} estimates in rodents; TD_{50} is defined as the total dose of carcinogen in mg/kg body weight required to reduce by 50% the probability of the animal being tumor-free throughout a standard lifetime. For a larger group of monofunctional alkylating agents Vogel (1989) and Vogel et al. (1990) presented evidence that data on nucleophilic selectivity correlated well with two genotoxicity tests in *Drosophila*, i.e. tests on the mutation enhancement ratio in *exr*$^-$ genotypes relative to *exr*$^+$ genotypes and on the relative clastogenic activity. Unlike the monofunctional agents, crosslinkers seem to show no correlation between nucleophilic selectivity and hypermutability in *exr*$^-$ cells

or tumor induction. Their relative carcinogenic potencies are higher than those of monofunctional agents with equal s-values. Probably, this will be due to the fact that cross-linking agents can induce chromosomal aberrations and produce efficiently deletion mutations at low dose levels. This data indicate that cells may "survive" the consequences of cross-linkage. This conclusion might be contrary to the widespread opinion that the formation of cross-links by bifunctional alkylating agents might cause limited genotoxicity.

Vogel et al. (1990) further demonstrated that *Drosophila* can be used to determine *in vivo* mutational spectra of potential carcinogenic agents, i.e. the frequency of base-pair substitutions, deletions or double mutations induced by an alkylating agent.

Concerning the mustards, much of the early work on the genotoxicity of these compounds has been well documented in reviews (Loveless, 1966; Auerbach, 1976; Fox and Scott, 1980). More recently, the genotoxicity of nitrogen mustard has been studied by Vogel (1989) and Vogel et al. (1990). Kunz and Mis (1989) reported that nitrogen mustard induces all types of base-pair substitution as well as a few deletions and a considerable amount of double mutations.

As the mustard-induced effects on the DNA of cells, such as nuclear pycnosis, inhibition of cell division, chromosome fragmentation and gene mutation, resembled those induced by γ-irradiation, mustards were initially classified as radiomimetic (Harold and Ziporin, 1958). Loveless (1966), however, pointed out that the term had only limited value, since there are also considerable differences between mustard- and γ-irradiation-induced effects.

The International Agency for Research on Cancer has classified sulfur mustard and some of the nitrogen mustards as being causally associated with cancer in humans (IARC, Suppl.4, 1982). Tumors like Bowen's disease, basal cell carcinoma and respiratory neoplasms have been described in former workers of poison gas factories during World War II (Easton et al., 1988). The formation of second tumors has been reported in patients treated with nitrogen mustard-derived antitumor agents (for review see Colvin, 1982).

Cytotoxicity is usually measured in a clonogenic assay as the ability of cells to replicate. Concentrations of sulfur mustard as low as 2 μM caused 1% survival of HeLa cells (Crathorn and Roberts, 1966). At that low concentrations of sulfur mustard, DNA synthesis was also reduced, whereas synthesis of protein and RNA were affected at concentrations of sulfur mustard at least one order of magnitude higher (Harold and Ziporin, 1958; Lawley and Brookes, 1965,1968; Crathorn and

Roberts, 1966; Vaughan et al., 1988). As a result, it was observed that cells become enlarged "giant" cells, due to the fact that they could not divide, while RNA and protein synthesis continued at normal rates (Roberts et al., 1971a; Ku and Bernstein, 1988). Despite the fact that monofunctional DNA alkylations produced by sulfur mustard greatly exceed cross-links in number, they are considered to be less cytotoxic than DNA cross-links. Lawley et al. (1969) compared the actions of the monofunctional semi-sulfur mustard and the bifunctional sulfur mustard on bacteriophage T7, and showed that the formation of a cross-link was more cytotoxic than a single substitution reaction. Finally, cytotoxicity of the mustards depends on the position of the cell in the cell cycle at the time of treatment. Cells that are in the G1 phase of the cell cycle are most sensitive, whereas cells in mid to late S phase are most resistant (Evans and Scott, 1969; Roberts et al., 1971a; Savage and Breckon, 1981; Murray and Meyn, 1986).

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3 INJURIES PRODUCED BY SULFUR MUSTARD

3.1 Occurrence and use of sulfur and nitrogen mustards

Sulfur mustard has a reputation as a chemical warfare agent (Prentiss, 1937; Lohs, 1963). The compound was first synthesized in the second half of the 19th century (Meyer, 1887) and thereafter the vesicant properties rapidly became clear. During World War I, methods for industrial preparation of sulfur mustard were developed and in July 1917 the German army used sulfur mustard as a chemical weapon. Even when most countries abandoned the first use of chemical agents by signing the Geneva Convention of 1925, sulfur mustard remained a threat and was used in later military conflicts. Exposure to sulfur mustard is not immediately lethal, but eye lesions, respiratory insufficiency and extensive blistering are highly incapacitating. Despite extensive research, even today adequate protection or therapy against sulfur mustard does not exist.

In the development of cancer chemotherapy sulfur mustard has played an important role. Almost 60 years ago sulfur mustard was used in the treatment of tumors in experimental animals and even in man (Adair and Bagg, 1931; Berenblum, 1935). However, sulfur mustard appeared to be too toxic for application in chemotherapy and experiments were continued with the less toxic nitrogen mustard. In the sixties the first successes in the clinical treatment of cancer using alkylating derivatives of nitrogen mustard were seen, when these compounds were applied either alone or in combination with other agents. To date, nitrogen mustard and its derivatives, such as melphalan, chlorambucil and cyclophosphamide, belong to the most useful alkylating agents (reviewed by Colvin, 1982; Calabresi and Parks, 1985).

3.2 Local injury

Sulfur mustard as well as the nitrogen mustards cause local damage to the tissues with which they come in contact, such as the eyes, the skin and the respiratory tract (Vedder, 1925; Cullumbine, 1944; Renshaw, 1946; Aasted et al., 1987; Requena et al., 1988; Willems, 1989). Both the liquid and the vapor of the mustards may affect the eyes, producing conjunctivitis. On the skin, mustards produce lesions

varying from mild erythema to vesication, or even necrosis. Of all mustards, sulfur mustard causes the strongest vesicant effects. The acute effects of sulfur mustard on the skin will be described more in detail in chapter 4. Long term effects of sulfur mustard exposure have been observed in former workers of poison gas factories during World War II. These included hyper- and hypopigmented spots on the skin, persistant ulcerations and tumors, such as Bowen's disease and basal cell carcinoma (Inada et al., 1978; Klehr, 1984).
By inhalation, mustard vapor produces an inflammatory reaction in the respiratory tract. Many individuals, employed in the handling of sulfur or nitrogen mustard in poison gas factories and exposed to small quantities of the vapor over a long period of time, developed a residual chronic bronchitis (Morgenstern et al., 1947; Perera and Thomas, 1986). In these men and in soldiers exposed during World War I, an elevated incidence of malignant respiratory neoplasms was observed (Beebe, 1960; Wada et al., 1968; Kurozumi et al., 1977; Manning et al 1981; Easton et al., 1988).

3.3 Systemic injury

The systemic action of sulfur and nitrogen mustards, observed upon animal or human intoxication has been excellently reviewed by Anslow and Houck (1946). Therefore, a very brief description of this subject will follow. Once sulfur mustard or nitrogen mustard has entered the body, the compound is rapidly distributed over all tissues (Boursnell et al., 1946; Anslow et al., 1948; Zhang, 1987; Klain et al., 1988). Fast dividing tissues, particularly bone marrow and lymphoid tissue, are the most sensitive to mustard damage (Needham et al., 1947). As a result, leukopenia and immunosuppression may occur. In some cases, severe leukopenia combined with large infections may cause acute death (Alexander, 1947; Willems, 1989). The effects on hematopoietic tissue resembles that of injury due to radiation.
Other systemic effects that have been observed following exposure to mustards are gastro-intestinal and neural toxicity. The latter effects are difficult to demonstrate and are most pronounced following exposure to nitrogen mustard. Probably, the parasympathic nervous system might be affected. Furthermore, repeated exposures to low concentrations of mustards may cause hypersensitivity (Morgenstern et al., 1947; see references in Colvin, 1982).

3.4 Detoxicification and excretion

Little is known about the toxicokinetics of sulfur mustard and its metabolites in animal or man. It is assumed from investigations in the rat, that hydrolysis and conjugation of the β-chloroethyl groups to glutathione are the principal routes of degradation of sulfur mustard in the body. The sulfur atom of the metabolites might be either or not oxidized to sulfone (Davison et al., 1961; Roberts and Warwick, 1963; Hambrook et al., 1987; Klain et al., 1988). Within 1 day after contamination, approximately 80 - 90% of the sulfur-containing residues from sulfur mustard are excreted in the urine and could be identified as the above-mentioned metabolites. Remarkably, only a minor part of sulfur mustard was recovered as its hydrolyzed product, thiodiglycol, whereas sulfur mustard in aqueous solution is very susceptible to hydrolysis. Three months after injection of radiolabeled ^{35}S sulfur mustard in rats, some radioactivity was still detectable in urine samples (Hambrook et al., 1987). This may indicate that it takes three months to degrade and remove ^{35}S sulfur mustard bound to cellular macromolecules in the body. Another explanation is, that the highly lipophilic sulfur mustard is initially stored in body fat, from which it is slowly released. The presence of sulfur mustard in lipid compartments of the body has been reported by Drasch et al. (1987).

Very few data exist on the metabolic pattern of nitrogen mustard. The compound may be hydrolyzed (Bartlett and Swain, 1949) or enzymatically metabolized (Skipper et al., 1951). In mice, the radiolabeled ^{14}C of the methyl group of nitrogen mustard was recovered as exhaled carbon dioxide, which might be indicative for enzymatic demethylation. In contrast with sulfur mustard, the involvement of glutathione in the metabolism of nitrogen mustard has not been demonstrated yet.

3.5 Prophylaxis and therapy

Ideally, effective protection against systemic intoxication by sulfur mustard might be achieved when the toxic molecules are prevented to bind to the most vulnerable cell components, DNA and proteins. This goal might be reached in two ways. On the one side protective measures, such as masks, protective clothing and topical skin protectants might prevent direct exposure to sulfur mustard. On the other hand, the introduction of scavengers, nucleophilic groups for which sulfur mustard has great affinity, could be effective. Many compounds have been tested *in vitro* on their

capacity to protect against sulfur mustard cytotoxicity (for review see Whitfield, 1987). The most useful scavengers appear to be sulfur containing compounds: soft nucleophiles that will be easily attacked by the mustards (Coles, 1984-85). Among them are cysteine, sodium thiosulfate, glutathione and β-mercaptoethylamine and its derivatives. With the exception of glutathione, which was not effective, these drugs were only moderately effective in animals. If they were administered shortly before or after lethal doses of sulfur mustard or nitrogen mustard, they diminished mortality (Whitfield, 1987). Vojvodic and coworkers (1985) also demonstrated that systemic effects might be treated in an early stage with dexamethasone, promethazine, vitamin E, heparin and sodium thiosulfate. The administration of these drugs in rats, 30 minutes after intoxication with sulfur mustard or nitrogen mustard, enhanced the survival times of the animals and caused a decrease of the pathological changes in their organs. The combined administration of sodium thiosulfate and dexamethasone appeared most effective. The use of these drugs has also been recommended to treat early symptoms in human casualties (Fischbeck, 1969; Weger, 1980). In a later stage, symptomatic treatment is the only therapy possible. Recently, Wormser (1991) reported that a non-toxic and non-irritant iodine/povidone-iodine containing preparation effectively protected guinea pig skin from sulfur mustard damage, when applied up to 60 minutes after exposure.

As far as the skin is concerned, the latency period of several hours between the irreversible binding of sulfur mustard to cellular macromolecules and the onset of blistering, might be utilized to intervene in the biochemical events that underly blister formation. Unfortunately, little is known of these processes, so that selective intervention is difficult. Several years ago it was postulated that administration of nicotinamide might prevent blister formation (Papirmeister et al., 1985). However, the results of investigations reported in this thesis, showed that this hypothesis is not longer tenable. To date, formation of blisters cannot be prevented and their treatment is confined to prevention against secondary infections and the protection of the slowly recovering epidermis.

3.6 References

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4 DERMAL TOXICITY OF SULFUR MUSTARD

4.1 Skin structure and function

One of the major functions of the skin is to provide a barrier to protect the body against environmental hazards, such as bacteria, foreign materials, radiation and mechanical factors. Other functions are thermoregulation, the control of liquid and electrolyte loss, sensory functions, immunological defense and social communication. Only a brief description of skin structure and function will be given here, since the subject is covered very well in many handbooks and reviews (Goldsmith, 1983; Ackerman, 1978).

At the surface of the skin, the *stratum corneum* provides a physical barrier, which is made up of tightly packed layers of dead, cornified cells. This cornified layer is the result of a specific terminal differentiation process that the cells of the epidermis, the keratinocytes, undergo to become completely filled with keratin filaments, i.e. highly insoluble, disulfide-crosslinked, proteins (Steinert, 1975; for review see Bowden et al., 1987; Fuchs, 1990). The intercellular space between the cells of the *stratum corneum* is filled with alternating aqueous and lipid layers, the latter consisting mainly of free fatty acids, cholesterol and ceramides. The lipid content and the morphology of the *stratum corneum* are the major determinants of skin barrier function (Elias, 1983; Golden et al., 1987; Potts and Francoeur, 1990, 1991). The lipids are formed in the *stratum granulosum*, the layer of differentiating cells immediately underlying the *stratum corneum* (Elias et al., 1979; Holbrook, 1989). Below the *stratum granulosum* lies the *stratum spinosum*, largely composed of postmitotic cells. Only the cells of the innermost basal layer, the *stratum basale*, have the capacity for mitosis. The cells that are formed here, are slowly moved upwards to the skin surface and are ultimately shed as dead horny cells from the *stratum corneum*. The capacity of basal cells to divide is thought to be responsible for the re-epithelialization of skin wounds. The morphology of the human epidermis parallels that of a few other mammals with a thick epidermis, like the pig, but differs significantly from species with a thin epidermis, such as the usual laboratory rodents (Fig. 4.1).

The basal keratinocytes rest on a basement membrane, that serves as a selective membrane regulating water loss, ion exchange and protein transfer through the skin.

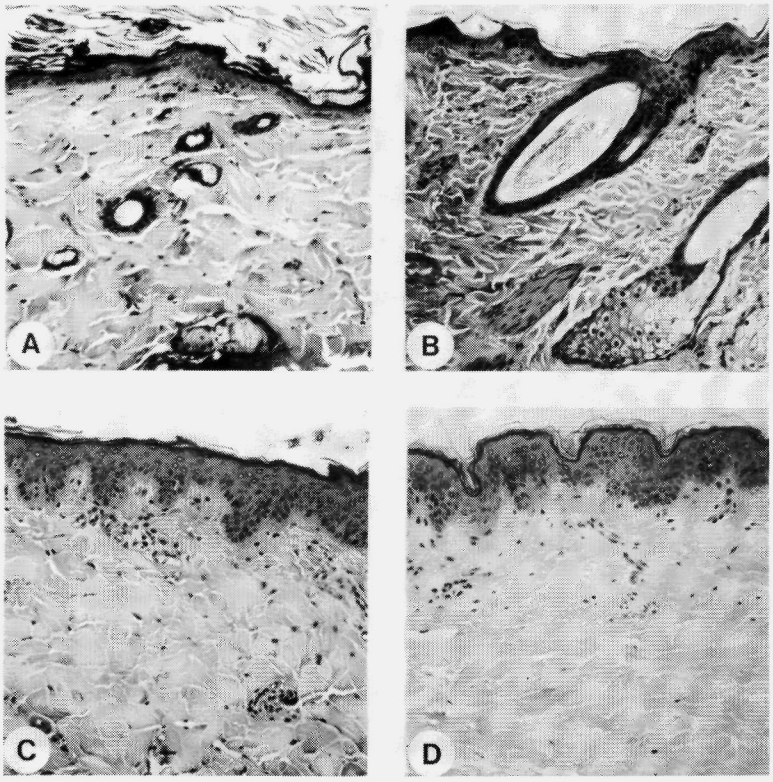

Figure 4.1. Light microscopic sections of skin originating from a) rat, b) horse, c) pig and d) man.

The basement membrane zone is important in the epidermal-dermal adherence. It is composed of various proteins, glycoproteins and proteoglycans that form a complex meshwork structure, based on protein-protein as well as protein-carbohydrate interactions. The ultrastructure of the basement membrane zone has been elucidated with electron microscopic studies (for reviews see Briggaman, 1983; Katz, 1984). A schematic representation of the dermal-epidermal junction is given in Figure 4.2. Proceeding from epidermis to dermis, the basement membrane zone consists of a) the basal keratinocyte plasma membrane, b) the *lamina lucida*, c) the *lamina densa* or *basal lamina* and d) the *subbasal* or *reticular lamina*. Together, the *lamina lucida* and the *lamina densa* form the basement membrane. Specialized attachment areas, hemidesmosomes, are tightly bound to the basal keratinocyte plasma membrane. They consist of an attachment plaque within the basal keratinocyte and anchoring filaments projecting outside this cell and establish a link

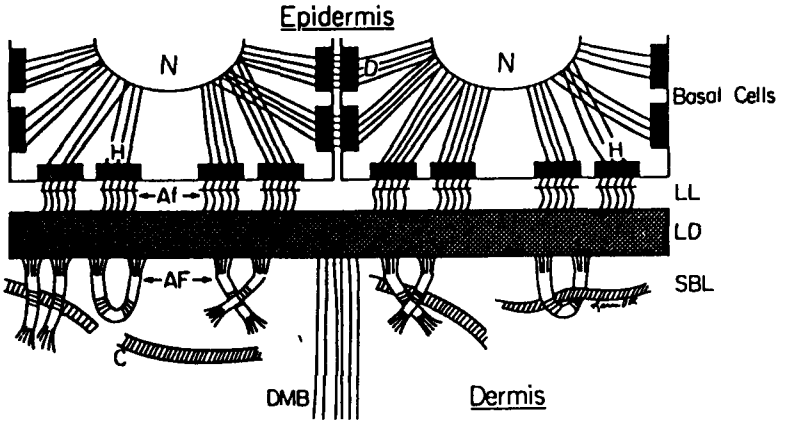

Figure 4.2. Diagrammatic representation of the epidermal-dermal junction. Adopted from Schechter, 1989. Symbols represent epidermal nucleus (N), desmosomes (D), hemi-desmosomes (H), lamina lucida (LL), lamina densa (LD), subbasal or reticular lamina (SBL), anchoring filaments (Af), anchoring fibers (AF), dermal microfibril bundles (DMB) and collagen fibers (C). Lines in basal cells of epidermis represent keratine filaments and border around the basal cells is the plasma membrane.

between the epidermal cytoskeleton and the collagen matrix of the dermis via the basement membrane. The anchoring filaments cross the *lamina lucida*, a 35-nm clear zone under the basal keratinocyte membrane and protrude into the *basal lamina* or *lamina densa*, the electron dense granular layer of the basement membrane. The *basal lamina* is connected to the dermis by anchoring fibrils, interstitial collagen fibres and dermal microfibril bundles, located in the *subbasal* or *reticular lamina*. In recent years, a number of specific components of the basement membrane zone has been identified, as well as their functional significance in most cases (for reviews see Sage, 1982; Donaldson and Mahan, 1988; Timpl, 1989; Uitto et al., 1989). It has been demonstrated that most of the basement membrane components are synthesized by dermal fibroblasts as well as by epidermal keratinocytes (Delvoye et al., 1988).

One of the structural elements of the basement membrane is type IV collagen, the major component of the *lamina densa* (Stanley et al., 1980). Type IV collagen interacts by means of its attachment sites with other basement membrane components including type VII collagen and laminin.

Type VII collagen has been identified as the biochemical building block of the anchoring fibrils (Sakai et al., 1986; Keene et al., 1987). Anchoring fibrils are

preferentially found in the dermis opposite hemidesmosomes and may specify the location of regenerating hemidesmosomes (Briggaman and Wheeler, 1975; Gipson et al., 1983).

Laminin is a large (900 kD) glycoprotein, with domains that bind to cells, type IV collagen and heparan sulfate proteoglyan (Woodley et al., 1983). Until recently, controversy existed in the literature as to the localization of laminin (Foidart et al., 1980; Laurie et al., 1982; Fleischmajer et al., 1985). Very recently, Horiguchi et al. (1991) demonstrated that laminin is present in the *lamina lucida* as well as in the *lamina densa*.

Heparan sulfate proteoglycan and possibly other protein-associated glycosaminoglycans are associated with anionic sites on the *lamina densa* (Stanley et al., 1982; Caughman et al., 1988; Fine and Couchman, 1988; Couchman et al., 1990). It is assumed that they regulate the charge-selective permeability of the basement membrane (Kanwar et al., 1980; Kazama et al., 1989).

Another component of the basement membrane zone is bullous pemphigoid antigen, a 230 kD molecule that is identified by its reactivity with bullous pemphigoid antibodies from serum of patients with the autoimmune blistering disease, bullous pemphigoid. It has been localized at or near the surface of basal cells and may be associated with the hemidesmosomes (Westgate et al., 1985; Mutasim et al., 1985, 1989; Thacher and Hefti, 1991).

Fibronectin is a large glycoprotein (500 kD) that is involved in cell attachment to extracellular matrices during wound repair (Couchman, 1990; Clark, 1990). As the normal basement membrane becomes established, fibronectin markedly diminishes (Clark et al., 1982). The exact localization of fibronectin in the basement membrane zone is not yet clear. Probably, fibronectin is present in the *lamina lucida* (Fleischmajer and Timpl, 1984).

Several other proteins that are involved in epidermal-dermal adherence have been identified. Amongst them are entactin, type V collagen, nidogen (Wikner et al., 1987; Alstadt et al., 1987) and a family of cell-surface receptor glycoproteins that may mediate cell adhesion to extracellular substrates such as basement membrane components (Hynes, 1987). These receptors mostly belong to the integrin family of large transmembrane heterodimers. Studies of normal skin have demonstrated that integrin receptors at special sites of the basal cell plasma membrane establish a link between the cytoskeleton, the plasma membrane, and the extracellular matrix. An integrin-type receptor ($\alpha_6\beta_4$) has been localized at the surface of basal keratinocytes in contact with the basement membrane (De Luca et al., 1990; Klein et al., 1990;

Carter et al., 1990).
The dermis is a dense fibroelastic tissue, within which the following structures are embedded: hairs, sweat glands, sebaceous glands, nerves, and blood and lymph vessels. The fibroblast is the cell that produces the fibers (collagen, reticulin and elastin) and the ground substance (hyaluronic acid, chondroitin sulfate and dermatan sulfate) of the dermis. The dermis can be morphologically subdivided into two distinct layers: the superficial papillary dermis, and the deeper, thicker reticular dermis. The collagen fibres in the reticular dermis are organized into bundles with a distinct orientation, which provides the skin with its characteristic biomechanical properties.
Compared with other mammals, the papillary dermis of man is highly vascularized. An overview of the structure of the human cutaneous vasculature has been presented by Braverman (1989). The dermal vasculature supplies nutrients to the metabolically highly active epidermis and the epidermal appendages, plays a central role in inflammatory skin reactions and is involved in a major way in the regulation of the human body temperature. In many mammals, the thermoregulatory function of the skin vasculature is confined to few skin sites with no or less hair, such as the tail and the ears. The ability to produce considerable amounts of sweat as a major component of thermoregulation is unique to man, horses and cattle (Scott, 1988).

Summarizing, compared to animal skin, the human skin has an unique structure and function. The lack of a hair coat on the human body may account for the relatively thick epidermis, the extensive dermal vasculature and the well-developed system for eccrine sweat secretion. Only the skin of the domestic pig shows similarities to human skin in some respects: the relative thickness of the epidermis, the sparsity of hair, the presence of a papillary dermis and a comparable vascularization (Forbes, 1969).

4.2 Skin toxicity

4.2.1 *Introduction*

Despite of the protection of the skin surface by the *stratum corneum*, the skin is a route of entry for many chemicals that cause systemic effects following uptake into the vascular system (Scheuplein and Bronaugh, 1983). It is assumed that substances which are both water- and lipid- soluble may easily penetrate the skin, because

the *stratum corneum* has both hydrophilic and lipophilic regions. Following passage through the *stratum corneum* transport in the epidermis occurs via the intercellular spaces and via the epidermal cells themselves. For lipophilic and large molecules significant penetration may occur via the transappendageal route, i.e. via the sweat glands, the hair follicles and the sebaceous glands. Vapors and gases may penetrate the skin if they are sufficiently soluble in the *stratum corneum*. One of the factors that may enhance percutaneous absorption is moisture, as it decreases the effectiveness of the barrier function and increases the contact between the agent and the skin surface (Bird, 1981). Another factor that may greatly influence the passage of chemicals into the skin is the vehicle in which a chemical is dissolved. In recent years, it became clear that the process of percutaneous absorption is not limited to diffusion of chemicals, but that also metabolism of chemicals may be involved. During percutaneous absorption, chemicals such as benzo(α)pyrene, may be metabolized by biotransformation systems that are present in the skin (Kao et al., 1985; for review see Kao and Carver, 1990).

Upon topical exposure, penetration of chemicals through the skin will be often accompanied by local damage. Several chemicals may cause only skin reactions and no systemic effects upon contact with the skin. The response of skin to toxic stimuli can be roughly divided into two classes: the allergic type in which the immune system of the body is involved, and the irritant type without immunologic factors. Below, the various aspects of irritant contact dermatitis will be illustrated in a short overview. More detailed information can be found in several reviews on this subject (Prottey, 1978; Parish, 1985; Marks and Kingston, 1985).

4.2.2 *Irritant contact dermatitis*

Primary irritants produce irritant contact dermatitis if they come in contact with skin in sufficient concentration for a long enough period. Hydration of the skin is an important predisposing factor (Bird, 1981). Characteristic of this type of reaction is that soon after exposure to the agent, the skin response can be observed at the site of contact. In general, the response comprises an inflammatory reaction of the dermis and various types of damage to the epidermis. The most serious lesions are erosive responses following strong stimuli, such as concentrated alkali and acid solutions or severe burns due to heat or radiation. In those cases, the full-thickness skin will be destroyed, resulting in permanent scarring following repair.

If no direct necrosis is induced, as with mild stimuli or less agressive agents, the

skin mostly reacts to the inflicted damage by an inflammatory reaction of the dermis. This reaction is characterized by one or more of the following events: blood vessels dilate and become more permeable; plasma extravasates into the extracellular spaces; inflammatory cells infiltrate the dermis. The inflammatory response is probably mediated by a variety of endogenous factors, such histamine, arachidonic acid metabolites, cytokines and lysosomal enzymes, arising from mast cells, macrophages, endothelial cells, leucocytes, fibroblasts or keratinocytes (Smuckler, 1983; Parish, 1985). There is increasing evidence that the epidermal cells play an active role in the development of the inflammatory response to irritant chemicals (Willis et al., 1991). The role of the inflammatory mediators and their very complex interactions are not clear yet (Kupper, 1989; Sauder, 1989).

4.2.3 *Blister formation*

Blister formation may be considered as a particular form of irritant contact dermatitis. Blisters may be defined as fluid-filled cavities in or just below the epidermis, formed by spontaneous separation through or near the epidermal-dermal junction or between epidermal cells (Beerens, 1977). Characteristic for blister formation is the loss of adherence between epidermal cells or between epidermis and dermis, combined with the filling of the resulting cavity with fluid. Blistering or vesication may have different causes, such as heat, cold, viral infection, suction, friction, radiation and chemicals. Representatives of vesicating chemicals are the sulfur and nitrogen mustards, arsenicals such as Lewisite (dichloro(2-chlorovinyl)arsine) (Prentiss, 1937; Klaassen, 1985), ammonium hydroxide (Frosch and Kligman, 1977) and cantharidin (Bagatell and Stoughton, 1964; Swinyard and Pathak, 1985). Moreover, several mechanobullous disorders exist. For example, in patients suffering from epidermolysis bullosa, which is a group of inheritable blistering diseases, blistering occurs in response to minimal mechanical trauma (Briggaman, 1983).

In most animal species no blisters are formed or can hardly be recognized by macroscopic observation. Remarkably, only since improved histologic and immunologic techniques were available, bullous disorders have been described in several domestic animals (Scott et al., 1980; Bruckner-Tuderman et al., 1991). The classical explanation of the inability of animal skin to form full-filled blisters is, that hairs may anchor the epidermis to the dermis, thus preventing separation. Additionally, other factors may be involved. For example, the thin animal epidermis will be easily destroyed by a blistering stimulus such as heat. Or, due to the limited blister fluid released from the dermal vasculature, animal skin forms only a flaccid, not easily

detectable, blister. In man, the extensive dermal vasculature and sweat produced in the eccrine sweat glands may contribute to the volume of the blister fluid.

The morphological changes in epidermis following a blister-inducing stimulus has been studied by light as well as electron microscopy (Pearson, 1964, 1965; Einbinder et al., 1966; Beerens et al., 1975; Takigawa and Ofuji, 1977; Briggaman et al., 1984; Papirmeister et al., 1984a, 1984b). These studies show that the plane of separation within the blister varies with the blistering agent. A histologic classification based on the plane of separation was made and comprises intraepidermal and subepidermal blisters (Ackerman, 1978). For example, blisters due to suction, mild freezing, severe heat (95 °C), epidermolysis bullosa lethalis, several proteases and sulfur mustard show separation at the level of the basement membrane, evolving in subepidermal blisters. In contrast, blisters resulting from moderate and mild heat, cantharidin and pemphigus vulgaris show disruption of basal and suprabasal cells, leading to intraepidermal blisters. These findings suggest that blisters can result from different mechanisms.

In understanding the molecular basis of subepidermal blistering, the interaction between the basement membrane and the basal layer keratinocytes of the epidermis is of major importance. The nature of the dermal-epidermal attachment has not yet been clarified. The observation that the dermal-epidermal adherence is strongly influenced by skin temperature, suggests that epidermis and dermis are connected by a viscous bond (Lowe and van der Leun, 1968; van der Leun et al., 1974b). Furthermore, it has been suggested that dermal-epidermal adherence is possibly governed by ionic interactions (Scaletta and McCallum, 1972; Beerens, 1977). Finally, there is also evidence that hemidesmosomes are the sites of actual dermal-epidermal adherence, because suction blister formation started with detachment of hemidesmosomes (Kiistala and Mustakallio, 1967; van der Leun et al., 1974a; Beerens et al., 1975, Beerens, 1977). Until now, the biochemical interactions that are involved in dermal-epidermal adherence are for the greater part unknown. There are some findings suggesting that proteinases are involved in blister formation, but their role is not clearly understood (Beloff and Peters, 1945; Einbinder et al., 1966; Matsumoto and Hashimoto, 1986; see for review Schechter, 1989). On the other hand, if one assumes that dermal-epidermal adherence results from an active process of synthesis and degradation of basement membrane zone components, disappearance of basement membrane zone structures could also be caused by

impairment of this process by the toxic action of the vesicant on the epidermal basal keratinocytes.

4.3 Models in dermatotoxic research

4.3.1. *Introduction*

In toxicity studies, human experiments are only permitted with agents with a known absent or only slightly harmful effect. Therefore, the percutaneous absorption and the skin damaging potential of chemical and physical agents are predominantly examined in models that resemble human skin characteristics as close as possible. These include animal studies and studies with *in vitro* skin models.

To study percutaneous absorption of chemical compounds, a skin model with an intact dermal vasculature is preferable to others, which implies the use of animals. Although the *in vivo* tests are usually performed in guinea pigs, it is assumed that the results obtained from studies with the pig and the monkey will be the most predictive for man, as their skin resemble most the human skin in their kinetics of percutaneous absorption (Wester and Maibach, 1983). However, these animals cannot be used in routine screening tests. A promising alternative model seemed to be the athymic nude mouse grafted with human skin. However, contrary to what was expected, evaluation showed that such grafted human skin is not suitable to predict penetration rates of agents in man (Scott and Rhodes, 1988; van Genderen, 1990).

For screening purposes *in vitro* tests are often performed. A frequently used technique is the skin diffusion cell technique. In a small chamber, test compounds are applied on a stretched piece of viable human or animal skin. The amount of test substance that is recovered in a receptor fluid circulating underneath the skin is taken as a index of the penetration rate (Hawkins and Reifenrath, 1984; Holland et al., 1984). However, this model has several drawbacks: a) the lack of microcirculation through the skin; b) the receptor fluid is usually a buffer instead of blood; c) the results obtained with model compounds appeared to be hardly predictive for the in vivo situation (Kao and Carver, 1990). A novel model for penetration studies may be the isolated, blood-perfused pig ear. The value of this model for percutaneous absorption studies is currently investigated in our laboratory (de Lange *et al.*, 1991).

To establish the skin damaging potential of noxious agents, experimental animals

have been long the only available model. In recent years, there has been growing interest in *in vitro* models for dermatotoxic research, such as skin slices in organ culture, keratinocyte cultures or living skin equivalents. So far, these *in vitro* models have been mainly used for mechanistic studies rather than for screening tests. Moreover, no *in vitro* test exists that can reproduce or mimick the acute inflammatory response of skin, in which systemic factors are involved (Parish, 1985, 1986).

4.3.2 *Keratinocyte cultures*

Human keratinocytes and those of several other species, such as rat, mouse, guinea pig and pig, can be cultured (Rheinwald and Green, 1975; Liu and Karasek, 1978; Vaughan et al., 1981; Fusenig and Worst, 1975; Hennings et al., 1980; Delescluse et al., 1977; Regauer and Compton, 1990). Cultures can be initiated with keratinocytes from the epidermis itself or from hair follicles (Weterings et al., 1981; Lenoir et al., 1988). Additionally, transformed human keratinocyte lines exist, such as the SVK14 cell line, immortalized by infection with Simian virus SV40 (Steinberg and Defendi, 1983). The method developed by Rheinwald and Green (1975) appeared to be the most suitable to culture primary keratinocytes of adult human skin. They co-cultured low numbers of keratinocytes with lethally irradiated mouse 3T3 cells as a "feeder layer". The precise function of this feeder layer has not yet been elucidated. Probably, lethally irradiated 3T3 feeder cells produce growth factors like eicosanoids, as well as basement membrane-like proteins, such as type IV collagen. When keratinocyte colonies become confluent, local stratification occurs at the central parts of the colonies. Although several markers of terminal differentiation can be demonstrated, in this submerged culture system keratinocytes do not reproduce the complete differentiation pattern of normal epidermis (Fuchs and Green, 1980; Asselineau et al., 1986). It is assumed that cultures obtained in this way represent the viable, basal and suprabasal, layers of the epidermis *in vivo*. Improved differentiation can be obtained by high concentrations of calcium and low concentrations of retinoids in the culture medium. Rosdy and Clauss (1990a) reported near optimal differentiation of keratinocytes that were cultured on a polycarbonate filter membrane in defined medium and exposed to the air-liquid interface (see also below in the section on living skin equivalents). In the past decade, chemically defined media such as MCDB 153 have been developed to exclude the influence of unknown factors such as serum and metabolites of 3T3 feeder cells on keratinocyte cultures (Tsao et al., 1982; Boyce and Ham, 1983).
Human keratinocyte cultures have enabled numerous mechanistic studies on cell

biology. To a smaller extent, keratinocyte cultures have been used to study the effects of drugs and toxicants, such as antimicrobial agents (Cooper et al., 1990), wound dressings (Rosdy and Clauss, 1990b), anthralin (Reichert et al., 1985; Reichert and Schmidt, 1990), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Milstone and LaVigne, 1984; Greenlee et al., 1985; van Pelt, 1990), benzo(α)pyrene (Parkinson and Newbold, 1980), organic peroxides (Kappus and Artuc, 1987), skin penetration enhancers (Ponec et al., 1989, 1990) and sulfur mustard (Mol et al., 1989; Smith et al., 1990).

Some investigations with screening assays, not intended to produce detailed data on the mechanisms of cytotoxicity but rather to show general toxic effects following chemical exposure, have shown that human epidermal keratinocytes and human fibroblasts respond in a comparable degree to exposure to some skin penetration enhancers (Ponec et al., 1989, 1990). In contrast, Babich et al. (1989) tested the relative toxicities of some pesticides and some organotins to keratinocytes and fibroblasts and found different sensitivities. Furthermore, they demonstrated that the metabolic competence of keratinocytes greatly determines their sensitivity to an indirect acting cytotoxic agent, such as benzo(a)pyrene. This supports the view that the use of keratinocytes is essential to study the mechanisms behind specific toxic effects of agents on the skin and cutaneous metabolism of xenobiotics (Pham et al., 1990; Kao and Carver, 1990).

4.3.3 *Skin organ cultures*

The great advantage of organ culture of skin slices is the possibility to study toxic effects in skin pieces with intact tissue morphology and similar cellular interactions as *in vivo*. However, microcirculation is missing, which implicates the absence of immunological and inflammatory responses and physical forces such as tissue pressure. On the other hand, the presence of a fully differentiated epidermis, a basement membrane zone and dermal structure offers advantages when compared with the keratinocyte cultures. The aim of using organ culture techniques is to maintain skin slices *in vitro* for several days, with preservation of their viability and skin characteristics.

Several short-term culture systems for skin pieces from mouse (Kao et al., 1983), guinea pig (Kondo and Hozumi, 1986), rabbit (Rutten et al., 1990), pig (Chapman et al., 1989) or man (Reaven and Cox, 1965; Tammi et al., 1979; Yasuno et al., 1980) have been published. In general, skin pieces are kept in medium either floating on paper "rafts", or placed on grids covered with filter paper. The main

problems were epibolic outgrowth and degeneration of the epidermis in an early stage of culture. Although no technique has become standardized yet, skin organ cultures have been used to investigate the mechanism of action of several chemicals on the skin, such as tributyltinchloride (Middleton, 1981), benzo(α)pyrene (Kao et al., 1984), retinoic acid (Tammi et al., 1985, 1989), coal-tar (Schoket et al., 1988, 1990), benzo(α)pyrene (Weston et al., 1982; Watson et al., 1989) and sulfur mustard (Dannenberg et al., 1985; Moore et al., 1986; Nakamura et al., 1990; Mol et al., 1991b). The chemical can be applied on the skin *in vivo* or on freshly excised skin. Subsequently, the skin is incubated in culture medium for a given time period, at the end of which the skin and supporting culture medium are analyzed for evidence of skin damage. Different parameters, mainly indicative for epidermal damage, can be used to assess the chemically-induced effects. These include histological examination and biochemical methods, such as enzyme leakage, DNA and protein synthesis, ATP and lactate contents, or glucose metabolism. Rikimaru et al. (1991) used human skin in organ culture to identify early mediators of inflammation following exposure to sulfur mustard.

In particular, skin organ cultures may be very helpful to investigate the pathophysiology of blister formation induced by proteolytic enzymes (Hino et al., 1982a, 1982b; Einbinder et al., 1966; Briggaman et al., 1984), by blister fluid or serum of patients with blistering diseases (Matsumoto and Hashimoto, 1986; Naito et al., 1989) or by sulfur mustard as mentioned above.

The applicability of skin slices in organ culture to screen the irritant potential of chemicals, has been evaluated using a small number of chemicals. The results indicated that not enough sensitivity could be achieved to discriminate mildly and moderately acting agents (Oliver and Pemberton, 1986).

4.3.4 *Cultured skin equivalents*

Keratinocytes become more completely differentiated if they are cultured on a substrate, acting as a dermal equivalent, and are exposed to air. Suitable matrix substrates are collagen gels, whether or not populated with fibroblasts (Coulomb et al., 1984; Asselineau et al., 1986) and de-epidermized dead dermis (Regnier, 1984; Ponec, 1989). Keratinocytes grow over the surface of the matrix, which is either floating or positioned on a metal grid in the culture medium. The epidermis formed under these conditons resembles in its morphological and functional aspects the epidermis *in vivo* (Asselineau, 1986; Lenoir et al., 1988; Mak et al., 1991). However, an imperfect basement membrane zone is formed. Organotypic cultures provide an

excellent tool to analyse the processes involved in epidermal morphogenesis and terminal differentiation (Ponec, 1989; Fuchs, 1990). It is also a useful technique to study the mutual influence of keratinocytes, fibroblasts and other cells of the skin (Coulomb et al., 1989; Haake and Scott, 1991; Sanquer et al., 1990).

A quite different application is the closure of large or slowly healing full-thickness wounds, such as burns and ulcers, with skin equivalents. Initially, interest was focussed on the application of pure keratinocyte sheets for grafting (Green et al., 1979; O'Connor et al., 1981; Gallico et al., 1984; Teepe et al., 1990; Hefton et al., 1986; Woodley et al., 1988; Herzog et al., 1988; Compton et al., 1989). Although successful results were reported, the need for both dermal and epidermal components in the healing of full thickness wounds has been recognized (Burke et al., 1981; Heck et al., 1985; Cuono et al., 1987; Boyce and Hansbrough, 1988; Yannas et al., 1989). The observations that fibroblasts play an important role in dermo-epidermal interactions and that fibroblasts and keratinocytes communicate via cytokines may be in favour of this view (Krueger et al., 1989; Delaporte et al., 1989). The problem of replacing large amounts of lost dermis has been approached in a number of studies in animals and man. These studies included allogenic dermis with cultured autologous keratinocytes (Cuono et al., 1987; Langdon et al., 1988), a collagen-glycosaminoglycan matrix (Heimbach et al., 1988; Boyce and Hansbrough, 1988; Murphy et al., 1990), and collagen gels covered with growing keratinocytes (Ramirez Bosca et al., 1988; Nanchahal et al., 1989; Mol et al., 1991a). The last two methods enable fabrication of autologous full-skin substitutes, as autologous fibroblasts can be incorporated in the dermal substrates.

4.4 Pathophysiology of sulfur mustard on the skin

Skin is an important target organ for sulfur mustard because of the sensitivity of frequently dividing basal epidermal cells. Moreover, sulfur mustard penetrates the skin rapidly. Before the carcinogenic properties of sulfur mustard were known, vesication of the human skin as result of exposure to this agent was investigated on human volunteers (Cullumbine, 1944; Renshaw, 1946; Nagy et al., 1946). Most of the liquid form of sulfur mustard applied to the skin evaporates (80%) and of the 20% that penetrates, 10 - 15% is fixed in the skin, while the remainder passes rapidly into the circulation, causing systemic toxicity. In contrast, pig skin possibly acts as a

reservoir in which free sulfur mustard is retained (Renshaw, 1946), a phenomenon that might be related to differences in lipid content and composition between human and pig skin. Saturated vapor of sulfur mustard is absorbed at a rate of 1.4 μg/ cm^2/ min at 22°C. At 30°C the rate of absorption is approximately 2.7 μg/ cm^2/ min. Similarly, only 10 - 15% of the sulfur mustard molecules absorbed from the saturated vapor will be bound to skin constituents. Skin reactions produced by the vapor of sulfur mustard on human skin are shown in Table 4.1. The first visible skin reaction is seen after a latency period of 1 - 2 hours and blistering is even delayed for at least 12 hours post-exposure. Healing of wounds is slow and takes 3 to 4 weeks. This might be attributed to persistent DNA damage of keratinocytes at the wound edges and of hair follicle and sweat gland cells at the base of the wound, from where normally re-epithelialization starts. Renshaw (1946) related the severity of skin damage to estimated amounts of sulfur mustard fixed per cm^2 skin. He assumed that 0.1 - 1.0 μg sulfur mustard bound per cm^2 skin would cause mild injury, resulting in erythema with or without edema. An amount of 1.0 - 2.5 μg of sulfur mustard bound per cm^2 skin would induce blister formation, whereas more than 2.5 μg sulfur mustard bound per cm^2 skin would lead to necrosis at the site of contact and circumferential vesication.

It appeared that skin of hot and sweaty sites of the body, such as the axillae and genitals, are more sensitive than that of other body sites (Renshaw, 1947). This is in accordance with general observations that the rate of blister formation is determined by skin temperature (van der Leun et al., 1974b) and by humidity (Pearson, 1964) (see also section 4.2).

Until now, it is unclear how the process of blister formation is triggered by sulfur mustard. The fact that the formation of large fluid-filled blisters is unique for human skin, and cannot be studied in animals, might have limited the research on this topic. Studies of skin injury due to sulfur mustard exposure in animals have shown that it is possible to evoke microblisters, but large blisters are not formed (McAdams, 1956; Mitcheltree et al., 1989; Marlow et al., 1990; Mershon et al., 1990). The pathology of microblisters, developed in skin of pigs and hairless guinea pigs and in human skin transplanted on athymic nude mice has been described at the light microscopic and electron microscopic level (Papirmeister et al., 1984a, 1984b; Mitcheltree et al., 1989; King and Monteiro-Riviere, 1990; Marlow et al., 1990; Petrali et al., 1990). Sulfur mustard-induced damage is especially pronounced in the

Table 4.1. Effects of exposure of human skin to saturated vapor of sulfur mustard at 30°C. According to Cullumbine, 1944; Nagy et al., 1946; and Renshaw, 1946.

Exposure time (min)	Ct[1] (mg.min/m^3)	μg fixed per cm^2 skin[2]	Effects on skin	Time of appearance after exposure (hrs)
1	1400	0.32	Light erythema	1
2	2800	0.64	Erythema	2 - 3
3.5	4900	1.13	Erythema and swelling	8 - 12
5	7000	1.62	Pinhead vesication	13 - 23
6	8400	1.94	Vesication	16 - 48
8	11,200	2.59	Vesication and necrosis	48 - 72

[1]The vapor concentration of sulfur mustard at 30°C is 1.4 g/m^3. Ct is the product of sulfur mustard concentration and exposure time. [2]For the estimation of the amount of sulfur mustard fixed per cm^2 skin it is assumed that a) the penetration rate of sulfur mustard in human skin at 30°C is 2.7 μg/min/cm^2 and b) 12% of the absorbed sulfur mustard is fixed in the skin.

keratinocytes of the basal layer and of the hair follicles. The earliest morphological changes are condensation of nuclear chromatin and blebbing of the nuclear membrane, followed by formation of large perinuclear vacuoles. At this stage, the endoplasmic reticulum and mitochondria in the cytoplasm of the basal cells become swollen. Subsequently, in the basement membrane-zone, damage of the basal cell membrane and breakage of hemidesmosomes is observed, resulting in detachment of the basal cells from the dermis. The plane of separation between epidermis and dermis is through the *lamina lucida*. To link the morphological findings with biochemical results, Papirmeister et al. (1985) proposed a hypothesis on the mechanism of blister formation. In brief, alkylation of DNA bases would induce depletion of cellular NAD^+, at vesicating doses of sulfur mustard. This depletion would stimulate the hexose monophosphate shunt, which in turn would result in the activation of proteolytic enzymes. Subsequently, these enzymes break down protein components of the basement membrane-zone and cause dermal-epidermal separation. Although lowering of NAD^+ levels, due to vesicating doses of sulfur mustard has been demonstrated (Gross et al., 1985; Meier et al., 1987; Mol et al., 1989, 1991b), the crucial role of NAD^+ depletion appeared to be questionable and the last part of the hypothesis remains to be validated. At present, sulfur mustard-induced vesication cannot be fully explained by the processes that are hypothesized by Papirmeister and his coworkers.

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5 QUANTIFICATION OF SULFUR MUSTARD-INDUCED DNA INTERSTRAND CROSS-LINKS AND SINGLE-STRAND BREAKS IN CULTURED HUMAN EPIDERMAL KERATINOCYTES

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Submitted for publication

ABSTRACT

Although alkaline elution is the most sensitive method to determine quantitatively DNA interstrand cross-links, the reliability of data obtained is diminished when treatment with a bifunctional alkylating agent also induces DNA single-strand breaks (SSB) and/or alkali-labile sites (ALS). However, this disadvantage can be obviated, if the frequency of SSB and/or ALS induced by the bifunctional agent is known. Following correction for these SSB and/or ALS, more accurate estimations of the number of cross-links will be obtained. In the present study, the induction by sulfur mustard, *bis*-(β-chloroethyl)sulfide, of DNA interstrand cross-links and their repair has been studied in cultured human epidermal keratinocytes. Correction for SSB and/or ALS induced by sulfur mustard, determined separately with a highly sensitive immunochemical method, resulted in a substantial increase in the calculated number of cross-links. The results indicate that sulfur mustard, per μM added, induces immediately after exposure approximately 0.05 SSB (and/or ALS)/ 10^9 dalton of DNA and approximately 0.12 cross-links / 10^9 dalton of DNA. Although efficient removal occurs during the first 24 hr post-exposure, a low number of cross-links appeared to remain, even in cells exposed to sulfur mustard concentrations as low as 1 μM.

INTRODUCTION

Sulfur mustard (*bis*-(β-chloroethyl)sulfide) is an aliphatic mustard that spontaneously forms the highly reactive episulfonium ion in aqueous solution. This reactive intermediate binds very rapidly to different cellular macromolecules, including DNA. Like other bifunctional alkylating agents, such as nitrogen mustard and its derivatives, sulfur mustard causes multiple types of DNA damage, among these DNA-DNA cross-links and monoalkylation adducts. DNA cross-links can be formed between the N-7 positions of two guanines, either adjacent in the same strand or situated in opposite strands, yielding respectively intrastrand (Lawley *et al.*, 1969; Walker, 1971) or interstrand crosslinks (Brookes and Lawley, 1961, 1963; Crathorn and Roberts, 1966; Reid and Walker, 1969). Total DNA-DNA cross-links represent only 17% of all DNA alkylations by sulfur mustard, one-third of them being interstrand crosslinks (for review see Papirmeister *et al.*, 1991). If one of the β-chloroethyl groups on sulfur mustard is rendered inactive, f.e. by hydrolysis, single substitutions will occur. The majority of sulfur mustard DNA alkylation products are monoadducts, predominantly at the N-7 position of guanine or the N-3 position of adenine. As has been suggested for other bifunctional anti-tumor agents, the presence of DNA interstrand cross-links is positively correlated to inhibition of DNA synthesis and cell proliferation, presumably by preventing strand separation (Papirmeister and Davison, 1964; Lawley and Brookes, 1965; Loveless, 1966; Roberts, 1978; Colvin, 1982; Calabresi and Parks, 1985). As a result, the cytotoxic response of cells to sulfur mustard will be greatly dependent on their capacity to overcome sulfur mustard-induced DNA damage. Removal of sulfur mustard-induced DNA damage during the first 24 hr post-exposure has been shown for several mammalian cell types (Lawley and Brookes, 1965, 1968; Kohn *et al.*, 1965; Crathorn and Roberts, 1966; Venitt, 1968; Chun *et al.*, 1969; Reid and Walker, 1969; Walker and Reid, 1971; Roberts *et al.*, 1971a,b).

In a previous study with cultured human epidermal keratinocytes, it was observed that, even following exposure to concentrations as low as 0.5 and 1 μM, sulfur mustard-induced inhibition of DNA synthesis and cell proliferation was not reduced after a recovery period of 24 hr (Mol *et al.*, 1991). These results motivated us to study the formation and removal of bifunctional DNA-adducts following sulfur mustard exposure.

The alkaline elution assay is a sensitive method for quantifying DNA interstrand

cross-links in cells exposed to bifunctional alkylating agents. This assay involves the induction of a limited number of single-strand breaks (SSB) in the DNA, followed by the determination of the elution of single-strand fragments. The elution is retarded to a greater extent when more interstrand cross-links are present. Consequently, in this assay the unintentional presence of DNA SSB and of alkali labile sites (ALS) giving rise to SSB upon alkali treatment, interferes with the assessment of DNA interstrand cross-links. In that case, quantitation of DNA interstrand cross-links might be unreliable. In several studies this interference has been recognized, but to what degree the results were influenced was not well established (Ewig and Kohn, 1977, 1978; Szmigiero et al., 1984).
Evidence that sulfur mustard induces both DNA cross-links and DNA SSB and/or ALS comes from a study of Papirmeister *et al.*, (1985) with DNA *in vitro*. Therefore, the number of DNA SSB and/or ALS were determined separately using a highly sensitive immunochemical method that does not significantly suffer from interference by the simultaneously induced cross-links (van Loon *et al.*, 1991). The data obtained were used to correct the estimates of sulfur mustard-induced DNA interstrand cross-links obtained by alkaline elution assay.

MATERIALS AND METHODS

Materials. Sulfur mustard (*bis*-(β-chloroethyl)sulfide) was synthesized by the Organic Chemistry Division of the TNO Prins Maurits Laboratory, Rijswijk, The Netherlands. Purity was >99%. The monoclonal antibody D1B was produced as described by van der Schans *et al.* (1989). The following chemicals were obtained commercially: media powders from Flow Laboratories, McLean, VA; fetal calf serum, proteinase K, 4-methylumbelliferyl phosphate and bisbenzimide (H33258) from Boehringer Mannheim, Mannheim, FRG; hydrocortisone from Sigma Chemical Co., St. Louis, MO; epidermal growth factor and gentamicin from GIBCO Europe, Breda, The Netherlands; cholera toxin from Calbiochem-Behring, La Jolla, CA; alkaline phosphatase conjugated goat anti-mouse Ig from Kirkegaard and Perry Laboratories, Inc. Gaithersburg, Md, USA.

Epidermal cell culture. Human keratinocytes were cultured as described elsewhere (Mol *et al.*, 1989). In brief, epidermal cells of mammary skin that was obtained from plastic surgery, with informed consent of the patients, were cultured according to the feeder layer method of Rheinwald and Green (1975), with a slight modification. The

culture medium was a mixture of Dulbecco's modification of Eagle's medium and Ham's F12 (3:1), supplemented with 5% fetal calf serum (FCS), 0.4 μg hydrocortisone/ml, 10^{-10} M cholera toxin, 10 ng epidermal growth factor/ml, and 50 μg gentamicin/ml. Just before confluency, the remnants of the irradiated 3T3 mouse fibroblast feeder layer were removed by vigorously pipetting. The next day cells were subcultured after trypsinization with a 0.3% trypsin/1% EDTA solution. The secondary cultures were used one day after reaching confluency, usually on day eight of culture. The cultures consisted mainly of proliferating basal keratinocytes. Cell numbers per culture were reproducible as established by counting.

Cell treatment. Cultures were washed with Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) and incubated for 30 min with various concentrations of sulfur mustard in PBS at 25°C. Sulfur mustard was dissolved first in dry acetone. The final concentration of the solvent in the exposure fluids was 1%. Control cultures were exposed to the same amount of acetone. It was assessed that acetone did not change background values of DNA interstrand cross-links or DNA SSB and/or ALS. The solutions of sulfur mustard in PBS were used immediately after preparation to avoid variations in concentration caused by hydrolysis of sulfur mustard. After exposure, cultures were washed with PBS and either used for immediate analysis or incubated with fresh medium at 37°C for 24 hr.

Immunochemical quantification of DNA SSB and/or ALS. To quantify sulfur mustard-induced DNA SSB and/or ALS, the immunochemical method described by van Loon *et al.* (1991) was used with slight modifications. After exposure to sulfur mustard the keratinocyte cultures were trypsinized for 30 min with a 0.3% trypsin/1% EDTA solution at 4°C. To prepare the partially unwound DNA samples, about 9.10^4 human keratinocytes suspended in 150 μl PBS at 0°C were injected into 800 μl 1.3 M NaCl, that had been adjusted to pH 11.9, using a Radiometer (Copenhagen, Denmark) pH meter equipped with a B-glass electrode without correction for ionic strength, at 20°C. After 16 min incubation at 0°C the mixture was neutralized with 145 μl 250 mM NaH_2PO_4 and the DNA fragmented by sonication (Ultrasonics W-370, USA, with microtip, output level 2.5) for 10 s to prevent rewinding of the single strand regions. The ELISA was performed as follows. Duplicate aliquots of 20 μl of each DNA sample were added to 120 μl 1.3 M NaCl adjusted to pH 11.0 and duplicate aliquots of 20 μl of a 10-fold dilution of the DNA samples in 1.3 M NaCl, pH 12.1, were added to 120 μl of 1.3 M NaCl, pH 12.1 in the first "horizontal" row of wells of a 96-

well microtiter plate. These solutions were diluted serially 6 times in the following rows of the plate. The last row, containing all test solutions except DNA samples, was used to determine the background. Next, each solution was neutralized with 20 μl 250 mM NaH_2PO_4 and incubated for 15 min at room temperature for DNA adsorption. After washing with PBS, the wells were incubated successively with 150 μl of PBS with 1% FCS for 1 hr at 37 °C and 100 μl of D1B monoclonal antibody specific for single stranded DNA diluted 200-fold in PBS containing 0.01% sodium dodecyl sulfate and 0.1% FCS for 45 min at room temperature. After washing with 0.05% Tween 20 in PBS (PT), the wells were incubated with 100 μl of alkaline phosphatase-conjugated goat anti-mouse Ig, diluted 1 : 1000 in PBS + 0.05% Tween 20 + 5% FCS for 45 min at 37°C. Then, the wells were washed 3 times with PT and once with 0.1 M diethanolamine (pH 9.8) and incubated for 1 hr at 37 °C with 100 μl 0.1 mM 4-methylumbelliferyl phosphate in 10 mM diethanolamine (pH 9.8), containing 1 mM $MgCl_2$. The fluorescence was recorded and the percentage of DNA single-strandedness was calculated according to van Loon *et al.* (1991).
The sulfur mustard-induced SSB and/or ALS were calculated by means of a calibration curve for SSB induced by ionizing radiation (^{60}Co-γ-source), included in each experiment. It was assumed that 4 Gy of ionizing radiation induces 1 SSB/10^9 dalton of DNA.
Since only up to about 10% of the DNA is made single-stranded in this unwinding assay, it can be calculated that at the concentrations of sulfur mustard applied, the DNA interstrand cross-links induced will not significantly reduce the extent of unwinding (see Discussion).

Alkaline elution with fluorometric quantitation of DNA in cultured epidermal cells. Although alkaline elution is a method to assay DNA SSB and/or ALS, it can be modified to detect the presence of DNA interstrand cross-links by studying the effect of the induction of a known number of SSB by γ-rays. When DNA interstrand cross-links are present, interconnected single-stranded DNA fragments will not separate upon the alkaline denaturation, which results in a slower elution and, consequently in a seemingly smaller number of SSB. To calculate DNA interstrand cross-links in sulfur mustard-exposed cultured human keratinocytes, the method described by Plooy *et al.* (1984) was used, modified as explained in the "Appendix".
The alkaline elution was performed as described by Schutte *et al.* (1988), with slight modifications. After exposure to sulfur mustard the keratinocyte cultures were

trypsinized for 30 min with a 0.3% trypsin/1% EDTA solution at 4°C. Cells were suspended in RPMI medium with 10% fetal calf serum and half of the cell suspension was irradiated at 0°C with 4 Gy of ^{60}Co-γ-rays in a Gammacell 100, Atomic Energy of Canada Ltd, Ottawa, Canada. Approximately 5.10^5 cells in a volume of 0.5 ml RPMI were added to 0.5 ml of 0.2% sarkosyl, 2 M NaCl and 0.02 M EDTA, pH 10.0 on polycarbonate membrane filters (Millipore, pore size 5 μm). The solution was removed by passage through the filter by gravity. Then, 3 ml of a solution containing 0.5% sodium dodecyl sulphate, 0.01 M Na_2EDTA, 0.01 M NaCl, 0.01 M Tris and 0.5 mg proteinase K/ml (final pH 8.0), were added very carefully to lyse the cells further and to remove proteins possibly crosslinked to DNA. After 1 hr at 20°C the lysis buffer was removed as above. The DNA on the filter was washed twice with 5 ml of 0.02 M Na_2EDTA, pH 10.0, and then eluted through the filter at 30 μl/min with 0.06 M NaOH and 0.01 M Na_2EDTA, pH 12.6. Six 2.5-h fractions of 4.5 ml were collected. The filters with the remaining DNA were transferred into vials and after addition of 4.5 ml elution buffer, irradiated with 100 Gy of ^{60}Co-γ-rays. Subsequently, each fraction of 4.5 ml, containing either eluted DNA or DNA released from the filter by irradiation were neutralized to pH 6.9 - 7.2 with 0.8 ml of the following buffer: 4 M NaCl, 0.6 M NaOH, 1.0 M NaH_2PO_4 and 0.5 μg bisbenzimide (H33258)/ml. The fluorescence of the bisbenzimide/DNA complex was measured in a spectrofluorometer with excitation at 370 nm, at the emission wavelength of 430 nm. Elution patterns were constructed by plotting the logarithm of the fluorescence remaining on the filter as a function of the fraction number.

Calculation of the number of DNA interstrand cross-links. In the normal procedure, first the alkaline elution profiles of the irradiated control DNA are used to establish the elution volume that corresponds to 80% elution. Next, from the graphs for agent-exposed DNA the percentages of DNA still retained by the filter at the same elution volume are determined. Then, after construction of the theoretical curve for x/p vs. % DNA remaining, conform curve c in Fig. A1 of the "Appendix", the x/p values belonging to these values are read. To obtain the values for x, i.e. the number of cross-links per unit length of DNA, the values for p, the number of SSB present, should be known. In the normal procedure, p corresponds to the sum of background and radiation-induced SSB. However, when the genotoxic treatment induces a substantial number of breaks in addition to cross-links, the values for p between the irradiated control DNA and the irradiated treated DNA show too great a

difference to be neglected. In that case, the 80% elution point determined for the DNA from the irradiated control cells pertains to a too low number of breaks. In order to arrive at correct values for x, a corrected 80% elution point should be taken, belonging to a steeper alkaline elution curve. In our calculations, this corrected 80% elution point was obtained by constructing, for each concentration of sulfur mustard applied, a curve that corresponded to the elution of control DNA containing the normal amount of spontaneous plus irradiation breaks, augmented with the number of mustard-induced SSB and/or ALS in the treated DNA under consideration. The latter number had been determined experimentally as described in the section "Immunochemical quantification of DNA SSB". The corrected values for p, thus obtained, were then used in calculating x from x/p.

RESULTS

The total number of DNA SSB and ALS detected as SSB present in cultured human epidermal keratinocytes exposed to different concentrations of sulfur mustard is shown in Figure 1. When measured immediately after exposure the frequency of the SSB increased linearly with the concentration over the range studied. When DNA damage was determined after the cells had been incubated with fresh medium for 24 hr, diminished SSB and/or ALS values were obtained. The dose dependency

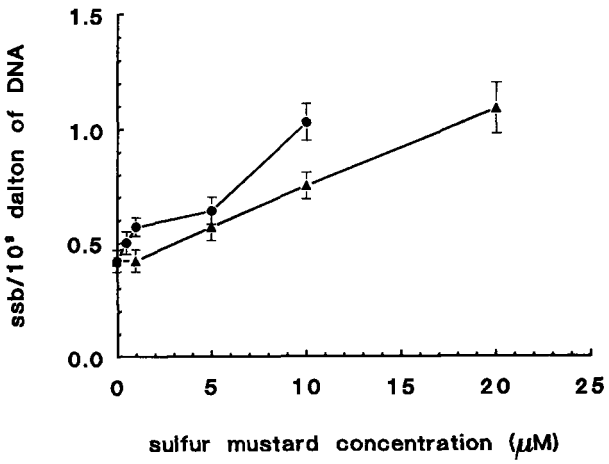

Figure 1. DNA SSB and/or ALS in cultured human epidermal keratinocytes following exposure to sulfur mustard for 30 min, as determined immunochemically. (●) immediately after exposure; (▲) 24 hr later. Data are given as the mean ± s.e.m. of 2 - 4 separate experiments with duplicate determinations of the same sample.

remained linear. In the assay after 24 hr, a longer concentration range could be used. At 20 μM sulfur mustard, directly after exposure too many breaks were present for reliable estimations. According to the linear dose-effect relationship established, sulfur mustard induces approximately 0.05 SSB/10^9 dalton of DNA per additional μM concentration, when determined immediately after exposure. In this respect, 1 μM sulfur mustard is equivalent to 0.20 Gy of ionizing radiation. When measured after 24 hr, approximately 0.03 SSB/10^9 dalton of DNA/μM could still be detected. These sulfur mustard-induced DNA lesions come on top of a background level of about 0.42 SSB/10^9 dalton of DNA. This background level is considerably higher than that observed in untreated human lymphocytes (van der Schans *et al.*, 1989). It is uncertain whether the lesions detected in untreated keratinocytes represent breaks already present, or that the SSB detected are to a large extent the consequence of assay conditions.

The number of DNA interstrand cross-links in sulfur mustard-treated cultured human epidermal keratinocytes was assessed by alkaline elution. When the interference by the SSB and/or ALS induced by the sulfur mustard was neglected, the number of cross-links calculated appeared to level off at the higher concentrations (Figure 2).

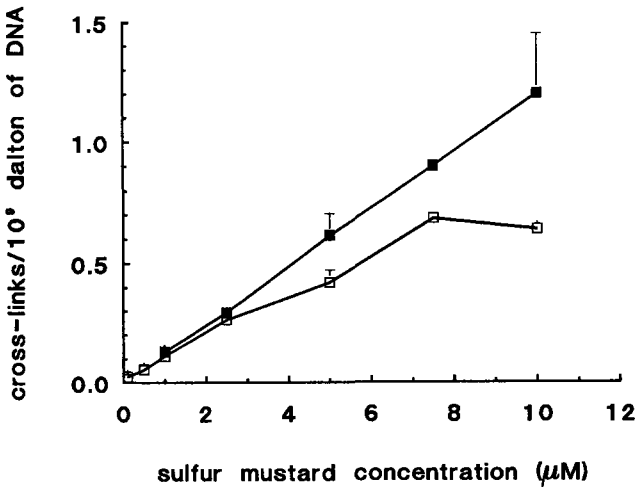

Figure 2. Induction of DNA interstrand cross-links in cultured human epidermal keratinocytes following exposure to sulfur mustard for 30 min, as determined by alkaline elution, immediately after exposure (□) not corrected for sulfur mustard-induced DNA SSB and/or ALS; (■) corrected for sulfur mustard-induced DNA SSB and/or ALS. Data are given as the mean numbers of cross-links/10^9 dalton of DNA ± s.e.m. of 1 - 5 separate experiments with triplicate determinations of the same sample. (One cross-link consists of two links, each with one strand of DNA)

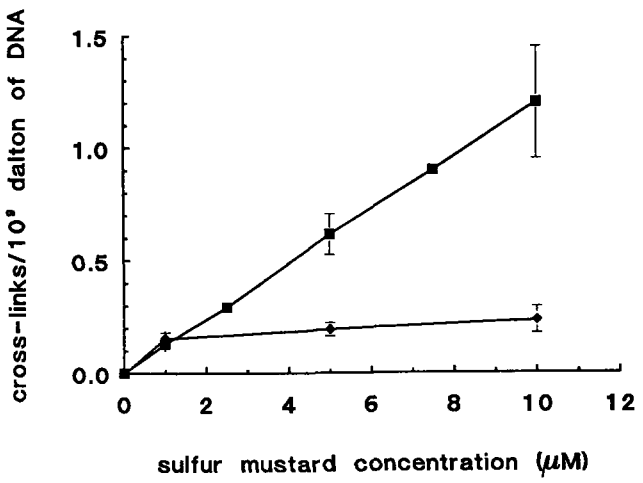

Figure 3. DNA interstrand cross-links in cultured human epidermal keratinocytes exposed to sulfur mustard for 30 min, as determined by alkaline elution, following recovery for 24 hr. (■) immediately after exposure (data from Fig. 2), (◆) after a 24 hr post-exposure incubation. Values have been corrected for DNA SSB and/or ALS present at the moment of sampling. Data (◆) are given as the mean ± s.e.m. of two separate experiments with triplicate determinations of the same sample.

However, if corrections were made for the presence of sulfur mustard-induced SSB and/or ALS in addition to the background and radiation-induced breaks, a concentration-dependent linear increase in the number of DNA interstrand cross-links induced by sulfur mustard was observed (Figure 2). From the data obtained it was estimated that immediately after exposure to sulfur mustard 0.12 cross-links / 10^9 dalton of DNA / μM were present. The cross-link assay was also performed after a 24-hr post-exposure incubation of the cells. Figure 3 presents the number of DNA interstrand cross-links, corrected for the sulfur mustard-induced SSB and/or ALS, still present after 24 hr. With concentrations of 5 and 10 μM a large reduction in the number of cross-links is seen (about 65 - 75%). Remarkably, in cells exposed to 1 μM sulfur mustard the same low level of cross-links was observed after a recovery period of 24 hr as was seen immediately after exposure. The accuracy of the assay does not permit a conclusion whether or not also in these cells (substantial) removal has occurred.

DISCUSSION

It is inherent in the alkaline elution assay that the number of DNA interstrand cross-links measured will be underestimated when the exposure of the DNA to the genotoxic agent has resulted in the induction of SSB and/or ALS concomitantly with the cross-link formation. However, for this interference can be corrected, provided that the number of SSB and/or ALS is known. This would demand a separate determination of the lesions. Although various methods are available to detect quantitatively SSB and/or ALS, detection of strand breaks in the presence of DNA interstrand cross-links is complicated. The denaturation-renaturation technique (Jolley and Ormerod, 1973), the alkaline sucrose gradient sedimentation (Erickson *et al.*, 1978) and the S1 nuclease digestion assay (Shafer and Chase, 1980) have low sensitivities. With alkaline elution the number of SSB and/or ALS cannot be quantified reliably in those cases that cross-links and SSB and/or ALS are simultaneously present (Ewig and Kohn, 1977, 1978).

The immunochemical method to quantify SSB described by van Loon *et al.* (1991)is more sensitive than the previous methods. Moreover, it can be used also when cross-links are present. Whereas a relative small number of SSB and/or ALS have a significant influence on the masking of DNA interstrand cross-links in the alkaline elution assay, in the immunochemical assay the interference of the cross-links in the determination of SSB and/or ALS is only marginal. This can be illustrated by the following calculation. Let us accept that treatment with 20 μM sulfur mustard induces 2.4 interstrand cross-links per 10^9 dalton of DNA, directly after exposure, as extrapolated from our results. The unwinding conditions of the immunochemical SSB assay are known from irradiation experiments to result in single-strandedness over a length of 10^8 dalton of DNA per SSB. Consequently, the chance that one cross-link is present in such a stretch of DNA amounts to 0.24. This means that 76% of the breaks can give rise to single-strandedness over the full stretch. Since the region to become single-stranded extends to either side of a break, the presence of one cross-link within this region will have its effect in one direction only. So, for the residual 24% of the breaks full unwinding in the other direction will still be possible. Since on the average, the cross-link will be located near the middle of the remaining half of the region, also some unwinding in that part will occur. Together, it is expected that less than 10% of the unwinding is prevented by the presence of cross-links when induced to the level considered. This means that at the lower sulfur mustard concentrations used in our studies, interference with the SSB assay leads

to an underestimation that can be neglected when compared to the experimental error of the method. An uncertainty in this argumentation remains the fact that it is not known whether the ALS present are converted into SSB to the same extent during the immunochemical assay as during the much more extensive alkaline exposure of the alkaline elution. If not, some undercorrection of p might have been applied, resulting in an underestimation of the number of cross-links. In principle, the number of SSB and/or ALS next to the number of DNA interstrand cross-links induced by sulfur mustard can also be derived from comparison of alkaline elution profiles of sulfur mustard-treated cells, either irradiated with 4 Gy of ionizing radiation or non-irradiated. However, the experimental variation in the data obtained in this approach were quite large, and the results were less reliable than those provided by the immunochemical assay (unpublished results). The present study demonstrates the great advantage of being able to correct for the SSB induced by the cross-linking agent sulfur mustard: there is a dramatic increase in the number of cross-links detected.

It is assumed that the lesions detected as SSB predominantly represent ALS caused by DNA repair activities. Papirmeister *et al.* (1985) reported that N-3 adenine adducts of sulfur mustard are rapidly removed from DNA, which may be responsible for the detection of SSB immediately after 30 min of exposure, since the depurinated sites in DNA are converted into SSB in alkali. The presence of SSB and/or ALS at 24 hr after exposure suggests ongoing repair activities, such as excision repair of N-7 guanine adducts. Our results with regard to the induction of SSB and/or ALS cannot be compared with data obtained with sulfur mustard by other investigators. Therefore, the results of the present experiments have been compared with those reported for nitrogen mustard (bis(β-chloroethyl)methylamine), which is closely related to sulfur mustard. Our results are in agreement with those of Shafer and Chase (1980), using the S1 nuclease assay, who calculated that the number of SSB induced in rat 9L cells by 1 μM nitrogen mustard was equivalent to 0.28 Gy of ionizing radiation.

The aim of the present study was to investigate the correlation between cytotoxicity and the persistence of cross-links at 24 hr after exposure to sulfur mustard. It was observed that following a recovery period of 24 hr DNA interstrand cross-links were present, although their number was less than that detected immediately after exposure. Even following treatment with a low concentration of sulfur mustard (1 μM) DNA interstrand cross-links did not completely disappear within 24 hr. Murnane and Byfield (1981) and Hansson *et al.* (1987) reported similar results for nitrogen

mustard. This might mean that some DNA interstrand cross-links cannot be removed and may contribute to the inhibition of DNA synthesis observed (Mol *et al.*, 1991). These results are in contrast with data reported earlier for sulfur mustard, indicating complete removal within 24 hr (Lawley and Brookes, 1965, 1968; Kohn *et al.*, 1965; Crathorn and Roberts, 1966; Venitt, 1968; Chun *et al.*, 1969; Reid and Walker, 1969; Walker and Reid, 1971; Roberts *et al.*, 1971a,b). Although the persistence of DNA interstrand cross-links can be considered as the main cause of inhibition of DNA synthesis and cell replication, it cannot be excluded that other factors are involved in the persistent inhibition of cell replication and DNA synthesis. Firstly, the fact that less DNA interstrand cross-links were detected at 24 hr post-exposure does not mean that the DNA lesions are repaired. Reid and Walker (1969) proposed a mechanism of removal of cross-links in which diadducts are first detached from one DNA strand. While the resulting bulky monoadducts attached to the other DNA strand will not be detected as interstrand cross-links, their presence may impair DNA replication. Secondly, the role of DNA interstrand cross-links in the inhibition of DNA synthesis may be of minor importance compared to DNA intrastrand cross-links, being the majority of sulfur mustard diadducts to DNA. These cross-links can not be detected by the alkaline elution method used here. Finally, sulfur mustard may induce a G2 block in the cell cycle, preventing mitosis, DNA synthesis and cell proliferation (Roberts *et al.*, 1986; Konopa, 1988).

ACKNOWLEDGEMENTS

The authors wish to thank Mr. R de Vries and Mrs. A.M.B.C. de Vries - van de Ruit for their technical assistance and Dr. F. Berends for critical reading the manuscript and his helpful suggestions.

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APPENDIX

MOLECULAR WEIGHT DISTRIBUTION OF FRAGMENTS IN ALKALI-DENATURED DNA SAMPLES WITH RANDOMLY INDUCED SINGLE-STRAND BREAKS AND INTERSTRAND CROSSLINKS

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In the appendix by Van der Schans *et al.* (1984), added to the paper of Plooy *et al.* (1984), curves have been derived and presented for the calculation of the average number of interstrand crosslinks related to the fraction of DNA eluted. The fraction of eluted DNA of cells pretreated with a crosslinking agent and subsequently irradiated was assessed at the 50% elution point of DNA of cells which have been only exposed to radiation. However, in the case of irradiated DNA, the point where 50% of the DNA has been eluted cannot be determined accurately because the amount of DNA in the first fraction of the elution cannot exactly be established with the method used to detect DNA (due to the presence of a small but varying amount of fluorescing material not related to DNA). The effect of this uncertainty on the calculation of the number of interstrand crosslinks can be reduced when the calculation is carried out for later fractions in the elution, i.e., at the 80% point.

For non-crosslinked DNA, the molecular weight of single-stranded DNA fragments that are passing through the filter at the point in the elution curve where 80.1% of the material has been eluted, is just equal to 3 times the number-averaged molecular weight of all fragments (m_n), when a random distribution of the breaks over the molecule is assumed. Since $m_n = M/p$, Eq. 4 of Van der Schans *et al.* (1984) can be written as:

$$X_m = \int_0^m m_n^{-2} \cdot e^{-m/m_n} \cdot (1 - e^{(x/p)(m/m_n)})dm \qquad \text{(Eq. 6)}$$

where X_m is the weight fraction of single strand fragments with a molecular weight

between 0 and m containing at least one link connecting it to another fragment. The value p is the average number of breaks and x is the average number of links, both per single-stranded DNA molecule with molecular weight M. Solution of this equation yields:

$$X_m = 1 - (m/m_n + 1)e^{-m/m_n} - (1 + x/p)^{-2} + (1 + x/p)^{-2} \cdot \{m/m_n + (m/m_n).(x/p) + 1\}e^{-(m/m_n)(1+x/p)}$$

(Eq. 7)

In the experimental determination of crosslinks, we use the percentage of total DNA already eluted at the point where non-crosslinked single-stranded material with $m=3m_n$ is expected to appear in the eluate (the 80% point). It is relevant, therefore, to ask what will be the elution behavior of the material in fraction X_{3mn}. Which part will acquire a molecular weight $>3m_n$ by the crosslinking? Analogous to Van der Schans *et al.* (1984), this question can be easily answered for two extreme situations:

1. All crosslinked fragments have acquired a total molecular weight exceeding $3m_n$ and are no longer eluted in the category $m<3m_n$. In this case, Eq.7 directly gives the weight fraction that disappears from this category. Hence, the original 80.1% eluted with $m<3m_n$ will be reduced to $0.801-X_{3mn}$.
2. Only the fragments crosslinked to pieces with $m>3m_n$ disappear from the category with $m<3m_n$. As the crosslinks have a random distribution and since - by definition - 0.199 of the material has a molecular weight $>3m_n$, in this case only 19.9% of the crosslinks will be effective in shifting the fragments to the category with $m>3m_n$.

This implies that in Eq. 7, x has to be substituted by the number of effective crosslinks, i.e., by 0.199x, in order to obtain the weight fraction of material disappearing from the population with $m<3m_n$.

In Figure A1, x/p has been plotted as a function of the weight fraction expected to be collected in the eluate at the point when 80.1% would have been eluted in the absence of crosslinks, for both cases. It will be clear that the real curve will lie somewhere between these two extremes.

For a better approximation of this real curve, it has to be considered which fraction

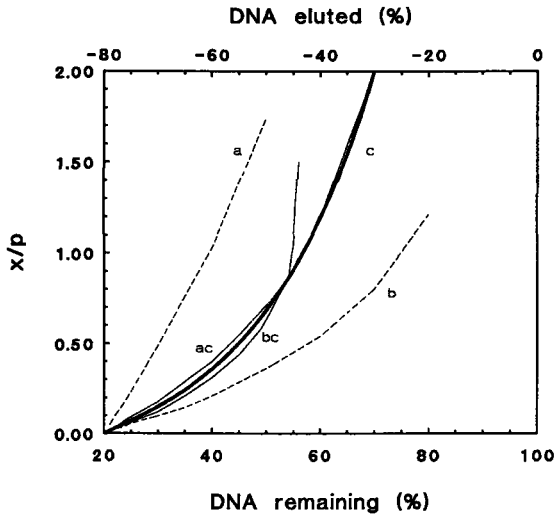

Figure A1. x/p as a function of the fraction of irradiated DNA treated with cross-linking agent eluted at the 80.1% elution point of irradiated untreated DNA (in which p represents the single-strand breaks already present in untreated cells plus those possibly induced by the agent and those caused by the irradiation). Curve a: 0.199 of the links in molecules with molecular weight $m<3m_n$ result in a retention beyond the 80.1% elution point; curve b: all links in molecules with $m<3m_n$ result in retention up to or beyond the 80.1% elution point; curves ac and bc: corrected curves a and b, respectively (as explained in the text); curve c: best estimate of the real curve on the basis of the subdivision of molecules with $m<3m_n$ into four weight categories.

of the molecules with $m<3m_n$ that are crosslinked to fragments of the same category will become larger than $3m_n$ because of this combination. As a first approach, this group of fragments can be subdivided into the following classes of molecules:

a1: with m between 0 and $0.75m_n$
a2: with m between $0.75m_n$ and $1.5m_n$
a3: with m between $1.5m_n$ and $2.25m_n$, and
a4: with m between $2.25m_n$ and $3m_n$.

Integration of Eq. 1 of Van der Schans *et al.* (1984) over these intervals, with the use of Eq. 3 from that paper, shows that 17.3% falls into category a1, 26.9% into

category a2, 21.5% into category a3 and 14.4% into category a4. As the number of links is proportional to the amount of DNA, the links will show the same distribution. In each class, 0.199 of the links will be connected to fragments with $m>3m_n$ (according to the above situation 2).

Combination of a fragment of category a1 with a fragment of each category a1, a2, or a3 will certainly result in material with $m<3m_n$. Combination of a fragment of category a2 with a fragment of category a4 will result in material with $m>3m_n$, whereas m will remain $<3m_n$ when two molecules of class a2 are crosslinked or if a fragment of category a2 is linked to a fragment of category a1 and so on. In the Table A1 the possible combinations are summarized. With the use of Eq.7 we can calculate the weight fraction of fragments containing a link in the categories a1, a2, a3 and a4, as a function of x/p.

Table A1. Possible combinations of linked fragments with m larger or smaller than $3m_n$

class	$m>3m_n$		$m<3m_n$	
a1: (17.3%)			a1+a1	
			a1+a2	
		(0.199x)	a1+a3	(0.657x)
a2: (26.9%)	a2+a4		a2+a1	
		(0.344x)	a2+a2	(0.442x)
a3: (21.5%)	a3+a3		a3+a1	
	a3+a4	(0.559x)		(0.173x)
a4: (14.4%)	a4+a2			
	a4+a3			
	a4+a4	(0.827x)		

As the breaks are random, it can be considered a matter of chance which two links (in different fragments) are connected together to form one cross-link. This implies that only 19.9% of the links in material of class a1 will result in material with $m>3m_n$. Consequently, in the formula used for the calculation of X_{a1}, x should be replaced by 0.199x in order to arrive at the fraction that with certainty is no longer be eluted before the 80.1% point. For the calculation of X_{a2}, x should be replaced by 0.344x; for X_{a3}, 0.559x and for X_{a4} by 0.827x.

Calculation of the sum of X_{a1}, X_{a2}, X_{a3} and X_{a4} with respectively 0.199x, 0.344x, 0.559x and 0.827x, for the various values of x/p between 0 and 2 yields a corrected version of curve a in Figure A1 (curve ac). In an analogous manner curve b can be corrected upwards by adding X_{a1} (0.657x), X_{a2} (0.442x) and X_{a3} (0.173x) to yield curve bc. In this way the distance between the two graphs can be narrowed from 14.7% at x/p=0.2 to 3.2%, whereas at x/p=0.86 the curves even cross each other. The latter has to be ascribed to an overcorrection of curve b, since it has not been taken into account that at high x/p values more than two different fragments can be linked together, resulting in molecules with $m>3m_n$. So, curve ac in Figure A1 is a fair approximation of the real curve in the range x/p 0.86-2, whereas for x/p 0-0.86 an average of the curves ac and bc (curve c) is a quite accurate one.
This curve can be described with the polynomial:

$$x/p = -0.723329+0.078693F-0.00359F^2+0.000094F^3-1.164573\times10^{-6}\times F^4+6.914941\times10^{-9}\times F^5-7.851405\times10^{-12}\times F^6$$

where F = percentage of DNA expected to remain on the filter at the point when 80% would have been eluted in absence of cross-links.
More refined calculations are without meaning, because, as mentioned above, in this approach the possibility is ignored that more than 2 fragments are linked together when multiple links per fragment are present, which will lead to inaccuracies in particular at higher values of x/p.

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6 CONCENTRATION- AND TIME-RELATED EFFECTS OF SULFUR MUSTARD ON HUMAN EPIDERMAL KERATINOCYTE FUNCTION

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Toxicology in Vitro, accepted

ABSTRACT

The concentration- and time-dependent effects of sulfur mustard on cultured human epidermal keratinocyte function were investigated with respect to cell proliferation, DNA and protein synthesis and the level of NAD^+ and ATP. If human epidermal keratinocytes were stimulated to proliferate by replating following exposure to sulfur mustard, inhibition of cell growth was observed after treatment of cells with 0.5 μM sulfur mustard or more. Similarly, DNA synthesis was concentration-dependently reduced following exposures to 0.5 μM sulfur mustard or higher. Inhibition of protein synthesis was only observed upon exposure to concentrations higher than 10 μM. These effects could be detected immediately after exposure and were constant for at least 24 hr. In contrast, parameters for cellular energy decreased with time following exposure. At 24 hr post-exposure a concentration-dependent depletion of NAD^+ and ATP was observed, starting at exposure levels of 50 μM sulfur mustard. Decrease of intracellular lactate dehydrogenase activity also occurred with exposures to 50 μM sulfur mustard or more. Furthermore, the results showed that sulfur mustard induces either persistent damage or damage that develops within 24 hr following treatment. The concurrent inhibition of DNA and protein synthesis combined with a decreased energy supply may play an important role in sulfur mustard-induced blister formation in skin.

INTRODUCTION

Human skin may be severely damaged upon contact with the bifunctional alkylating agent sulfur mustard (bis(β-chloroethyl)sulfide). In particular, the basal epidermal cells are grossly affected following topical application of sulfur mustard, as was observed in human skin grafted onto the nude mouse and in pig skin (Papirmeister *et al.*, 1984; Mitcheltree *et al.*, 1989). It was shown that sulfur mustard triggered a severe drop in NAD^+ levels of human skin grafted onto the nude mouse (Gross *et al.*, 1985), of human lymphocytes (Meier *et al.*, 1987), of cultured human epidermal keratinocytes (Mol *et al.*, 1989) and of human skin pieces in organ culture (Mol *et al.*, 1991). The latter experiments provided evidence that separation between epidermis and dermis occurs when skin is exposed to doses of sulfur mustard vapor that cause a 50% decrease of NAD^+ levels. These data supported the hypothesis of Papirmeister *et al.* (1985), that NAD^+ depletion is involved in blister formation. They suggested that the chromosomal enzyme poly(ADP-ribose)polymerase, activated by sulfur mustard-induced DNA single strand breaks, depleted cellular NAD^+ levels. Decreased concentrations of NAD^+ would disturb the cellular energy system and, in case of human skin exposure, might probably mediate blister formation. Following this hypothesis inhibition of nuclear poly(ADP-ribose) polymerase should prevent NAD^+ depletion and thereby, blister formation. However, when sulfur mustard-exposed cultured human epidermal keratinocytes or human skin pieces were incubated with nicotinamide, an inhibitor of poly(ADP-ribose)polymerase, no recovery of the cellular energy system or prevention of the epidermal-dermal separation was observed (Mol *et al.*, 1989, 1991). These results indicated that NAD^+ depletion by nuclear poly(ADP-ribose)polymerase is not likely to be the main factor leading to sulfur mustard-induced blister formation. To determine which other cell functions were affected by sulfur mustard following treatment with concentrations that induce NAD^+ depletion, general toxic effects of sulfur mustard on cell function of human epidermal keratinocytes were examined. Changes in cell proliferation, synthesis of macromolecules and cellular energy status were assessed. To follow the time course of the induction of the effects, cell functions were measured immediately and at certain time intervals up to 24 hr after exposure.

MATERIALS AND METHODS

Materials. 2,2'-dichlorodiethyl sulfide (sulfur mustard) was synthesized by the Organic Chemistry Division of the Prins Maurits Laboratory TNO, Rijswijk, The Netherlands. Purity was >99%. The following chemicals were obtained commercially: media powders from Flow Laboratories, McLean, VA; fetal calf serum, ATP bioluminescence HS Test-Combination, and chemicals for DNA and LDH assay from Boehringer Mannheim, Mannheim, FRG; hydrocortisone and chemicals for NAD^+ assay from Sigma Chemical Co., St. Louis, MO; epidermal growth factor and gentamicin from GIBCO Europe, Breda, The Netherlands; cholera toxin from Calbiochem-Behring, La Jolla, CA; L-[4,5-^{3}H]leucine (specific activity 74 Ci/mmol)and [methyl-^{3}H]thymidine (specific activity 25 Ci/mmol) from Amersham International, Amersham, UK.

Cell culture method . Human epidermal keratinocytes were cultured as described elsewhere (Mol *et al.*, 1989). In brief, epidermal cells of mammary skin, obtained with informed consent from patients undergoing plastic surgery, were cultured with a slight modification according to the feeder layer method of Rheinwald and Green (1975). Freshly isolated keratinocytes were seeded (2.10^4 cells/cm^2) on a feeder layer of irradiated 3T3 mouse fibroblasts (2.10^4 cells/cm^2). The culture medium was a mixture of Dulbecco's modification of Eagle's medium and Ham's F12 (3:1), supplemented with 5% fetal calf serum, 0.4 μg/ml hydrocortisone, 10^{-10} M cholera toxin, 10 ng/ml epidermal growth factor, and 50 μg/ml gentamicin. Just before the keratinocytes reached confluency the remnants of the lethally irradiated 3T3 mouse fibroblast feeder layer were removed by vigorously pipetting. The next day the keratinocytes were either used for experiments or subcultured by seeding 1.10^4 cells/cm on a fresh feeder layer. Secondary cultures were used one day after reaching confluency, usually at 8 days. The cultures consisted mainly of proliferating basal keratinocytes and cell numbers per culture were reproducible as measured by counting.

Cell treatment. Secondary cultures were used, except for the assay on cell proliferation (see below). Cultures were washed with Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) and incubated for 30 minutes with various concentrations of sulfur mustard in PBS at 22 °C. Sulfur mustard was dissolved in dry acetone and diluted to the desired concentration with PBS immediately before

use, to minimize variations in concentration due to hydrolysis of sulfur mustard. The final acetone concentration in PBS was 1%. Exposure of control cultures to 1% acetone in PBS had no effect on the various parameters measured. After exposure the cultures were washed with PBS and either analysed immediately or incubated with fresh medium at 37 °C for various periods of time.

Measurement of cell proliferation. Immediately after exposure to sulfur mustard or after a post-treatment incubation with medium supplemented with 5% serum for 24 hr, cells were harvested by trypsinization and subcultured as described above. Cells were viable as measured by trypan blue exclusion. Seven days after plating, the cells were trypsinized and counted in duplicate in a haemocytometer.

Measurement of DNA and protein synthesis. DNA synthesis was assessed by incubation of sulfur mustard-treated and control human epidermal keratinocytes cultures with complete growth medium containing 2 μCi [^{3}H]thymidine/1.5 ml for 4 hr at 37 °C. [^{3}H]thymidine incorporation was arrested by washing the cultures twice with cold PBS and once with cold 5% trichloroacetic acid (TCA). Then cold 5% TCA was added for 20 min at 4 °C. The precipitates on the culture dishes were washed with ethanol and solubilized during 10 min at 37 °C with a solution containing 0.1 M NaOH, 2% Na_2CO_3 and 1% SDS; the latter was added just prior to use. After addition of scintillation fluid (Ultima Gold) radioactivity was determined in a liquid scintillation counter. Results were expressed as dpm per μg DNA.
Protein synthesis was assessed by incubation with complete growth medium containing 5μCi [^{3}H]leucine/1.5 ml for 1 hr at 37 °C. The incorporated radioactivity was determined following the TCA procedure as described above.

DNA assay. The DNA content of human epidermal keratinocytes cultures was measured using a fluorimetric method (Otto et al, 1989). Human epidermal keratinocyte cultures were washed with PBS, drained and stored frozen at -20°C until analyzed. After thawing, cells were lysed by incubation with a Dispase/Triton X-100 solution (2.4 mg Dispase/ml PBS containing 1% Triton X-100) for 20 minutes at 37°C. Then, to a 300 μl sample was added 100 μl each of 115 μg/ml pronase and 125 μg/ml RNAse in PBS, and 500 μl of the Dispase/ Triton solution. After incubation of the mixture for 60 min at 37°C, 500 μl of 15 μg/ml ethidium bromide in PBS was added. Fluorescence was measured on a Perkin-Elmer LS-2 filter fluorimeter with excitation and emission bandpass filters of 537 and 580 nm, respectively. A

calibration curve was obtained by treating calf thymus DNA (0 - 8 μg/ml) in a similar way.

NAD^+ assay. NAD^+ was extracted from human epidermal keratinocyte cultures with 0.5 M $HClO_4$ on ice during 20 min. The $HClO_4$ extracts were assayed for NAD^+ using an enzymatic cycling assay (Jacobson and Jacobson, 1976).

LDH and ATP assay. Human keratinocyte cultures were lysed by incubation with 1% Triton in PBS at 4 °C for 2 hr. LDH activity in the lysate was measured spectrofotometrically according to Bergmeyer *et al.* (1983). Sulfur mustard itself did not inhibit the enzymatic activity of LDH. ATP levels were measured using a luciferin/luciferase bioluminescence HS kit. The emitted light was measured in a liquid scintillation analyzer (Trio Carb 2000CA, United Technologies Packard).

RESULTS

The response of cultured human epidermal keratinocytes upon exposure to sulfur mustard for 30 min at 22 °C was investigated using assays of cell proliferation and metabolic function. If human epidermal keratinocytes were stimulated to divide by subculturing them immediately or 24 hr after treatment with concentrations of 0.5 μM sulfur mustard or higher, they showed a concentration-dependent decrease in cell proliferation compared to control cultures (Fig. 1). On day 7 after subculturing, the cell yield from cultures replated 24 hr post-exposure was at least 30% lower than was seen for cells replated immediately after exposure to sulfur mustard. Almost no cell proliferation was observed after replating of human epidermal keratinocytes that were exposed to 10 μM sulfur mustard.

Exposure of human keratinocyte cultures to sulfur mustard concentrations of 0.1 to 100 μM resulted in a concentration-dependent decrease in [^{3}H]thymidine incorporation into DNA (Fig. 2). Similarly, when cultures were incubated for 24 hr in medium with 5% serum after exposure to sulfur mustard, DNA synthesis in treated cultures was reduced compared to that in untreated cultures. Keratinocyte protein synthesis, measured as incorporation of [^{3}H]leucine into TCA-precipitable material, was inhibited concentration-dependently after exposure to concentrations higher than 10 μM sulfur mustard (Fig. 3). The effects of sulfur mustard on protein synthesis measured at 24

Figure 1. Inhibition of proliferation of human epidermal keratinocytes exposed to various concentrations of sulfur mustard for 30 min. The cells were replated either immediately (hatched bars) or 24 hr (open bars) after exposure and cell numbers per culture were counted on day 7 after replating. Each point represents the mean ± s.e.m. of 3 separate experiments, each with 3 cultures per treatment group.

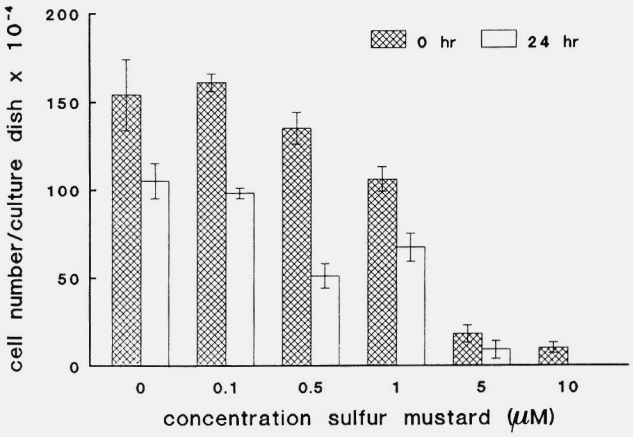
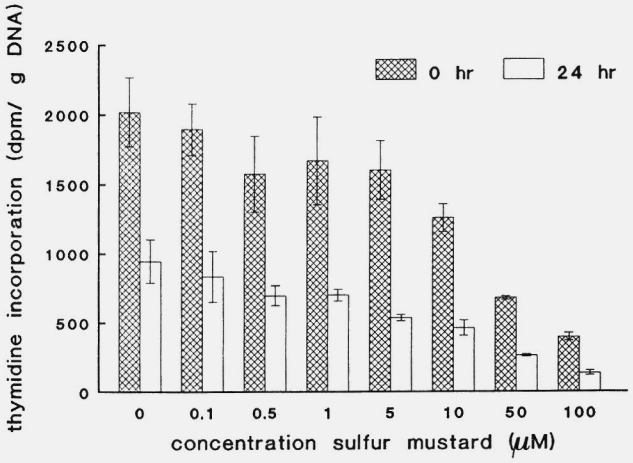


Figure 2. Inhibition of DNA synthesis in human epidermal keratinocytes due to exposure to various concentrations of sulfur mustard for 30 min. Incorporation of [^{3}H]thymidine into DNA either immediately (hatched bars), or 24 hr (open bars) after exposure. Data are expressed as the incorporated activity/μg DNA. Each point indicates the mean ± s.e.m. of 2 separate experiments, each with 3 cultures per treatment group.



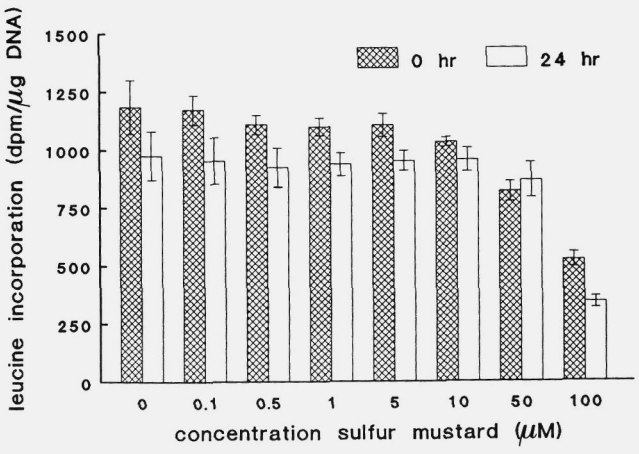


Figure 3. Inhibition of protein synthesis in human epidermal keratinocytes due to exposure to various concentrations of sulfur mustard for 30 min. Incorporation of [^{3}H]leucine into protein either immediately (hatched bars), or 24 hr (open bars) after exposure. Data are expressed as the incorporated activity/μg DNA. Each point indicates the mean ± s.e.m. of 2 separate experiments, each with 3 cultures per treatment group.

hr after exposure were not different from those measured immediately following treatment.

Compared to control cultures, no changes in the concentrations of NAD^+ and ATP or in the activity of intracellular LDH were observed immediately after exposure of human epidermal keratinocytes to sulfur mustard concentrations between 50 and 500 μM (results not shown). Therefore, we assessed these parameters at various time intervals following exposure to 200 μM sulfur mustard (Fig. 4). NAD^+ levels showed a decrease with time, starting from 1 hr after treatment, and nearly maximal depletion was observed at 6 hr after exposure. In contrast to the time-dependent decrease in intracellular NAD^+ content, ATP concentrations decreased only slightly during the first 6 hr after exposure to 200 μM sulfur mustard. Similarly, the intracellular LDH activity in cells exposed to 200 μM sulfur mustard was approximately equal to that in control cultures or even slightly elevated (up to 110%) during the first 6 hr following exposure. However, the intracellular LDH activity was reduced to 60% of control value at 24 hr after exposure. When measured 24 hr post-exposure, cellular NAD^+ and ATP contents and the intracellular LDH activity of cultures exposed to concentrations of sulfur mustard lower than 100 μM were not further decreased than

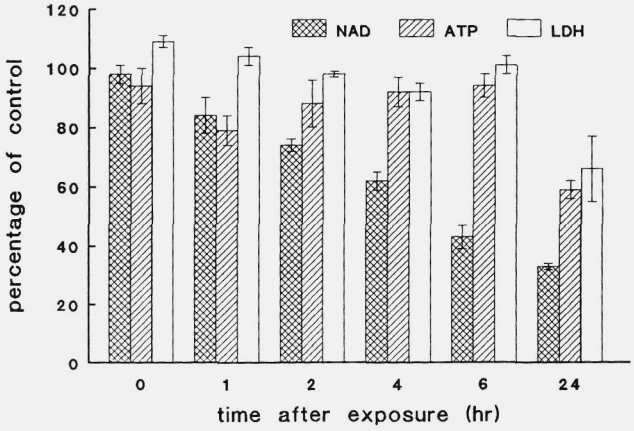


Figure 4. Decrease in time of the NAD⁺ and ATP concentrations and the intracellular LDH activity of human epidermal keratinocytes exposed to 200 μM sulfur mustard for 30 min. Data are expressed as percentage of control values. Each point represents the mean ± s.e.m. of at least 2 separate experiments, each with 3 cultures per treatment group.

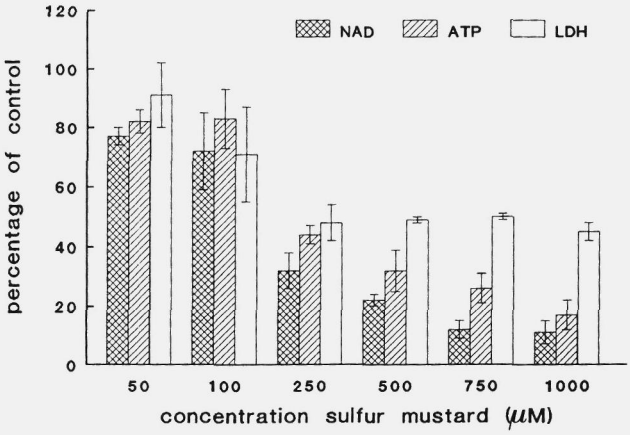


Figure 5. Decrease of NAD⁺ and ATP concentrations and the intracellular LDH activity of human epidermal keratinocytes at 24 hr following exposure to various concentrations of sulfur mustard. Data are expressed as percentage of control values. Each point represents the mean ± s.e.m. of at least 2 separate experiments, each with 3 cultures per treatment group.

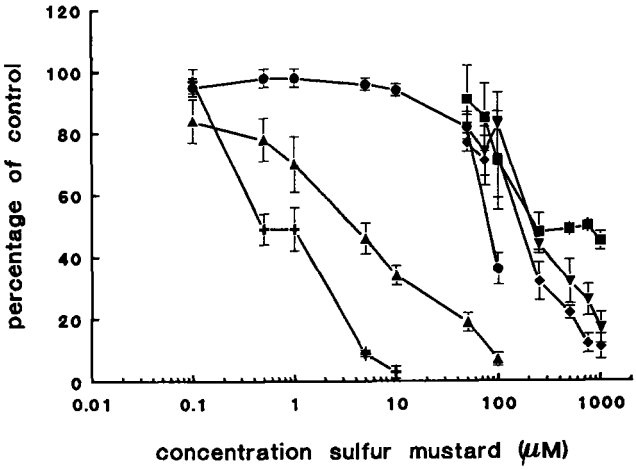


Figure 6. Effects on cell proliferation (✚), DNA synthesis (▲), protein synthesis (●), NAD^+ concentration (◆), ATP concentration (▼) and intracellular LDH activity (■) following exposure of human epidermal keratinocytes for 30 min to various concentrations of sulfur mustard, measured after a 24 hr post-incubation period. Data are expressed as a percentage of control values. Each point represents the mean ± s.e.m. of at least 2 separate experiments, each with 3 cultures per treatment group.

to 70 - 80% of control values (Fig. 5). A considerable decrease in cellular NAD^+ and ATP levels and LDH activity was observed in cultures exposed to concentrations of sulfur mustard of 250 μM and higher. It seemed that following treatment with concentrations of sulfur mustard higher than 250 μM the decreases leveled off slowly. Particularly, it was not possible to induce a decrease of more than 50% in the LDH activity, even with concentrations as high as 1000 μM sulfur mustard.

Since exposure of human epidermal keratinocytes to different concentrations of sulfur mustard appeared to induce either persistent damage (inhibition of cell proliferation and macromolecular biosynthesis) or damage that developed with time following treatment (decrease of NAD^+ and ATP levels and intracellular LDH activity), the sensitivity of each parameter to sulfur mustard exposure has been compared on the basis of the results measured at 24 hr post-treatment (Fig. 6). It appeared that sulfur mustard exposure had the most severe implications on proliferation and DNA synthesis, whereas protein synthesis and NAD^+ and ATP levels were only slightly affected following exposure of human epidermal keratinocytes to concentrations up to 50 μM sulfur mustard. All measured parameters were severely suppressed upon treatment of the cultures with sulfur mustard concentrations of 250 μM or more.

DISCUSSION

The aim of the present study was to investigate the toxic effects of the bifunctional alkylating agent sulfur mustard on the basal epidermal keratinocytes and their capacity to recover from this damage, because these effects probably play a role in the formation of blisters on sulfur mustard-exposed human skin. The development of effective measures to prevent vesication partially depends on an understanding of the mechanism of action of sulfur mustard.

Cultures were exposed for 30 min to sulfur mustard concentrations between 0.1 and 1000 μM to establish the concentrations and time periods at which distinct cell injuries become manifest. Due to the alkylating action of sulfur mustard DNA is one of its main cellular targets. The observations that DNA synthesis and cell proliferation were most sensitive to sulfur mustard were in agreement with results of numerous former studies (Herriott, 1951; Harold and Ziporin, 1958; Roberts *et al.*, 1971a; for an extended review see Papirmeister *et al.*, 1991). The remarkable reduction of cell proliferation capacity and DNA synthesis in the "24 hr" control cultures compared to the "0 hr" control cultures (Figs. 1 and 2) might be due to the transition of cells from proliferation to differentiation upon reaching confluency.

Despite the existence of repair processes, which may remove mono- and bifunctional alkylation products from sulfur mustard-damaged DNA in mammalian cells (Reid and Walker, 1969; Roberts *et al.*, 1971b), neither cellular proliferation nor DNA synthesis by human keratinocyte cultures were improved following incubation in fresh medium for 24 hr after treatment with sulfur mustard (Figs. 1 and 2). This finding indicates that DNA damage could not be repaired completely within 24 hr.

Inhibition of protein synthesis in human keratinocyte cultures was observed following exposure to concentrations at least one order of magnitude higher than those needed to inhibit DNA synthesis (Fig. 6). Similar results have been reported for other cell types (Harold and Ziporin, 1958; Lawley and Brookes, 1965, 1968; Crathorn and Roberts, 1965; Vaughan *et al.*, 1988). Although protein synthesis, measured as [^{3}H]leucine incorporation, was not inhibited following sulfur mustard exposures below 10μM, a possible effect of sulfur mustard on the synthesis of regulatory, labile, proteins could not be excluded. The results further showed that, in turn, protein synthesis was slightly more sensitive to the toxic action of sulfur mustard than cellular NAD^+ and ATP levels (Fig. 6). The concentration-dependent decrease in NAD^+ preceeded a concentration-dependent decrease of the energy status of sulfur

mustard-injured cells, measured as ATP levels (Fig. 4).

It was striking that the activity of LDH could only be reduced to maximally 50% of the control values at 24 hr after exposure. A similar phenomenon was observed for the consumption of glucose over a 24 hr period by sulfur mustard-treated keratinocytes (Mol *et al.*, 1989). Using high concentrations of sulfur mustard in both cases, it was not possible to cause a reduction of more than 50% of control values. These findings could not be interpreted satisfactorily.

The present results include a dosimetry study for sulfur mustard-induced toxicity in human epidermal keratinocytes. The results indicate that upon exposure to vesicating concentrations that induce a 50% NAD^+ depletion, i.e. higher than 250 μM, extensive DNA damage exists, protein synthesis is inhibited and energy supply is reduced. No recovery of human epidermal keratinocytes from sulfur mustard-induced damage was observed within 24 hr following exposure. Moreover, it cannot be excluded that other vital cell functions, not included in this study, are impaired. These results demonstrate that metabolic cell death due to sulfur mustard not only results from energy depletion, but may also be correlated with inhibition of protein synthesis. This finding may provide an explanation why raising NAD^+ levels by incubation with nicotinamide hardly reduced epidermal-dermal separation in human skin pieces exposed to vesicating doses of sulfur mustard (Mol *et al.*, 1991). Although nicotinamide partially prevents depletion of NAD^+ levels, other vital cell functions, such as protein synthesis, may remain damaged. The results of the present study on concentration-related aspects of the action of sulfur mustard on human epidermal keratinocytes show that in addition to energy depletion, the inhibition of protein synthesis has to be considered as a potential cause of sulfur mustard-induced skin injury.

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7 NAD^+ LEVELS AND GLUCOSE UPTAKE OF CULTURED HUMAN EPIDERMAL CELLS EXPOSED TO SULFUR MUSTARD

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Toxicol. Appl. Pharmacol. **98,** 159-165 (1989).

ABSTRACT

In cultured human epidermal cells exposure to the vesicant sulfur mustard (HD) causes a decrease of the NAD^+ content, which depends on the dose and the time period between exposure to HD and NAD^+ measurement. Presumably, this NAD^+ loss is due to activation of the enzyme NAD:protein ADP-ribosyltransferase (ADPRT) and may lead to glycolysis inhibition, disturbance of energy metabolism, and eventually cell death. Since prevention of this NAD^+ depletion could lead to cell survival, HD-exposed cultures have been incubated with nicotinamide, a precursor of NAD^+ and an inhibitor of ADPRT. Although a reduction in NAD^+ levels of the cultures can be prevented, the uptake of glucose, which was taken as a measure for cellular viability, appears to be inhibited in cultures in which the NAD^+ levels are at the 100% level at 4 hr after exposure. Therefore, prophylactic or therapeutic measures that are focused on maintenance of NAD^+ levels in order to preserve energy supplies do not protect human epidermal cells in culture that have been exposed to HD. These experiments indicate that mechanisms other than NAD^+ depletion may play an important role in HD-induced cell injury in human skin.

INTRODUCTION

The action of sulfur mustard (HD), a potent bifunctional alkylating agent, results in the formation of covalent linkages with nucleophilic groups in the cell. An important nucleophilic target in cells is DNA (Wheeler, 1962), although abundant binding to proteins such as keratin (Peters and Wakelin, 1947), collagen (Pirie, 1947), and enzymes (Wheeler, 1962) also occurs. Moreover, it has been demonstrated that HD inhibits glycolysis (Needham *et al.*, 1947; Bailey and Webb, 1948). Initially, it was suggested that this inhibition might be the result of inactivation of the enzyme hexokinase. Later, when a characteristic decrease of the NAD^+ content of HD-treated cells and tissue was found, this depletion was thought to be the cause of the impaired glycolysis (Wheeler, 1962; Papirmeister *et al.*, 1985), which eventually could lead to cell death. Although it appears from the above that HD has multiple targets, the potential beneficial effects of increasing NAD^+ levels seemed worth testing. Recently, it has been shown that increased levels of NAD^+ can be obtained in HD-treated human leukocytes by treatment with nicotinamide or nicotinic acid and it was suggested that cell death could be prevented when cells should be able to maintain their NAD^+ levels (Meier *et al.*,1987a).

HD may severely damage the human skin upon exposure. Until now, no adequate prophylactic or therapeutic measures exist to reduce HD damage to human skin. In this study the effects of treatment with nicotinamide or nicotinic acid on HD-exposed human epidermal cells have been examined. The investigations have been carried out on human epidermal cells in culture, because the epidermis is the first target of HD upon contact with the skin and severe injury to the epidermal cells has been observed (Papirmeister *et al.*, 1984a, b). In those cases, in which the NAD^+ levels were maintained by means of incubation with nicotinamide, the viability of the cultures was assessed by measurement of the glucose uptake from the medium. Glucose uptake was used as a parameter in this investigation, since usual parameters such as colony formation efficiency and dye exclusion could not be used due to the culture technique and the nature of the epidermal cells.

METHODS

Materials. Medium and fetal calf serum were obtained from Flow Laboratories

(Irvine, Scotland, UK), epidermal growth factor was purchased from Bethesda Research Laboratories (Gaitesburg, MD), cholera toxin from Calbiochem (La Jolla, CA), and gentamycin from GIBCO (Madison, WI). Hydrocortisone, nicotinamide, nicotinic acid, and chemicals for NAD^+ assay were obtained from Sigma (St. Louis, MO) except Na-bicine, which was obtained from Janssen Chimica (Beerse, Belgium).

Cell culture method. Human keratinocytes were obtained from adults who underwent mammary reduction and were grown according to the feeder-layer technique (Rheinwald and Green, 1975). The culture medium was a mixture of Dulbecco's modification of Eagle's medium and Ham's F12 (3:1), supplemented with 5% fetal calf serum, 0.4 μg hydrocortisone/ml, 10^{-10}M cholera toxin, 10 ng epidermal growth factor/ml, and 40 μg gentamycin/ml. The cells were incubated at 37°C and gassed with a mixture of 10% CO_2 in air. After a first passage the cultures were grown to confluence, which was reached at Day 8 after subculturing. The cultures consisted predominantly of monolayers and it was clear from DNA measurements that cell numbers per culture were reproducible.

Cell treatment. Confluent cultures of human epidermal cells were washed twice with PBS and then exposed during 30 min to different concentrations of HD in Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) at 25°C. HD was dissolved first in dry acetone and the final concentration of acetone in PBS was 1%. Because of the rapid hydrolysis of HD following dilution in PBS, the HD solution in PBS were applicated to the epidermal cell cultures immediately after preparation. Control cultures were exposed to the same amount of acetone and were found not to differ in NAD^+ content and glucose uptake from cultures exposed to PBS alone. After exposure to HD the cultures were washed with PBS and placed back into the incubator with complete medium. NAD^+ was measured after an incubation time of 4 hr and glucose uptake was determined after an incubation time of 24 and 48 hr.

Incubations with nicotinamide or nicotinic acid. To examine the effects of nicotinamide or nicotinic acid on the NAD^+ contents of the cells after exposure to HD, the confluent cultures were incubated in complete culture medium supplemented with nicotinamide or nicotinic acid in different concentrations during various times before and after exposure. NAD^+ was measured by the assay described below.

Assay of NAD$^+$. NAD$^+$ was extracted from the cultures with 0.5 M $HC1O_4$ on ice during 20 min. The $HClO_4$ extract was removed by centrifugation and the supernatants were assayed for NAD$^+$ using an enzymatic cycling assay (Jacobson and Jacobson, 1976).

Glucose uptake. To obtain an indication about the viability of the human epidermal cell cultures, the glucose uptake following HD treatment was calculated from the decrease in the glucose contents in the media of the cultures. After exposure, the cultures were incubated with complete medium and the glucose contents were determined at 24 and 48 hr. A glucose analyzer (Yellow Springs Instruments Co., Inc., Yellow Springs, OH) was used to determine the glucose concentration in the medium.

RESULTS

When human epidermal cells were exposed during 30 min to HD this resulted in a progressive decrease of the NAD$^+$ content in the cultures following termination of the exposure. In Fig. 1 this decrease is shown for a time period of 24 hr after the incubation with 0.1, 0.25, 0.5, or 1.0 mM HD. At 24 hr after exposure to HD the NAD$^+$ levels of epidermal cell cultures that were treated with 0.1 mM HD did not fall below

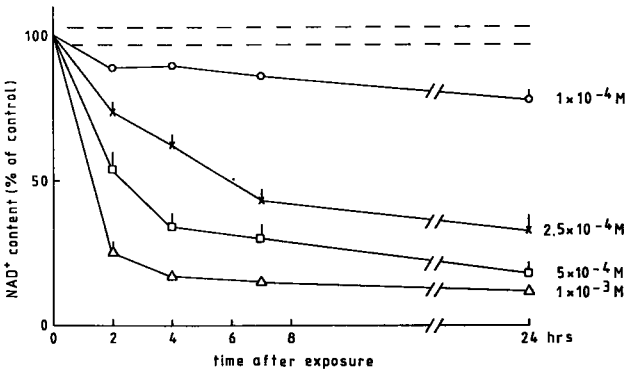

Fig. 1. The time-related decrease in NAD$^+$ levels of human epidermal cell cultures following exposure during 30 min to 0.1, 0.25, 0.5, or 1.0 mM HD. Each point represents the mean ± SE of six cultures.

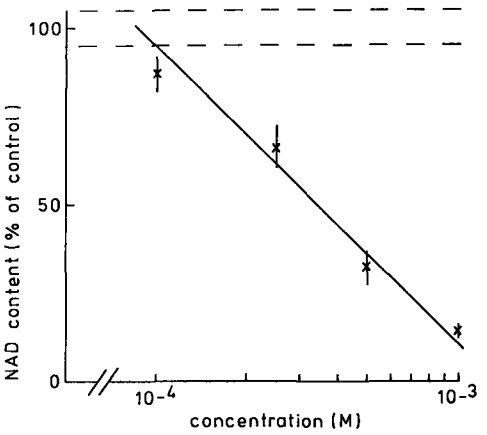

Figure 2. The effect of varying concentrations of HD on NAD⁺ levels of human epidermal cell cultures at 4 hr following exposure during 30 min to HD. Each point represents the mean ± of six cultures.

80% of the control level. In cultures that were exposed to 0.25 mM HD or more, the NAD^+ content dropped to 40-20% of control values at 24 hr after exposure. At approximately 4 hr after exposure, the NAD^+ decrease appeared to be related to the HD concentration when measured after exposure to HD concentrations between 0.1 and 1.0 mM HD (Fig. 2).

In an attempt to maintain the NAD^+ levels of cells after exposure to HD, nicotinamide was added to the culture medium during different time periods before or after HD exposure. Pretreatment during 24 hr of human epidermal cell cultures with medium containing nicotinamide caused an increase of the NAD^+ content in the cultures to maximally 160% of the control value (Fig. 3A). Following exposure during 30 min to 0.25 mM HD, these pretreated cultures were switched from nicotinamide containing medium to standard medium. The initial raise of the NAD^+ levels did not prevent their depletion, determined 4 hr after termination of HD exposure (Fig. 3C). However, in control cultures that were pretreated with nicotinamide, and exposed to PBS only, the raised NAD^+ levels dropped within 4 hr to normal values (Fig. 3B).

In a subsequent series of experiments the cultures received nicotinamide containing medium before as well as after the exposure to HD. Figure 3E shows that the presence of nicotinamide in the medium before and after HD exposure prevented the cellular NAD^+ depletion, when measured at 4 hr after termination of the HD exposure. In control cultures that were exposed to PBS only following pretreatment with nicotinamide, the NAD^+ levels did not change (Fig. 3D).

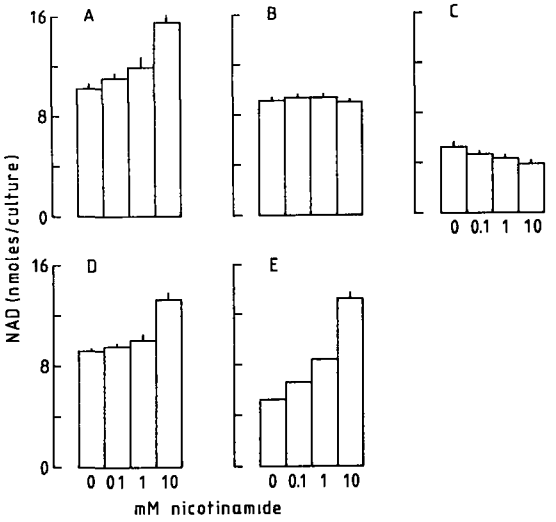

Fig. 3. The effect of incubations with nicotinamide on the NAD⁺ content of human epidermal cell cultures and the effects of HD. All cultures were preincubated during 24 hr with different concentrations of nicotinamide as mentioned under A. Results represent the means ± SE of six cultures. (A) NAD⁺ contents of cultures that were incubated during 24 hr with varying concentrations of nicotinamide. (B) NAD⁺ contents of nicotinamide-pretreated cultures that, following exposure to PBS during 30 min, were subsequently incubated with normal medium. NAD⁺ was assessed at 4 hr. (C) The effects of 0.25 mM HD during 30 min in nicotinamide-pretreated cultures. After HD exposure the cultures were kept in normal medium. NAD⁺ was assessed after an incubation time of 4 hr. (D) NAD⁺ contents of nicotinamide-pretreated cultures that, following exposure to PBS during 30 min, were subsequently incubated with medium containing the same concentrations of nicotinamide as during pretreatment. NAD⁺ was assessed at 4 hr. (E) The effects of 0.25 mM HD during 30 min in nicotinamide pretreated cultures. After HD exposure the cultures were kept in medium containing the same concentrations of nicotinamide as during pretreatment. NAD⁺ was assessed after an incubation time of 4 hr.

When pretreatment with nicotinamide was omitted and nicotinamide was added to the cultures after HD exposure only, the NAD^+ levels were also well preserved. In Fig. 4 the results are presented of NAD^+ assessments in cultures which after exposure to 0.25 and 10 mM HD were treated for 4 hr with medium containing 0.1-40 mM nicotinamide. When determined 4 hr after a 30-min exposure to 0.25 mM HD 100% preservation of the NAD^+ content is obtained with 10 mM nicotinamide (Fig. 4A). Similarly, after exposure to 1.0 mM HD the measured NAD^+ levels do not differ from control values when the medium contained 40 mM nicotinamide (Fig. 4B). However, no beneficial effects were observed with nicotinic acid. Pretreatment during 24 hr of the cultures with medium containing nicotinic acid caused no increase of the NAD^+ content (Fig. 5A). In cultures in which the NAD^+ content was decreased due to exposure to 0.25 mM HD, addition of nicotinic acid to the culture medium caused no raise of the NAD^+ levels (Fig. 5B). To investigate how important the cellular NAD^+ levels are for the survival or viability of these cells, the uptake of glucose by the HD-treated human epidermal cell cultures over a 24- and 48-hr

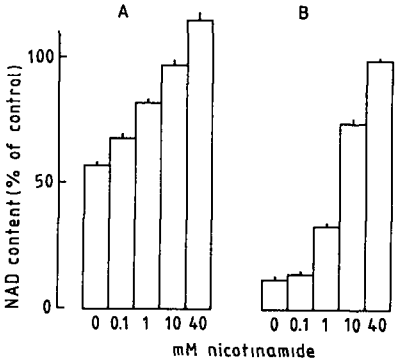

Fig. 4. The effect of nicotinamide incubations on the NAD⁺ content of human epidermal cell cultures at 4 hr following an exposure during 30 min to 0.25 mM HD (A) and 1 mM HD (B). Each point represents the mean ± SE of six cultures.

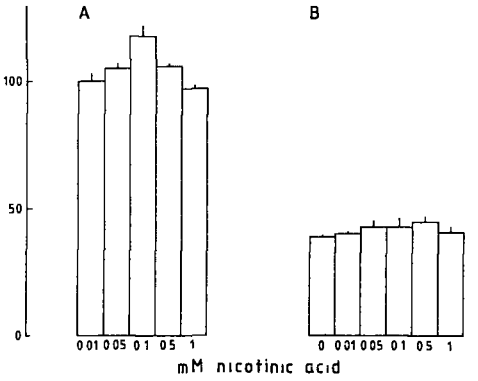

Fig. 5. (A) The effect of incubations during 24 hr with varying concentrations of nicotinic acid on the NAD⁺ contents of human epidermal cell cultures. (B) The effects of nicotinic acid incubations following an exposure during 30 min to 0.25 mM HD on the NAD⁺ contents of human epidermal cell cultures. NAD⁺ was assessed after an incubation with nicotinic acid during 4 hr.

period after exposure was measured. Figure 6 demonstrates that in the period between 0 and 24 hr after exposure to HD maximally a 50% inhibition of the glucose uptake could be achieved in human epidermal cell cultures that were exposed to concentrations of 0.25 mM HD or higher. When the cultures were exposed to lower concentrations a dose-related response was observed. Figure 7 shows that the glucose uptake over 24 hr of cultures that were treated with 0.1 to 40 mM nicotinamide after exposure to 0.1, 0.25 or 1.0 mM HD was not different from that of cultures that did not receive nicotinamide. Moreover, it must be noted that incubations with 10 and 40 mM nicotinamide of control cultures showed that these concentrations of nicotinamide per se reduced the glucose uptake of the cultures.

DISCUSSION

A reduction of the NAD⁺ levels has been observed after exposure of cells or tissues to HD (Gross *et al.*, 1985; Meier *et al.*, 1987a). As has been proposed for other DNA-damaging agents, loss of NAD⁺ may result from enhanced synthesis of poly-ADP ribose by the chromosomal enzyme NAD:protein ADP-ribosyltransferase

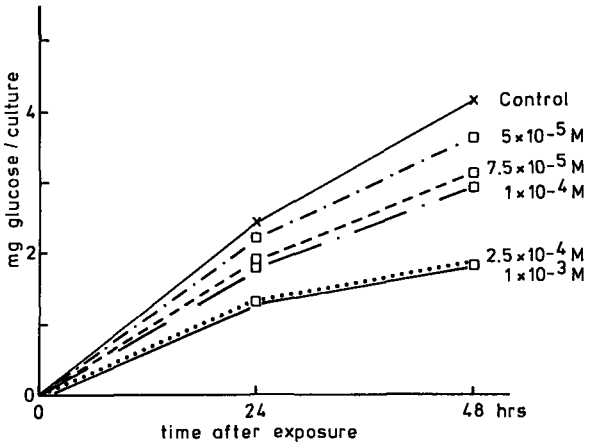

Fig. 6. The mean (± SE) glucose uptake of human epidermal cell cultures up to 48 hr after an exposure time of 30 min to varying concentrations of HD. Values represent the means ± SE of six cultures. The bars indicating the SE fall within the symbols.

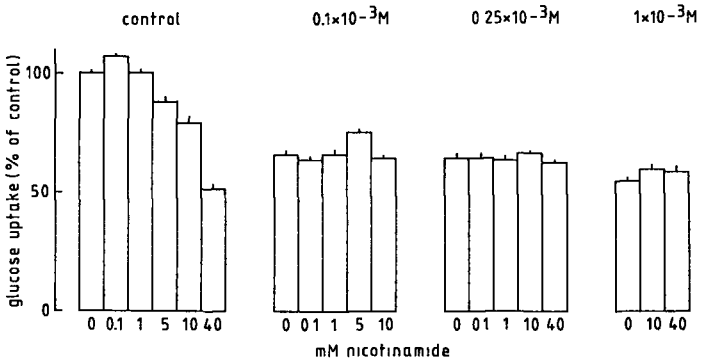

Fig. 7. The mean (± SE) glucose uptake over a 24-hr period of human epidermal cell cultures that were incubated with nicotinamide containing medium following an exposure during 30 min to HD. Each point represents the mean ± SE of six cultures.

(ADPRT). This enzyme is involved in repair mechanisms of DNA strand breaks caused by alkylating agents (Berger *et al.*, 1979; Jacobson *et al.*, 1980; Sims *et al.*, 1982). It is hypothesized that this NAD^+ decrease may lead to cell death by inhibition of the tricarboxylic cycle due to the absence of the essential cofactor NAD^+. If this hypothesis holds, NAD^+ concentrations at levels capable of maintaining aerobic glycolysis may be a possible way to intervene in the development of HD-induced cell death (Berger, 1985; Meier *et al.*, 1987a). The present results, obtained with human epidermal cells, demonstrate that incubations with nicotinamide can prevent

decreases in NAD^+ concentrations of cultures at 4 hr following HD exposure (Fig. 4). However, cell viability as measured by the glucose uptake from the medium over a 24-hr period is not better in HD-exposed cultures incubated with nicotinamide than in cultures that are not incubated with nicotinamide following exposure (Fig. 7). From morphological examinations after a 24-hr period (not shown), it was also clear that cultures which were treated with nicotinamide had no better condition than untreated ones. These results suggest that the decrease in glucose uptake of cells following HD exposure is unrelated to the lack of NAD^+ since preservation of the cellular NAD^+ at control levels does not increase glucose uptake from the medium. Additionally, it was found that loss of NAD^+ was not observed in cultures at 4 hr after exposure to 0.05 mM HD (Fig. 2), whereas the glucose uptake over a 24 hr period is already inhibited at this concentration (Fig. 6).

Another mechanism, perhaps crosslinking of enzymes, may be responsible for the inhibition of glucose uptake.

The observation that the glucose uptake of the cultures during the first 24 hr following HD exposure was inhibited to maximally 50% of the control value provides evidence that initially one route of glucose conversion is not affected. The suggestion that the hexose monophosphate shunt remains active, whereas the tricarboxylic cycle is impaired by lack of NAD^+ (Papirmeister *et al.*, 1985), does not seem plausible since $NADP^+$ will also be exhausted rapidly, because of the dependency of the pyridine nucleotides within their pool (Kaplan, 1985). It is possible that the anaerobic glycolysis, which is the main route of glucose conversion in epidermal cells (Rongone, 1983), is not affected in the first hours following exposure; this will be further investigated.

The NAD^+ precursor nicotinic acid had no influence on the cellular NAD^+ levels (Fig. 5). In addition, the observed rapid fall of cellular NAD^+ after change from medium containing nicotinamide to normal medium (Fig. 3B) suggests that the main mechanism of action of nicotinamide during incubation will be the reversible inhibition of the enzyme ADPRT and that synthesis of NAD^+ from nicotinamide as precursor will hardly occur. This may explain why a protective effect of nicotinamide pretreatment was not observed in the nicotinamide-pretreated cultures that were incubated with normal medium after exposure to HD solutions (Fig. 3C). On the other hand, the inhibition of the enzyme ADPRT by nicotinamide also implicates that nicotinamide incubations during the repair period may disturb the process of excision repair, which depends on poly-ADP-ribosylation. Thus, although NAD^+ levels could be preserved by nicotinamide incubations following HD exposure, DNA repair

might be impaired. Further, it was observed in the present experiments that concentrations of nicotinamide of 5mM and more appeared to suppress the glucose uptake over 24 hr of unexposed control cultures (Fig. 7). However, concentrations of nicotinamide lower than 5 mM have only a NAD^+-preserving effect on cultures that were exposed to HD concentrations lower than 0.25 mM. But in those cases nicotinamide incubations have little use, because the NAD^+ content decreases only slightly after HD exposure. Cytotoxic effects of nicotinamide on cultured cells have also been reported by Berger and Sikorski (1980) and Cleaver *et al.* (1985).

The results presented here are in contrast with those of Meier *et al.* (1987a), who observed that incubation of a suspension of human leukocytes with 0.01 mM nicotinic acid causes an increase in NAD^+ content and prevented the loss of NAD^+ following exposure to 1 mM HD. These effects of nicotinic acid were not obtained with the human epidermal cell cultures. They also found that incubation of human leukocytes with 0.1 mM nicotinamide shortly before or during exposure to 1 mM HD not only prevented NAD^+ depletion but also enhanced their NAD^+ contents. Such a "superinduction" was not found in the human epidermal cell cultures used here. The reason for this discrepancy may be that leukocytes are nondividing cells, whereas the epidermal cells in culture are active in replication, and thereby more susceptible for DNA damage or NAD^+ shortage. Further, Meier *et al.* (1987b) demonstrated that nicotinamide incubations preserved the viability of the leukocytes as measured by trypan blue exclusion. This again may indicate that accumulation of DNA strand breaks is not immediately harmful to resting leukocytes. It must be noted that no metabolic viability was assessed.

It has to be concluded from the present experiments that the fall in NAD^+ concentrations of cultured human epidermal cells is not crucial in the course of events that occur after exposure to HD solutions. Therefore, prophylactic or therapeutic measures that are focused on maintaining NAD^+ levels to preserve energy supplies are unlikely to be successful for the protection of human epidermal cells against HD.

ACKNOWLEDGEMENTS

These experiments have been carried out with the excellent technical assistance of Mr. R. de Vries. The authors are also grateful to Dr. P.H.M. Lohman and Dr. O.L. Wolthuis for critically reading the manuscript.

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8 EFFECTS OF NICOTINAMIDE ON BIOCHEMICAL CHANGES AND MICROBLISTERING INDUCED BY SULFUR MUSTARD IN HUMAN SKIN ORGAN CULTURES

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Toxicol. Appl. Pharmacol. **107**, 439 - 449 (1991)

ABSTRACT

Blister formation due to sulfur mustard (HD) exposure was studied in an *ex vivo* human skin model. Pieces of fresh human skin were exposed to HD vapor and subsequently incubated in medium for 48 hr. During this culture period cellular NAD^+ levels and uptake of glucose from the medium decreased relative to the duration of the exposure to HD. In light microscopic sections of skin that was exposed to HD for 6 min, clefts of variable size could be clearly observed between epidermis and dermis after a 48-hr culture period. Immunohistochemical staining of collagen type IV demonstrated that separation occurred between the basal membrane and the basal keratinocytes. The described *ex vivo* human skin model mimicked the *in vivo* process quite well. Since it was reported that nicotinamide could protect cellular NAD^+ levels after HD exposure, HD-treated skin pieces were incubated in medium containing 10 mM nicotinamide. Although this incubation caused an elevation of cellular NAD^+ levels and of glucose uptake from the medium compared to control values, it did not result in a substantial reduction of cell death or microblister formation as observed by light microscopy in tissue sections. It was concluded that depletion of cellular NAD^+ levels is not the only cause of HD-induced cell death.

INTRODUCTION

Sulfur mustard (HD) is a powerful vesicant. Either direct contact with the compound or exposure to vapor may lead to severe and slow-healing lesions. As with other vesicating agents, the mechanism underlying the process of vesication by HD is unknown. Morphological changes in the epidermal-dermal zone, induced by HD, have been described in human skin transplanted onto nude mice on the light and electron microscopic level (Papirmeister *et al.*, 1984a, 1984b). Other investigations were focused on the alkylating properties of the compound, but the relationship between alkylation and blister formation, if it exists, has not been elucidated. A role of plasminogen activator in the separation of dermis and epidermis was hypothesized recently (Papirmeister *et al.*, 1985), whereas approximately 40 years ago protease activity was also supposed to be involved in blister formation (Renshaw, 1946). To investigate the biochemical processes as well as the morphological changes in blister formation induced by vesicating agents such as HD, a skin model that morphologically resembles the *in vivo* tissue is needed. The use of animals is limited to a few species that vesicate following exposure to HD, such as the hairless guinea pig, the pig and the human skin transplanted nude mouse (Marlow *et al.*, 1989; Mitcheltree *et al.*, 1989; Gross *et al.*, 1985). An alternative model to study HD-induced blister formation may be *ex vivo* human skin. Organ cultures of skin from murine, pig or human origin during various time periods have been described recently (Moore *et al.*, 1986; Chapman *et al.*, 1989; Tammi *et al.*, 1989). With this technique biochemical and morphological changes that are comparable to those *in vivo* can be studied in skin.

Exposure to HD is known to activate the NAD^+-requiring enzyme poly(ADP-ribose)polymerase, which eventually depletes the NAD^+ levels (Papirmeister *et al.*, 1985). This depletion may lead to death of basal epidermal cells and, thereby, to the generation of the epidermal-dermal cleft. Restoration of NAD^+ concentrations at levels that maintain the energy providing system might be a way to intervene in this process of blister formation. Incubations with nicotinamide, an inhibitor of poly(ADP-ribose) polymerase and a precursor of NAD^+, protected the NAD^+ levels and the viability of HD-exposed human leukocytes (Meier *et al.*, 1987), whereas only a slightly positive effect was observed in HD-treated cultured human epidermal cells (Mol *et al.*, 1989). This difference might be attributed to the proliferative activity of the cultured epidermal cells, in contrast to the leukocytes which are resting cells. Since the rate of cell division of the basal cells in freshly excised human skin is slow

compared to that of cultured epidermal cells, it might be expected that the results obtained with the cultured epidermal cells are not predictive for the effects that nicotinamide might have on *in vivo* skin. Hence, the response of pieces of human skin exposed to HD vapor and subsequently to nicotinamide treatment is assessed biochemically by measuring the NAD^+ content of the skin and the glucose uptake from the medium. Moreover, morphological changes in the skin 24 and 48 hr following exposure to HD were evaluated qualitatively by light microscopy and immunohistochemical staining, including the effects of nicotinamide incubations on the skin damage.

MATERIALS AND METHODS

Chemicals. HD, 2,2'-dichlorodiethyl sulfide, was synthesized by the Organic Chemistry Division of the Prins Maurits Laboratory TNO (Rijswijk, The Netherlands). Purity was >99%. DMEM powder and Ham's F12 powder were purchased from Flow Laboratories (McLean, VA); fetal calf serum, ATP, NADP, and hexokinase were bought from Boehringer Mannheim (Almere, The Netherlands); hydrocortisone, nicotinamide and the chemicals for NAD^+ assay, except Na-bicine, were obtained from Sigma Chemical Co. (St. Louis, MO); Na-bicine was from Janssen Life Sience Products Olen, Belgium). Cholera toxin was bought from Calbiochem-Behring, La Jolla, CA; epidermal growth factor and gentamicin were puchased from GIBCO/BRL (Breda, The Netherlands); imidazol was from Merck (Darmstadt, F.R.G.). Human anti-collagen type IV antibody was obtained from Organon Teknika (Turnhout, Belgium) and horseradish-peroxidase conjugated IgG rabbit anti-mouse antiserum from Dakopatts (Glostrup, Denmark).

Exposure to HD vapor. Breast skin obtained from cosmetic corrections was cut into round pieces (20 mm in diameter) and placed with the dermal side down in petri dishes on a small drop of medium to prevent dehydration of the dermis. The surface of the skin pieces was blotted dry before it was exposed to saturated HD vapor. To expose the skin to saturated HD vapor a special device was used; a schematic diagram is shown in Fig. 1. Onto a piece of filter paper (B) on the inner side of the plastic cap (A) an excess of 3 μl (3.8 mg) of liquid HD was applied. The air within the cylinder (D), with a volume of approx 2 ml, became saturated with HD vapor at

30°C to a concentration of 1.44 μg/ml. The exposed area of skin was 0.8 cm^2. Skin (E) was exposed to the HD vapor for 2, 4, 5, 6, 8, 10 or 16 min. This corresponds to Ct dosages between 5.4 and 43.2 $\mu g.min/cm^2$, calculated on the assumption that the penetration rate of saturated HD vapor at 30 °C is 2.7 $\mu g/cm^2$/min (Nagy *et al.*, 1946).

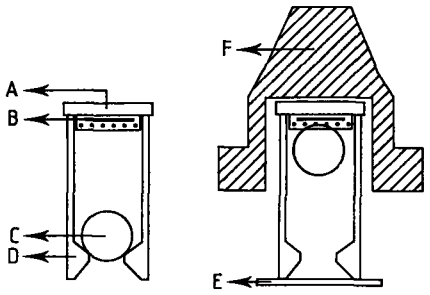
Organ culture of skin pieces. Following exposure, pieces of skin (5 x 5 mm) were excised from the exposed area with a double cutting knife, consisting of two surgical blades screwed on a metal rod at a distance of exactly 5 mm. Skin pieces were floated with the dermal side down on culture medium (0.5 ml medium/well of a 24-well cluster plate) and incubated at 37°C in an atmosphere of 10% CO_2/90% air for time periods up to 48 hr. The humidity was >98%. The culture medium was a mixture of DMEM/F12 (3:1), 5% fetal calf serum, 0.4 μg hydrocortisone/ml, 10^{-10}M cholera toxin, 10 ng epidermal growth factor/ml and 50 μg gentamicin/ml. To examine the effects of nicotinamide the medium was supplemented with 10 mM nicotinamide for 24 hr.

Assay of NAD^+. After varying incubation periods, the skin pieces were frozen in liquid nitrogen and transferred into an icecold 0.5 M $HClO_4$ solution. The skin was extracted for 24 hr at 4°C. The $HClO_4$ extracts were assayed for NAD^+ using an enzymatic cycling assay (Jacobson and Jacobson, 1976).

Glucose uptake. Skin pieces were incubated with medium as described above. At 24 hr skin pieces were blotted dry and incubated in fresh medium for a second 24-hr period. The glucose contents of the media collected after both incubation periods were determined by a spectrophotometric microassay (Cairns, 1987). The glucose uptake by the human skin pieces was calculated from the decrease in glucose content in the media of the organ cultures.

Light microscopy and immunohistochemistry. Skin pieces that were organ cultured for 24 or 48 hr were rinsed in PBS and fixed by immersion in Zenker's fixative for 24 hr at 4°C. Then, skin specimens were stored in 10% phosphate-buffered formalin until they were embedded in paraffin. The mercury precipitates that were present in the paraffin sections due to the use of Zenker's fixative, were removed before staining with hematoxylin and eosin (Kiernan, 1981). Sections of three specimens per treatment, which were obtained in separate experiments, were examined qualitatively.

Fig. 1. Schematic diagram of the device to expose skin pieces to saturated HD vapor. The top of a glass cylinder (D) was closed with a plastic cap (A). At the inner side of the plastic cap a piece of filter paper (B) was placed, onto which liquid HD was applied. The bottom side of the glass cylinder had a smaller diameter and could thus be closed by a stainless steel globule (C). When the air within the cylinder had become saturated with HD vapor, the cylinder was placed onto the skin (E) and the globule was lifted with a magnet (F) for varying time periods.



Representative photographs were made with a Zeiss photomicroscope. For immunohistochemistry tissue samples were frozen in liquid nitrogen and stored air-thight at -20°C. Cryostat sections (6 μm) were incubated with human anti-collagen type IV antibody. The antigen-antibody complex was visualized by incubation with horseradish peroxidase-conjugated IgG rabbit anti-mouse antiserum according to the instructions of the manufacturer.

RESULTS

When fresh human skin that was obtained from mammary reduction was exposed for 2, 4, 6, 8 or 10 min to saturated HD vapor at 30 °C, the result was a progressive decrease of the NAD^+ content in the tissue compared to that of fresh, unexposed skin. In Fig. 2 it is shown that the NAD^+ content, measured 4 hr after exposure, was related to the exposure time and thereby indirectly to the dose of HD. For the two selected exposure times of 4 and 10 min the levels of NAD^+ remained low for at least 24 hr (Fig. 3). It was observed that NAD^+ levels of untreated skin pieces gradually increased during the 24 hr incubation period. The NAD^+ content of untreated, fresh skin pieces was used as control.

The uptake of glucose from the medium by the skin pieces during 0-24 and 24-48 hr of culture was taken as a measure of the effects of HD on the energy metabolism of the skin (Fig. 4). Data were related to the glucose uptake of untreated skin pieces. Inhibition of glucose uptake was seen when exposures lasted longer than

4 min. For skin pieces that had been exposed for 16 min, the glucose uptake over 0-24 and 24-48 hr was inhibited to approx. 40 and 25% of the control values, respectively. The glucose uptake did not become completely inhibited within 48 hr following HD exposure, whereas skin after incubation in liquid nitrogen (assumed to be "dead") used no glucose. Specific inhibition of the glycolytic pathway by adding sodium fluoride (NaF) to the medium of skin pieces not exposed to HD, showed a decrease of glucose consumption during both incubation periods.

In a subsequent series of experiments, the culture medium in which excised pieces of skin were placed following a 5 min exposure to saturated HD vapor, was supplemented with 10 mM nicotinamide. The effects of nicotinamide on the NAD^+

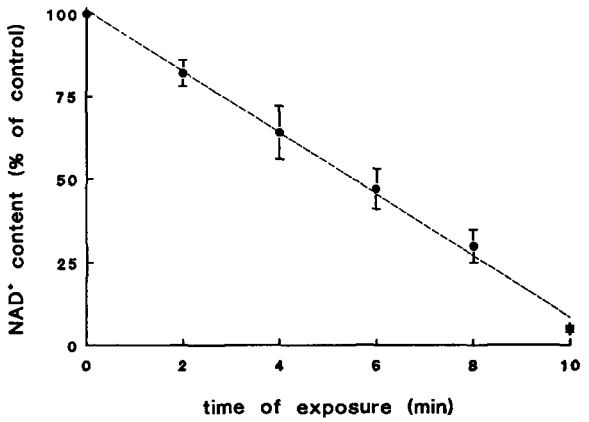


Fig. 2. The effect on the NAD^+ levels of human skin in organ culture measured 4 hr after exposure for 2 to 10 min to saturated HD vapor. Unexposed, fresh skin pieces were used as controls. (Mean ± S.E.M., n=6 for each point)

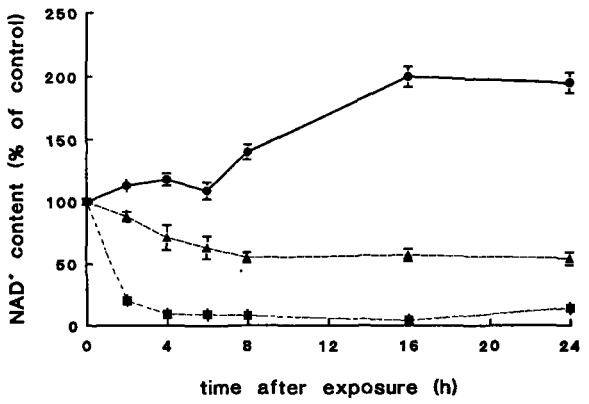


Fig. 3. The NAD^+ content of human skin pieces over 24 hr following exposure for 0 (●), 4 (▲) or 10 (■) minutes to saturated HD vapor. The NAD^+ content of unexposed, fresh skin pieces was used as control. (Mean ± S.E.M., n=6 for each point)

content after 4 hr and the glucose uptake over a period of 24 hr are shown in Fig. 5 (left and right panels, respectively). Control values were obtained from untreated skin pieces that were incubated in medium without nicotinamide. Incubation in culture medium without nicotinamide caused a decrease in the NAD^+ content to 50% of the control value, whereas incubation in nicotinamide-containing medium caused the NAD^+ content of the skin to be restored to the level of untreated skin. Similar trends were obtained for the glucose uptake by the organ cultured skin. Skin not incubated with nicotinamide following HD exposure took up 70% of the amount of control skin, whereas uptake of glucose from the medium by skin that was exposed for 5 min to HD vapor and incubated with 10 mM nicotinamide was equal to that of control skin. Skin pieces that were organ cultured for 24 and 48 hr following exposure to HD vapor for 0, 2, 6 or 10 min were prepared for light microscopy and evaluated qualitatively. Human skin pieces that were not treated with HD kept their normal histological appearance when they were cultured for 24 hr (Fig. 6A). Skin pieces exposed for 2 min to HD vapor had no visible damage after 24 hr in culture (Fig. 6B). Extensive nuclear pyknosis was observed in the lower epidermal cell layers of skin that was exposed for 6 min to HD. Nuclei of upper epidermal cells were fractionated. Moreover, small spaces between basal epidermal cells and dermis had developed (Fig. 6C). In skin that was exposed to HD for 10 min and thereafter cultured for 24 hr, both cells with pyknotic or fractionated nuclei and those in which the nuclei had disappeared were seen. Minute spaces were present between basal cells and the dermis (Fig. 6D). In control skin that was cultured for 48 hr, perinuclear vacuoles had developed (Fig.7A). These vacuoles were not seen in epidermal cells of skin exposed to HD for 2 min. However, their nuclei were fractionated and a few pyknotic cells were observed (Fig. 7B). In contrast, the majority of the epidermal cells of skin exposed to HD for 6 min were pyknotic at 48 hr after exposure. The small spaces beneath the basal cells that were present 24 hr following 6 min of HD vapor exposure had coalesced to clefts of variable size, and several microblisters were observed (Figs. 7C and 7D). The roof of the blisters appear to be composed of necrotic, but intact, basal cells (Fig. 7E). Microblisters were not formed in 10-min-exposed skin; only small ruptures between necrotic basal cells and dermis were observed (Fig. 7F). Immunohistochemical staining of collagen type IV, one of the components of the basement membrane, showed that separation at the epidermal-dermal junction, as observed 24 hr after exposure for 5 min, occurred above the basement membrane, since collagen type IV was located at the base of the blister, i.e. at the top of the dermis (Fig. 8). Untreated skin pieces that were incubated for

Fig. 4. The glucose uptake of human skin pieces from 0 to 24 hr and from 24 to 48 hr after exposure for 2, 4, 6, 8 or 16 min to saturated HD vapor; the glucose uptake from 0 to 24 hr and from 24-to 48 hr of untreated human skin pieces after two times freezing and thawing rapidly ("dead"); the glucose uptake of untreated skin pieces during incubations with 0.01% ("F-1") or 0.02% ("F-2") sodium fluoride in medium. The glucose uptake of untreated skin pieces was used as control. (Mean ± S.E.M., n=6 for each point)

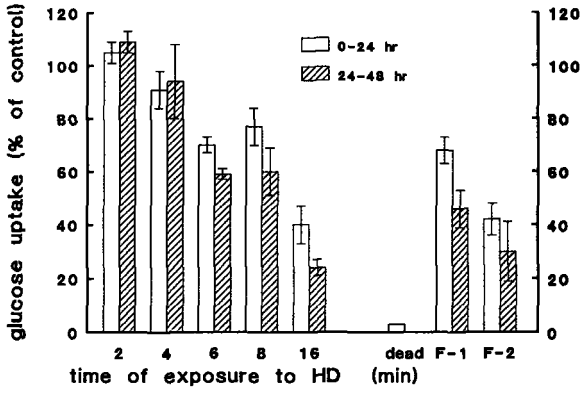
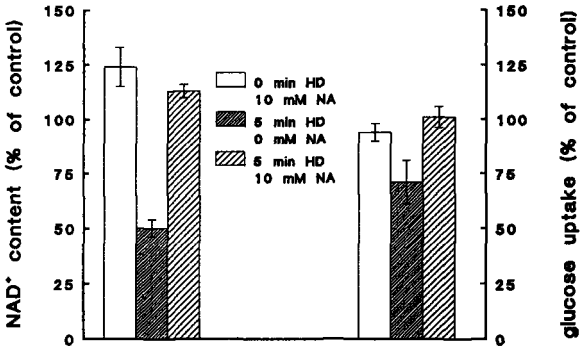


Fig. 5. The effect of incubation with medium containing 10 mM nicotinamide (NA) on the NAD⁺ content at 4 hr (left) and the glucose uptake from the medium over a period of 24 hr (right), following exposure of fresh human skin to saturated HD vapor for 5 min. The NAD⁺ content and the glucose uptake of untreated skin pieces were used as controls. (Mean ± S.E.M. of three separate experiments with n=6 each.)



24 hr in nicotinamide-containing medium had a normal histologic appearance (Fig. 9A). If skin was incubated in nicotinamide-containing medium for 48 hr, perinuclear vacuoles and pyknotic cells had developed (Fig. 9B). Despite the addition of nicotinamide to the culture medium, epidermal necrosis and formation of clefts at the

epidermal-dermal junction were not diminished, as observed at 24 or 48 hr following a 6-min exposure to HD (Figs. 9C and 9D).

DISCUSSION

The results reported here correlate with studies on human volunteers, in which it was noted that blisters were formed 24 - 48 hr following a 6-min or longer exposure to saturated HD vapor at 30°C (Cullumbine, 1944; Nagy, 1946). In previous studies it was estimated that the penetration rate of HD from saturated vapor into human skin at 30°C was 2.7 μg HD/cm^2/min (Nagy *et al.*, 1946) and that about 12% of the penetrated HD was fixed by skin constituents (Renshaw, 1946). On the basis of these assumptions, exposure for 2, 6 or 10 min to saturated HD vapor at 30°C caused fixation of 0.65, 1.95 and 3.24 μg HD per cm^2 of skin. Binding of 0.1-1.0, 1.0-2.5, and >2.5 μg HD per cm^2 human skin has been correlated with the development of erythema/edema, vesication and necrosis, respectively (Renshaw, 1946). The time course of the HD-induced damage and the histologic appearance of *ex vivo*-exposed skin after 48 hr of culture seem to correlate well with these stages of pathology of human skin *in vivo*. Light microscopic sections of organ cultured skin pieces that were exposed for 6 min or longer to saturated HD vapor (Figs. 7C - 7F) showed progressive damage that was similar to that resulting from HD vapor treatment of human skin grafted onto the nude mouse (Papirmeister *et al.*, 1984a). The microvesication of *ex vivo*-exposed human skin was also similar to that reported for *in vivo*-exposed pig skin (Mitcheltree *et al.*, 1989). However, pig skin seemed to be far less sensitive to HD than human skin. The observation that separation of the epidermis and dermis occurred above the basal lamina of the basement membrane (Fig. 8), is also in agreement with the studies on pig skin and transplanted human skin. Thus, it may be concluded that microblister formation in the organ cultured human skin simulates the *in vivo* process of blistering quite well. The fact that the separation between epidermis and dermis, which could be regarded as an important phase in blister formation, occurs in an *ex vivo* situation may indicate that only epidermal or dermal factors and no systemic factors are involved in the cleavage between the epidermis and the dermis. Most likely, the development of a full blister in humans is dependent on additional factors such as extravasation of lymph or blood constituents.

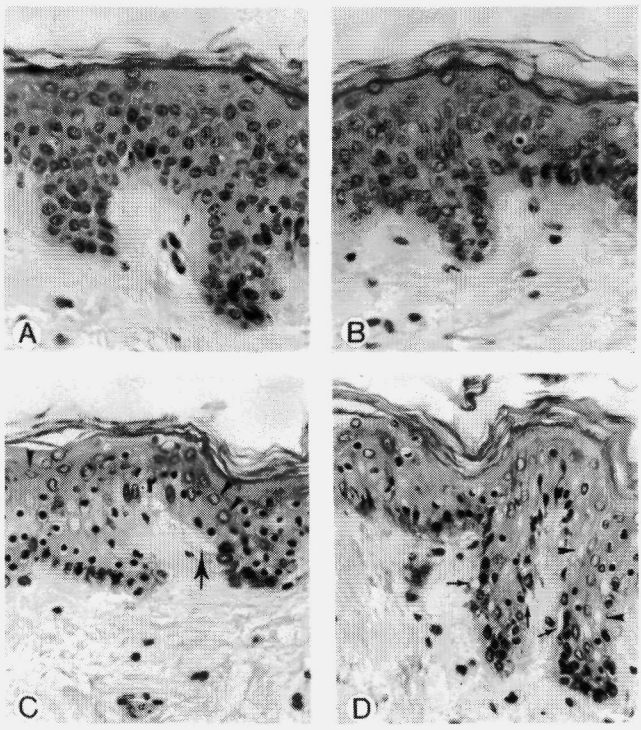


Fig. 6. Light microscopic views of skin pieces after a culture time of 24 hr. ***A.*** *Untreated skin. The structure of untreated skin is well preserved during culture time.* ***B.*** *Skin treated with HD vapor for 2 min. No visible changes are noted.* ***C.*** *Skin treated with HD vapor for 6 min. Extensive nuclear pyknosis is seen in the lower epidermal cell layers, whereas suprabasal cell nuclei are fractionated- (arrowheads). Initial separation occurs at the epidermal-dermal junction (arrow).* ***D.*** *Skin treated with HD vapor for 10 min. Nuclei of basal epidermal cells are pyknotic; nuclei of cells in the upper layers of the epidermis are fractionated or even lysed (arrowheads). Minute spaces are present between the basal epidermal cells and the dermis (arrows). (original magnification* ***A - D****: x 400)*

The present results show that formation of microblisters between the basal membrane and the basal keratinocytes occurred within 48 hr after exposure to HD vapor for 6 min (Figs. 7C - 7E). Following exposure for 10 min to HD only small vacuoles between epidermis and dermis were observed (Fig. 7F), possibly because cell death occurs too rapidly at high concentrations for further biochemical reactions to take place. This might mean that the HD blistering mechanism depends on active processes in cells that still have metabolic activity. Indeed, microvesication was seen in the transitional area of 10 min exposed and unexposed skin (not shown).

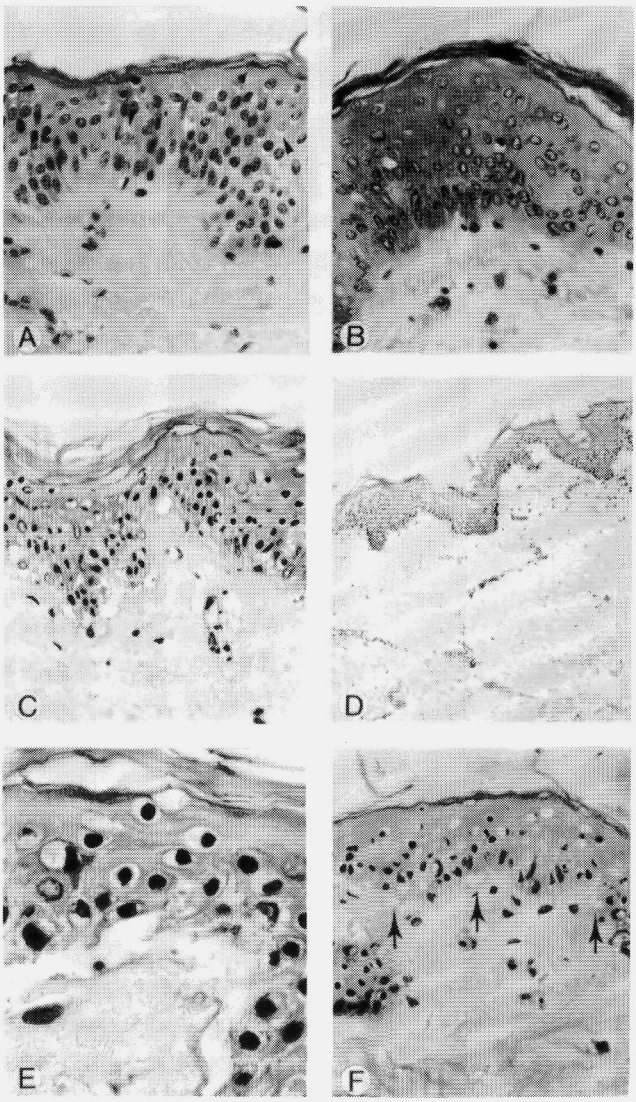


Fig. 7. Light microscopic views of skin pieces after a culture time of 48 hr. ***A.*** *Untreated skin. A few perinuclear vacuoles are observed (arrowheads).* ***B.*** *Skin treated with HD vapor for 2 min. Epidermal cell nuclei are fractionated.* ***C - E.*** *Skin treated with HD vapor for 6 min. The epidermis is severely damaged. Separation of epidermis and dermis occurs along the entire epidermal-dermal junction. The size of the space between epidermis and dermis is variable (C and D). The roof of the blisters appears to be composed of necrotic, but intact, basal cells (E).* ***F.*** *Skin treated with HD vapor for 10 min. Many cells with lysed nuclei are present in addition to pyknotic cells. However, only small ruptures occur between epidermis and dermis. (original magnification* ***A, B, C*** *and* ***F:*** *x 400;* ***D:*** *x 100;* ***E:*** *x 1000)*

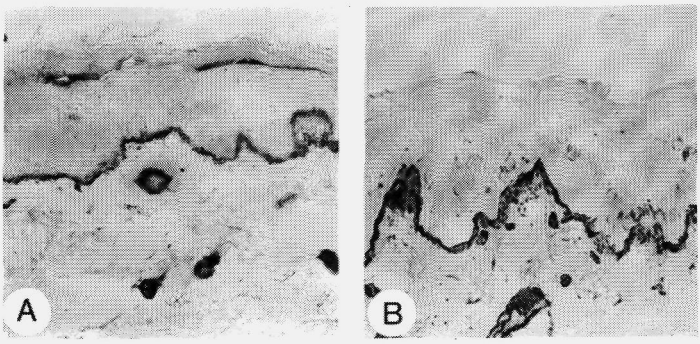


*Fig. 8. Immunohistochemical staining of collagen type IV in the basement membrane of untreated skin (**A**) and skin treated with HD vapor for 5 min (**B**), both after a culture period of 24 hr. (original magnification **A, B** : x 400)*

Circumferential vesication of severely exposed skin *in vivo* was already described by Renshaw (1946). Moreover, Mitcheltree *et al.* (1989) mentioned recently that blister formation was observed in the slightly contaminated periphery of pig skin that was treated with HD droplets, whereas only necrosis was seen in the central area.

In the study described here, the NAD^+ content of human skin was measured 4 hr following exposure to saturated HD vapor at 30°C (Fig. 2). The observed decrease is in agreement with observations that have been reported for human skin transplanted onto nude mice (Gross *et al.*, 1985), human leukocytes (Meier *et al.*, 1987) and cultured human epidermal cells (Mol *et al.*, 1989). Although the NAD^+ content of skin pieces that were exposed for 10 min was already exhausted at 4 hr after exposure, the glucose uptake over 24 hr is only 30% inhibited (Fig. 4). It islikely that the observed glucose consumption is caused by either anaerobic metabolism or conversion via the hexose-mono-phosphate shunt, since the aerobic glucose conversion is strongly inhibited by the NAD^+ depletion. Another possible explanation for the relatively high glucose uptake remaining after HD exposure might be that the dermal part of the skin was only slightly affected by the HD exposure and consumed glucose quite normally, whereas the epidermis was heavily affected and did not use glucose.

Cytotoxic effects of nicotinamide on glucose uptake have been shown for several cultured cells (Grunfeld and Shigenaga, 1984; Nadeau and Lane, 1989), but the concentration of nicotinamide used here did not inhibit the glucose uptake (Fig. 5, right). The addition of 10 mM nicotinamide to the medium in which the skin pieces were organ cultured following a 5-min exposure to HD, prevented the decrease of

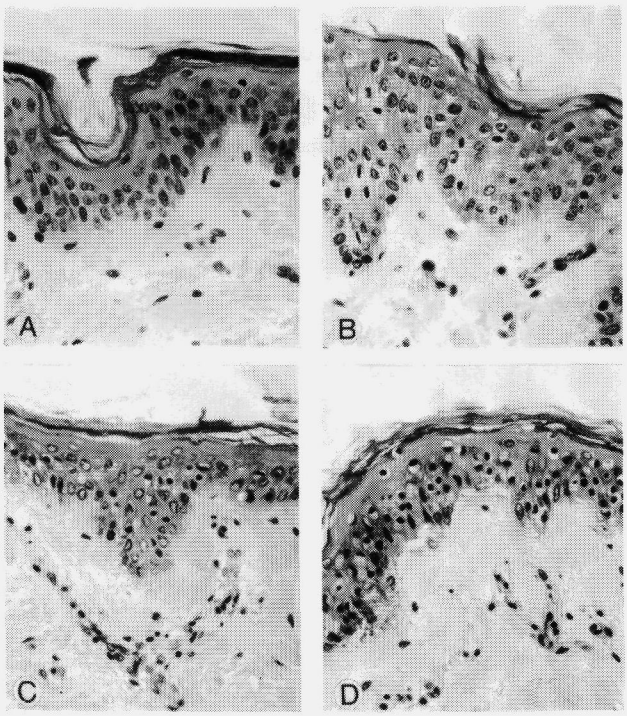


Fig. 9. Light microscopic views of skin pieces that were maintained in medium containing 10 mM nicotinamide. ***A.*** *Untreated skin, organ cultured for 24 hr. The skin has a normal appearance.* ***B.*** *Untreated skin, organ cultured for 48 hr. A few pyknotic cells and cells with perinuclear vacuoles are noted.* ***C.*** *Skin treated with HD vapor for 6 min and organ cultured for 24 hr. Nuclei of epidermal cells are fractionated or pyknotic. No spaces are observed between basal cells and dermis.* ***D.*** *Skin treated with HD vapor for 6 min and organ cultured for 48 hr. Severe pyknosis of the epidermal cells is observed and most of the basal cells are detached from the dermis. (original magnification* ***A - D****: x 400)*

the NAD^+ content and, in contrast to the results obtained with cultured human epidermal cells (Mol *et al.*, 1989), restored the glucose uptake during 24 hr to control levels. Thus, therapy with nicotinamide *in vivo* might have better results than was expected on the basis of earlier results with cultured human epidermal cells. However, despite the above mentioned observations, no substantial improvement was seen in the histological appearance of skin that was incubated in nicotinamide-containing medium following HD exposure (Fig. 9). The obtained results indicate that nicotinamide incubations might be beneficial tothe NAD^+ content and the glucose

utilization of HD-treated human skin, but they do not diminish the development of cell death visible by light microscopy. Thus, it appears that other factors than depletion of cellular NAD^+ levels contribute to HD-induced cell death.

ACKNOWLEDGEMENTS

The authors wish to thank Ms A.M.B.C. van de Ruit and Ms P.W. Valkenburg for their excellent technical assistance, and Dr. O.L. Wolthuis and Dr. P.H.M. Lohman for helpful discussions and critically reading the manuscript.

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9 GRAFTING OF VENOUS LEG ULCERS: AN INTRA-INDIVIDUAL COMPARISON BETWEEN CULTURED SKIN EQUIVALENTS AND FULL THICKNESS SKIN PUNCH GRAFTS

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J. Am. Acad. Dermatol. **24**, 77 - 82 (1991)

ABSTRACT

Skin equivalents that consisted of a non-contracted collagen gel populated with allogeneic fibroblasts and covered with autologous cultured keratinocytes were used for grafting venous leg ulcers. The results were compared in the same patient with those obtained with a routinely used standard method of grafting with autologous full thickness punch grafts. The skin equivalents and the punch grafts were grafted successfully in four of five patients. The median healing time of ulcers grafted with skin equivalents was 18 days, whereas that of ulcers covered with punch grafts was 15 days. The cosmetic appearance of the skin equivalent-grafted ulcers was better than that of the punch-grafted ulcers.

INTRODUCTION

Leg ulcers are frequent occurring and have a chronic, relapsing course. In most patients they are due to venous insufficiency. Normally, healing takes place by ingrowth of epithelium from the wound edges at a rate of approximately 1 mm a day. To accelerate healing, various grafting techniques have been developed. One of the oldest is the application of full thickness punch grafts[1].

Current interest has focused on the use of cultured epithelial cells for grafting. Cultured pure epidermal sheets have been used[2-6], although the need for both dermal and epidermal components in the healing of full thickness wounds has been recognized[7,8]. The importance of dermal control in the regulation of epidermal proliferation may be crucial in wound healing[9,10]. Collagen lattices that are populated with fibroblasts and covered with keratinocytes, have been successfully used in animals[11-15]. Recently, the use of cultured composite allografts in tattoo excisions was described[16]. In the present study, skin equivalents for grafting venous leg ulcers were evaluated. Healing of chronic leg ulcers after application of full- thickness punch grafts or cultured skin equivalents was compared in the same patient. The skin equivalent was composed of a noncontracted collagen gel populated with allogeneic fibroblasts and covered with autologous keratinocytes. The results have been compared in terms of healing time and cosmetic appearance.

PATIENTS

Consecutive patients with venous leg ulcers were included in this study. The ulcer had to be larger than 3 cm in diameter, or two comparable ulcers had to be present. Large ulcers were half covered with cultured skin equivalent and half with autologous punch grafts. When two ulcers were present in one patient, these were treated separately with these methods. Written informed consent was obtained from each patient. This study was approved by the medical ethical committee of the hospital.

MATERIALS AND METHODS

Preparation of the skin equivalent. Collagen type I from rat tail tendons[17] (3 mg/ml)

Fig.1. Top view of a skin equivalent after culture period of 12 days in a petri dish. Epidermal cells covering the collagen gel have been stained with Nile Blue sulphate.

and calf skin collagen types I and III (Flow; Irvine, Scotland) were mixed 1:1. Human foreskin fibroblasts, free of the human immunodeficiency virus, were originally derived from a donor. In Petri dishes (diameter 60 of 90 mm or 100x100 mm), springs were placed along the edges before the collagen solution containing 10^5 human fibroblasts/ml was poured out. After gelation the collagen gel was covered with culture medium containing lethally irradiated 3T3 mouse fibroblasts, thereby providing a feeder layer for the keratinocytes[18]. The latter were isolated from full-thickness punch biopsy specimens (ϕ 8 mm) that were taken from patients under local anaesthesia from the lateral thigh on the day of admittance. Subsequently, the keratinocytes were seeded on the gel and cultured according to methods described elsewhere[19]. Growth was evaluated microscopically. After 10 to 14 days the skin equivalents were just confluent and ready for grafting (Fig.1). It can be clearly seen that the spring is completely embedded into the gel. The surface of the gel is covered with one or two layers of keratinocytes (Fig. 2).

Pregrafting and grafting procedures. During the preparation of the skin equivalent the patient stayed in hospital; bed rest was mandatory. On the first day the ulcer was cultured. To clean the ulcer a pregrafting schedule was followed[20]. Moistened gauze pads were placed on the ulcer and renewed three times a day. The antiseptic solution of the gauze was changed every other day with first a 0.9% saline solution,

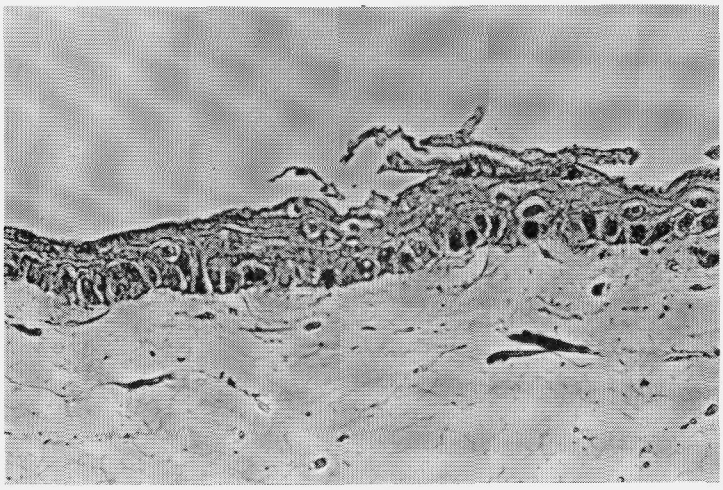

Fig.2. Paraffin-embedded vertical section of a skin equivalent shows fibroblasts in the collagen gel and one or two layers of keratinocytes on top (Hematoxylin-eosin stain; x400).

second a 2% acetic acid solution, and finally a 10% povidone-iodine solution. This regimen was continued until the wound was clean and granulating, generally after 7 to 12 days.

On the day of grafting half of the ulcer was covered with skin equivalent and the other half with full thickness punch grafts (ϕ 4 mm), freshly taken from the thigh; local anaesthesia was given. If the patient had two comparable ulcers, one was grafted with skin equivalent and the other with punch grafts. Before grafting small cuts were made in the gel to allow exudate to escape from the wound. The surrounding spring was removed and a sterile nylon mesh was adhered to the skin equivalent for safe transfer to the wound. The gel did not contract after removal of the spring. The nylon mesh was removed when the skin equivalent was in place (Fig. 3A).

The punch grafts (total thickness 1 mm) were placed on the granulating wound at regular distances of 5 mm (Fig. 3B). The grafted areas were covered with sterile petrolatum gauze and wrapped for fixation and protection. Bed rest was continued for at least 7 days. From the fifth day on, the grafted areas were inspected daily. When the ulcers were healed, nonelastic compression bandages were applied and the patient was allowed to ambulate.

RESULTS

Between April and June 1989 seven consecutive patients (4 women, 3 men) were included in the study. Their median age was 85 years (range 70 to 94 years). Table I summarizes the clinical data. Two patients (Nos 3 and 7) could not undergo

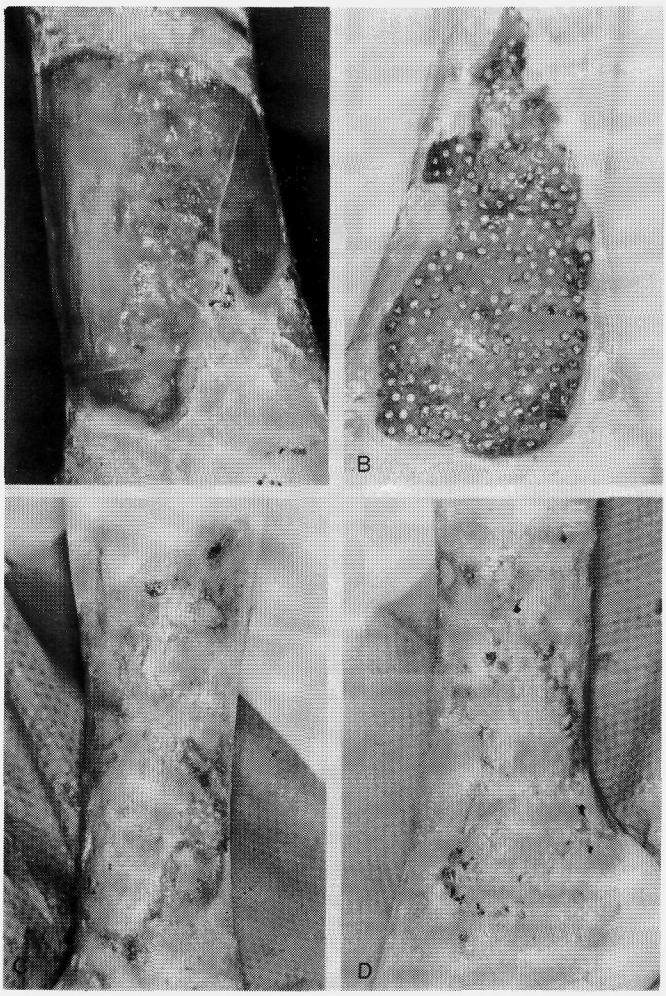

Fig.3. ***A****. Ulcer on lateral aspect of lower leg, covered with skin equivalent. Note uncovered area right (see text for explanation).* ***B****. Ulcer on the medial aspect of the same leg, covered with full-thickness skin punch grafts.* ***C****. Eighteen days after grafting with skin equivalent ulcer is closed.* ***D****. Fourteen days after grafting with full-thickness skin punch grafts ulcer is closed. (****A****, x1.2;* ***B****, x1.0;* ***C****, x0.9;* ***D****, x0.7.)*

grafting with skin equivalents because of bacterial contamination of the cultures. Punch grafts were used for their ulcers. Results of cultures before grafting showed a variety of bacterial contamination (no *Streptococcus species*), but no systemic or local treatment with antibiotics was needed. The size of the respectively grafted areas was comparable in each individual patient. In all patients the areas grafted with punch grafts were healed 2 to 4 days earlier than those grafted with skin equivalents; median healing time was 15 days compared with 18 days, respectively (Table 1). With both grafting methods, the healing time of the ulcers was independent of their size. In four of five patients the grafting of skin equivalents and punch grafts was successful; 100% reepithelialization was achieved. However, in patient 6 both techniques failed because the ulcer was located in an atrophic and sclerotic area.

In one patient the surface of the skin equivalent was too small to cover the entire ulcer (Fig. 3A). Initially no graft was placed on this part. When the bandages were removed on the fifth day, this area of the ulcer did not show reepithelialization. The decision was made to use punch grafts. On the eightteenth day the ulcer was healed completely (Fig. 3C). The surface of the ulcers that were grafted with skin equivalent was in level with the surrounding skin and its cosmetic appearance was better than that of the punch-grafted area, which showed a cobblestone appearance (Fig. 3C and 3D).

Table I. Skin equivalent grafting versus full-thickness skin punch grafts: clinical data

Patient	Number of ulcers	Skin equivalents		Punch grafts	
		Area grafted in cm^2	Healing time in days	Area grafted in cm^2	Healing time in days
1	2	81	18	72	14
2	1	12	18	12	15
3	1	-	-	150	29
4	2	12	19	7	16
5	2	9	12	6	10
6	1	4	28	6	26
7	2	-	-	24	19

- : infection of culture

However, skin from the skin equivalent was thinner and more fragile in the early postgrafting period compared with that resulting from punch grafts.

Follow-up. Ulcers grafted with both techniques were observed for 8 to 10 months. In patient 1 two small ulcers (diameter 1 cm) recurred in both grafted areas, whereas in the other patients the ulcers remained healed, regardless of the grafting technique (Fig. 4).

DISCUSSION

By intraindividual comparison in a controlled trial it has been shown that chronic leg ulcers healed completely within 20 days after grafting of skin equivalents. This healing required 2 to 4 days longer than that observed in ulcers that were grafted with autologous full-thickness punch grafts. Only one preliminary report on grafting of full thickness wounds with cultured skin substitutes has been published[16].

It is unlikely that epithelial cells from skin appendages contribute to the epithelialization of full-thickness ulcers. In large ulcers grafted with skin equivalents, islands of epithelium were clearly visible in the center of the wound 5 days after grafting. In smaller ulcers the origin of the epithelium remained unclear. If the ulcers were closed by epithelial ingrowth from the wound edges, this may be attributable to a stimulating effect of the skin equivalent. However, the observation that healing

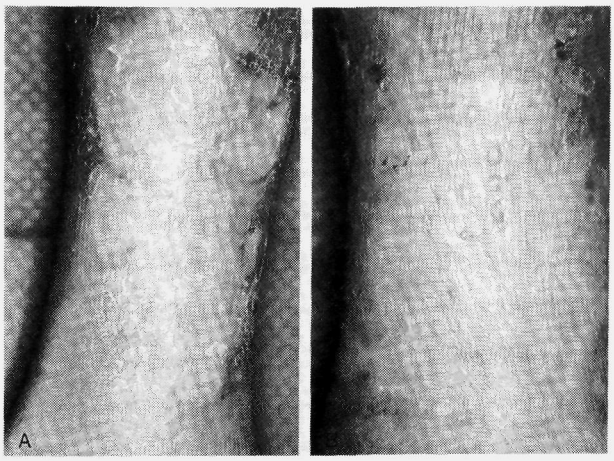

Fig.4. ***A****. Result of skin equivalent grafting technique after 4 months.* ***B****. Result of the punch biopsy grafting technique after 4 months. (****A*** *and* ***B****, x1.4.)*

time did not depend on the size of the ulcers confirms the concept that the wounds were probably closed by "take" of the skin equivalent.

For grafting of leg ulcers various methods have been described[21,22]. The use of sheets of cultured keratinocytes as grafting material is a relatively new technique. Grafting of leg ulcers with autologous epithelial sheets resulted in healing of respectively 33% and 65% of them [2,3]. Because there is a delay of 3 to 4 weeks between obtaining the biopsies and grafting, the use of allografts was investigated.Although this technique was applied successfully[4-6], it actually activates the wound edges by release of growth stimulating factors from the cultured sheets; one week after grafting the donor cells had disappeared[23]. These cells were probably lysed because tissue rejection is generally observed after 12 to 15 days. The average healing time of ulcers grafted with cultured allogeneic sheets is approximately 4 weeks[5,6] (i.e. 10 days longer than the healing time in the present study). Furthermore, with the methods herein, the time needed to manufacture a graft is reduced to a maximum of 14 days, which corresponds to the duration of the pregrafting procedure. Because the gel serves as a carrier, there is no need to wait until a cohesive multilayered epithelial sheet was formed. Thus, a comparatively large number of proliferative cells can be grafted. This may be an important reason for the rapid healing of the ulcers. Another disadvantage of autologous keratinocytes was assumed to be the low growth potential of keratinocytes from elderly persons. With the culture technique we used the keratinocytes of all patients grew well.

The skin equivalent used in this study was composed of a collagen gel populated with allogeneic fibroblasts and covered with autologous cultured keratinocytes. In laboratory studies the optimal conditions for growth of keratinocytes on a collagen gel were investigated. It was found that a 1 : 1 mixture of rat tail collagen and Flow calf skin collagen appeared most satisfactory [24]. Incorporation of human skin fibroblasts in the gel positively affected the growth of keratinocytes. Sher et al.[13] demonstrated in rats that skin equivalents made up of allogeneic cells bearing class I antigens were accepted; this suggested that allogeneic fibroblasts might be used for skin grafting. The present results show no clinical signs of rejection of the skin equivalents. To become independent of allogeneic cells, which bear the risk of viral infection, the procedure has now been modified by the use of autologous fibroblasts.

When skin equivalents were made without springs, the gels contracted. This was probably due to the action of fibroblasts in the gels and of keratinocytes on top[25]. Embedding a spring along the edges of the gel at the moment of casting prevented

the contraction. The use of non-contracted gels has practical advantages over three-dimensionally contracted gels. For example, the size of the gels is standardized and cell growth on top of the gel can be more easily observed microscopically.

Although the number of patients we treated is small, this study indicates that grafting of leg ulcers with cultured skin equivalents yields results that are as good as those obtained with punch grafts. However, the use of skin equivalents is quite laborious, needs sophisticated laboratory facilities and bears a risk of infection during culture. On the other hand, the advantage of grafting skin equivalents is the considerable reduction in donor skin area. Application of punch grafts, which are immediately available, will probably remain the treatment of choice. In those cases, however, in which a large donor area is needed, such as in patients with extensive leg ulcers or burns, the use of cultured skin equivalents may be of great value.

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10 GENERAL DISCUSSION

Sulfur mustard is one of the earliest known alkylating agents and has a strongly vesicating action on human skin. In particular, dividing cells are sensitive to the DNA damage resulting from alkylation by sulfur mustard. In case of skin exposure, the basal epidermal cells, which have mitotic capacity, are the most vulnerable to sulfur mustard-induced cytotoxicity. Although the toxic action of sulfur mustard on these cells is thought to be strongly related to blister formation, until now, only limited studies have been performed to examine the process of blister formation by sulfur mustard. Therefore, a broad study intended to understand the mechanisms of action involved in sulfur mustard-induced vesication was initiated. While in the past the various sulfur mustard-induced molecular and cellular lesions had been examined separately in a variety of cell types, in the present study the effects of sulfur mustard have been integrally investigated in the target cell of the skin, i.e. the human epidermal keratinocyte. Both cultured human epidermal keratinocytes and human skin pieces in organ culture were helpful *in vitro* models to study the toxic reaction to sulfur mustard in human skin. Dosimetry of the effects of sulfur mustard on epidermal keratinocyte cultures has been presented over a concentration range between 0.1 and 1000 μM sulfur mustard (chapter 6). Most sensitive to sulfur mustard exposure were DNA synthesis and cell replication. Significant inhibition of these cell functions was observed first at concentrations of sulfur mustard between 0.1 and 1 μM and persisted for at least 24 hr. The inhibition of DNA synthesis and cell replication was probably due to the formation of DNA interstrand cross-links, which could be detected immediately following exposure to these low concentrations (chapter 5). The number of cross-links increased linearly with the applied dosis over a range up to 10 μM sulfur mustard. Although considerable removal of these cross-links occurred during 24 hr following exposure, some cross-links remained detectable. It concerned a small, constant number of cross-links, which was independent of the sulfur mustard concentration applied. These "irreparable" cross-links might contribute to the persisting inhibition of cell replication after 24 hr.

Only at sulfur mustard concentrations nearly two orders of magnitude higher than those inducing damage to DNA, changes in other vital cell functions were observed. Exposure of keratinocyte cultures to sulfur mustard concentrations higher than 50 μM resulted in inhibition of protein synthesis, reduction of cellular NAD^+ and ATP levels and declined intracellular LDH activity and glucose utilization (chapters 6 and

7). Results of experiments with human skin pieces in organ culture provided evidence that formation of microblisters between the basal membrane and the basal keratinocytes occurred within 48 hr after exposure to sulfur mustard vapor for 4 to 8 min at 30°C (chapter 8). These vesicating doses of sulfur mustard vapor induced a progressive decrease of the NAD^+ content in the human skin pieces, measured 4 hr after exposure. A comparable depletion of the NAD^+ concentration was observed in cultured human keratinocytes exposed for 30 min to sulfur mustard concentrations varying between 250 and 600 μM (chapter 7). Based on this data it was estimated that exposure of cultured human keratinocytes to sulfur mustard concentrations between 250 and 600 μM causes cellular effects that are comparable to those occurring in the basal epidermal cells of intact human skin following exposure to doses inducing vesication. However, the loss of cellular NAD^+ is probably not the only cause of sulfur mustard-induced blister formation as was shown by experiments on the efficacy of the potential therapeutic agent nicotinamide to prevent NAD^+ depletion and thereby vesication. These data demonstrated that the hypothesis of Papirmeister et al. (1985), proposing sulfur mustard-induced DNA damage to be the initial cause of cellular energy depletion and, consequently, of vesication is only partially valid.

Results of the present study established that if epidermal keratinocytes are exposed to concentrations of sulfur mustard comparable with doses inducing vesication on intact human skin, DNA is extensively alkylated. In addition, based on the Swain-Scott constant s of the episulfonium ion of sulfur mustard (0.95), it may be expected that also many sulfur mustard-protein adducts has been formed. The considerable alkylation of both DNA and proteins will have profound effects on epidermal cell function. Two other potential factors in the pathogenesis of sulfur mustard-induced blisters may be the termination of protein synthesis and the enhancement of intracellular free Ca^{++} levels. Possible implications of these effects on blister formation will be discussed below.

Vesication is the most striking acute effect that follows exposure of man to sulfur mustard. Even today, it is not understood by what mechanism the blisters are induced. In essence, blister formation can be divided into two separate processes. One process leads to a rupture between epidermis and dermis; the other process is the filling of the blister cavity with fluid from the dermis. Electron microscopic studies have suggested that separation of the basal epidermal cells from the dermis is initiated by detachment from the basement membrane of the anchoring filaments,

which are part of the hemidesmosomes (Papirmeister et al., 1984b). Evidence for an important role of hemidesmosomes in epidermal-dermal adherence has been obtained from studies of Beerens and colleagues (1975) on the formation and repair of suction blisters in man. They showed that formation of suction blisters occurs by successive detachment of hemidesmosomes from the basement membrane. If suction was terminated before a fluid-filled blister was formed, they observed a rapid regeneration process that consists of two steps: a realignment of basal cells to the basement membrane accompanied by autophagocytosis of detached hemidesmosmes, and *de novo* formation of hemidesmosomes. Interestingly, Beerens (1977) also observed that suction exposure stimulated the regeneration of hemidesmosomes, even in nitrogen mustard-treated skin. However, in the latter case, the formation of new hemidesmosomes was incomplete, because they were not linked to the tonofilament network of the basal keratinocytes, as in control skin. These observations suggest a) that attachment of basal keratinocytes to the basement membrane is an active process, demanding energy and supply of ingredients for hemidesmosome formation and b) that nitrogen mustard intervenes in the mechanism of attachment. In addition to investigations on suction-induced blisters, Beerens (1977) studied the ultrastructural changes in the basement membrane zone of dog skin, exposed to nitrogen mustard. He observed a gradual degeneration of hemidesmosomes over 48 hours following exposure. Similarly, Feng et al. (1987) observed a reduction of filaments in cell-cell connection regions of sulfur mustard-treated cultured rat keratinocytes. They suggested that sulfur mustard may damage genes required for the generation of proteins involved in cell-cell adhesion. Another explanation may be the prolonged impediment of protein synthesis in the basal epidermal keratinocytes as demonstrated in the present study. If one assumes that the basement membrane zone, including the hemidesmosomes, is maintained by the balanced synthesis and degradation of the (glyco)protein constituents, prolonged inhibition of protein synthesis may lead to degeneration of the epidermal-dermal junction.

Epidermal keratinocytes synthesize basement membrane associated (glyco)proteins, but data on their turnover rates are not available. If the turnover rates should fit with the latency time for sulfur mustard-induced blisters to appear, this would support the role of protein synthesis in the maintenance of epidermal-dermal attachment structures.

The results of the present study have suggested that activation of nuclear poly(ADP-

ribose)polymerase is not likely to be the only factor responsible for NAD^+ depletion of cells. We postulate that NAD^+ depletion is also caused by the activation of another NAD^+ consuming enzyme, mono(ADP-ribose)transferase, which catalyzes mono-ADP-ribosylation of proteins (Vaughan and Moss, 1981; for review Althaus and Richter, 1987). Because activation of this enzyme is associated with cellular Ca^{++} levels (Richter et al., 1983; Duncan et al., 1988; see for review Richter and Kass, 1991), it is assumed that sulfur mustard causes an enhancement of free cytoplasmic Ca^{++}. Support for this postulate is obtained from the following. First, damage to the endoplasmic reticulum is clearly present amongst the early ultrastructural changes observed in basal epidermal cells treated with sulfur mustard (Papirmeister et al., 1984b). The primary cause of damage by sulfur mustard to the endoplasmic reticulum may be the preference of this lipophilic toxicant for the lipid bilayer of the membrane of the endoplasmic reticulum and the subsequent electrophilic attack of the membrane bound proteins. Since it has been shown that the endoplasmic reticulum is the major intracellular Ca^{++} store, damage to this structure may cause increased cytosolic Ca^{++} levels (Somlyo et al., 1985; see for review Richter and Kass, 1991). Second, although no clear data exist, it is supposed that sulfur mustard rapidly depletes cellular glutathione levels, if cells are exposed to concentrations of sulfur mustard higher than 50 μM (Gentilhomme and Thiriot, 1990). Such a depletion may result in an increase of intracellular Ca^{++} concentrations (Orrenius, 1985).

It has been established that under toxic conditions liver mitochondria have the capacity to sequester large quantities of Ca^{++} and may act as efficient buffer stores of Ca^{++} under toxic conditions. However, since mitochondria cannot store Ca^{++} in unlimited amounts, they release Ca^{++} at a rate comparable to its uptake. Since Ca^{++} uptake and release occur along different pathways, "cycling" of Ca^{++} across the mitochondrial membrane may take place (for reviews see Nicotera et al., 1990; Richter and Kass, 1991). In Ca^{++} cycling mitochondria a net loss of oxidized pyridine nucleotide has been observed, which was attributed to its hydrolysis into ADP-ribose and nicotinamide by mono(ADP-ribose)transferase. It has been suggested that a functional link exists between mitochondrial protein ADP-ribosylation and Ca^{++} release from these cell organelles (Hofstetter et al., 1981; Richter et al., 1985; see for review Richter and Kass, 1991). Due to sulfur mustard-induced enhancement of intracellular Ca^{++} levels, the mitochondrial bound mono(ADP-ribose)transferase would become activated. Extreme activation of mono(ADP-ribose)transferase will cause depletion of the mitochondrial NAD^+ pool and reduction of ATP synthesis (Richter and Kass, 1991).

It is generally accepted that intracellular Ca^{++} overload, in addition to mitochondrial damage, also induces cytoskeletal alterations and activation of Ca^{++}-dependent degradative enzymes, such as phospholipases, proteases and endonucleases (see for review Nicotera et al., 1990). In particular, activated Ca^{++} dependent proteases may affect the cytoskeletal proteins and membrane integral proteins, which may further contribute to weakening of the epidermal-dermal attachment structures.

For a better understanding of the biochemical mechanisms involved in vesication, it may be useful to compare the cellular processes involved in basal epidermal cell toxicity due to sulfur mustard with those of other stimuli causing vesication, such as heat and Lewisite (dichloro(2-dichlorovinyl)arsine). Although the exact mechanism of heat-induced cytotoxicity is unknown, major effects of heat on cells may be the denaturation and aggregation of cytoplasmic and nuclear proteins (Iliakis and Pantelias, 1989; see for review Dewey, 1989), damage to either plasma or internal membranes (Roti Roti, 1982; Stevenson et al., 1987), increase in the level of intracellular Ca^{++} (Calderwood et al., 1988) and protein phosphorylation, in particular of heat shock proteins (Landry et al., 1988). Moreover, Dewey (1989) observed several hours after heating of cells detachment of the cytoskeleton from the membrane and he suggested that this event might be important in (hemi)desmosome destruction.

With respect to Lewisite, little is known about the mechanism of action of this vesicant. Probably, it binds to thiol groups on proteins with two proximal thiol groups, in particular to pyruvate oxidase, an enzyme involved in glycolysis (Stocken and Thompson, 1949; Klaassen, 1985). It is unknown whether cellular glutathione levels might be depleted and, consequently, cellular Ca^{++} levels might be enhanced. Nor is it known whether Lewisite might affect DNA or chromatin.

In conclusion, one may speculate that a common cellular effect of heat and sulfur mustard is damage to the nucleus, comprising DNA, chromatin and nuclear matrix proteins. Beyond nuclear damage, destruction of cytoplasmic proteins will cause disturbance of numerous cell functions, amongst them inhibition of DNA and protein synthesis, damage to cellular membranes, and increase of intracellular Ca^{++} concentrations. It remains to be established to which extent the cellular toxic events are interrelated and in which way the attachment of basal cells to the basement membrane is eventually affected. Probably, none of these injuries by themselves will be the primary cause of vesication. Rather the concurrent impairment of many vital cell functions will result in a slowly progressing metabolic cell death and detachment of epidermis and dermis.

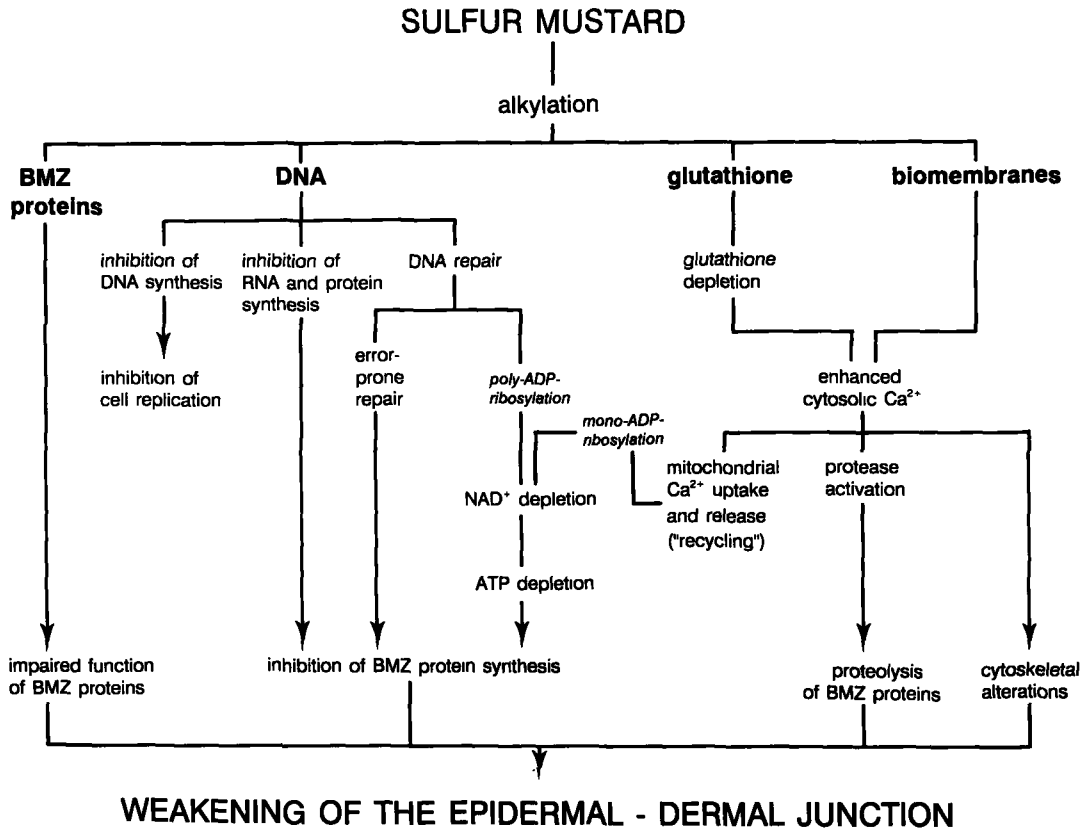

Figure 10.1. Hypothetical scheme of sulfur mustard-induced cytotoxicity leading to weakening of the epidermal-dermal junction and subsequent vesication. BMZ = basement membrane zone.

Summarizing, the following biochemical cascade causing separation of epidermal cells from the dermis is proposed. For clarity, a general scheme depicting reactions involved in sulfur mustard-induced cell injury has been presented in Figure 10.1. Due to alkylation by sulfur mustard, nuclear structures and cytoplasmic proteins of the basal cells may become extensively damaged. Free cytosolic Ca^{++} levels may become enhanced by different causes, probably by damage to the endoplasmic reticular and plasma membranes, or by glutathione depletion. Initially, the cytosolic Ca^{++} concentrations will be buffered by uptake into the mitochondria, but when Ca^{++} levels are extremely raised, mitochondria will "cycle" Ca^{++}, resulting in depletion of NAD^{+}, serious damage of the mitochondria and depletion of ATP. In addition to mitochondrial damage, enhanced cytosolic Ca^{++} concentrations result in cytoskeletal alterations and activation of proteases, which may have implications on the epidermal-dermal attachment structures. If one assumes that the firmness of the epidermal-dermal junction is the resultant of processes of synthesis and degradation of basement membrane zone proteins, an alternative cause for the loss of adherence of basal cells from the dermis can be given: the epidermal-dermal junction will be weakened by sustained inhibition of protein synthesis and energy depletion. Based on this hypothesis, future therapeutic measures might be directed against the elevation of free intracellular Ca^{++}, either by prevention of glutathione depletion or by loading cells with intracellular Ca^{++} chelators.

Finally, there is the intriguing question as to why human skin vesicates more easily than animal skin. The classic explanation of the unique blister response of human skin is that by the absence of hair in human skin the anchoring function of the hair follicles has been lost, leading to easy separation of epidermis and dermis. The observation that full blisters can be induced by heat on the hairy ears of mice, whereas only microvesicles developed upon sulfur mustard exposure of the rather hairless pig skin, may argue against this theory. An alternative explanation is the following. In the skin of pigs and hairless guinea pigs, minute vesicles can be observed microscopically between the epidermis and the dermis after exposure to sulfur mustard (Mitcheltree et al., 1989; Marlow et al., 1990). This demonstrates that in the skin of these animals weakening of the epidermal-dermal junction occurs in response to sulfur mustard exposure. Therefore, it can be concluded that the second step of blister formation, filling of the blister cavity with blister fluid, fails to occur in the skin of these animals, whereas in human skin filling of the blister occurs. The reason for this discrepancy may be that in human skin, contrary to animal skin,

an extensive microvasculature and numerous active eccrine sweat glands are present in the upper part of the dermis, which provide a potential source of blister fluid. The cavity between dermis and epidermis will be filled with edematous fluid from the dermis and with sweat from the sweat gland ducts that were torn when the basal cells detach from the basal membrane.

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11 SUMMARY

Sulfur mustard, bis(β-chloroethyl)sulfide, is a representative of the mustards, a group of alkylating agents containing at least one β-chloroethyl group attached to a sulfur or nitrogen atom. Its cytotoxic activity is mediated through covalent binding to important functional groups of cellular molecules, including nucleic acids, proteins and amino acids. Inhibition of DNA synthesis and cell replication were amongst the first known biochemical effects of sulfur mustard. Due to this observation sulfur mustard has played an important role in the first studies on genotoxicity and in the development of antitumor drugs. Despite ample investigations, its extremely high toxicity and vesicant properties, that had drawn attention since the first synthesis of the compound, still cannot be explained satisfactorily.

Sulfur mustard-induced vesication becomes manifest after a latent period of at least several hours. In essence, the formation of blisters on human skin can be divided into two separate processes. One process leads to the separation between the epidermis and the dermis, and the other leads to filling of the blister cavity with fluid from the dermis. The aim of the present study was to investigate the toxic effects of sulfur mustard on basal epidermal keratinocytes and their capacity to recover from this damage, as these cells probably play an important role in the first process. Since no animal species displays large fluid-filled blisters similar to that of sulfur mustard-exposed human skin, the phenomenon of vesication is preferentially investigated in human skin. Several *in vitro* models for human skin in dermatotoxic research are available. In the present study, cultured human epidermal keratinocytes and human skin pieces in organ culture have been used to investigate the mechanism of action of sulfur mustard and to evaluate a potential therapeutic agent against it.

DNA is considered to be one of the main cellular targets of sulfur mustard. Several years ago a hypothesis was put forward that linked sulfur mustard-induced DNA damage via inhibition of glycolysis to vesication. The hypothesis was based on the observations that a) for many DNA damaging agents repair processes are accompanied by activation of nuclear poly(ADP-ribose)polymerase, and b) for various vesicants, including sulfur mustard, a close relationship exists between inhibition of glycolysis and vesication. It was postulated that due to sulfur mustard-induced DNA damage, the nuclear enzyme poly(ADP-ribose)polymerase, which consumes NAD^+ as a substrate, was activated. Repair of severely damaged DNA

would exhaust the cell's NAD^+ supply, resulting in inhibition of glycolysis. A further cascade of biochemical events was proposed, eventually leading to vesication due to the release of proteolytic enzymes that hydrolyse proteins of the epidermal-dermal junction. The validity of this postulate has been partially examined.

Experiments with human skin in organ culture provided evidence that separation between epidermis and dermis occurs at doses of sulfur mustard that cause a decrease of NAD^+ levels. These data supported the afore-mentioned hypothesis that NAD^+ depletion is greatly involved in blister formation. If true, protection of cellular NAD^+ contents should prevent epidermal-dermal separation. Therefore, the efficacy was investigated of the potential therapeutic agent nicotinamide, which inhibits poly(ADP-ribose)polymerase. Although addition to the culture medium of nicotinamide prevented a severe drop of cellular NAD^+ levels, light microscopic preparations showed that microvesication still occurred. It was concluded that NAD^+ depletion resulting from activation of poly(ADP-ribose)polymerase during DNA repair, is probably only one of the mechanisms leading to sulfur mustard-induced vesication.

As a next step, it was investigated which other cell functions are affected upon sulfur mustard exposure. A dosimetry study for sulfur mustard-induced cytotoxicity was performed in cultured human epidermal keratinocytes. It appeared that cell replication and DNA synthesis were the most sensitive markers. As a bifunctional alkylating agent, sulfur mustard causes multiple types of DNA damage, among them DNA cross-links and monoalkylation products. Since it is generally assumed that formation of DNA interstrand cross-links is directly related to cytotoxicity, the formation and removal of DNA interstrand cross-links were measured. A linear relationship was observed between the number of DNA interstrand cross-links and the concentration of sulfur mustard used. The number of DNA interstrand cross-links was decreased following a post-exposure incubation for 24 hr as compared to those present immediately after exposure. However, a small number of residual DNA interstrand cross-links remained detectable, even following exposure to low sulfur mustard concentrations which induced only a small number of initial DNA interstrand cross-links. The lesions still present at 24 hr after treatment are likely to account for the persistent inhibition of cell replication and DNA synthesis.

Effects of sulfur mustard on protein synthesis and energy supply occurred at doses many times higher than those necessary for inhibition of cell replication. Effects on the cellular energy system were manifested by inhibition of glucose uptake, reduction of NAD^+ and ATP levels and decrease in the activity of the enzyme lactate dehydrogenase. It was striking that both the glucose uptake and the activity of

lactate dehydrogenase could only be reduced to maximally 50% of control values over a 24 hour period following exposure. This phenomenon could not be explained. No recovery of human epidermal keratinocytes from sulfur mustard-induced cell damage was observed within 24 hours following exposure. For a meaningful interpretation of the results from studies with cultured human epidermal keratinocytes it had to be assessed which concentrations used in the *in vitro* experiments were comparable to that causing vesication *in vivo*. Hence, based on the observation that epidermal-dermal separation in skin pieces in organ culture is coupled with NAD^+ depletion, it was estimated that exposure of cultured human epidermal keratinocytes to concentrations of at least 250 μM sulfur mustard correspond to doses leading to vesication in the intact skin.

The results of the dosimetry study indicated that upon exposure to concentrations of sulfur mustard higher than 50 μM DNA and protein synthesis are inhibited and energy supply is reduced. The importance of inhibition of protein synthesis being one of the key events involved in vesication has been discussed. If one assumes that the attachment between epidermis and dermis is the resultant of the synthesis and degradation of basement membrane zone proteins, prolonged inhibiton of protein synthesis may lead to detachment of epidermal cells from the dermis. However, it cannot be excluded that other vital cell functions, not included in this study, are impaired. For example, enhancement of intracellular free Ca^{++} concentrations may be involved in the toxic response of the cells on sulfur mustard and may have profound implications on vesication. Probably, none of these cellular injuries by themselves will be the primary cause of vesication. Rather the concurrent impairment of many vital cell functions will result in slowly progressing metabolic cell death and vesication, comparable to effects observed following heat exposure of cells. Based on the results of the experiments presented here, the hypothesis about the biochemical events that may occur in basal epidermal keratinocytes of the skin upon sulfur mustard exposure, eventually resulting in loss of adherence between epidermis and dermis has been modified. Future research will show whether the sulfur mustard-induced separation between epidermis and dermis mainly results from reduced synthesis of components of the basement membrane zone and from enhanced free intracellular Ca^{++} levels.

Apart from the study on the effects of sulfur mustard on the skin, a clinically important application of cultured human epidermal keratinocytes has been presented. The suitability of a human skin equivalent that provides a substitute for lost skin in the case of large, deep skin wounds, has been validated on patients with badly

healing venous leg ulcers. One of the advantages of this technique is that the area of the skin biopsy from which the culture was initiated was expanded about 40 times by culturing. The successful grafting of the skin equivalents on venous leg ulcers indicated that they may be of great value in patients with extensive deep skin defects such as third degree burns.

SAMENVATTING

Zwavelmosterd of bis(β-chloorethyl)sulfide is een alkylerend agens dat behoort tot een groep chemische stoffen die bekend staan als mosterdverbindingen. Deze groep stoffen wordt gekenmerkt door de aanwezigheid van tenminste één β-chloorethylgroep die gebonden is aan een zwavel- of stikstofatoom. In de cel bindt zwavelmosterd covalent aan belangrijke functionele groepen van moleculen zoals peptiden, eiwitten, RNA en DNA. Met name de binding aan DNA heeft gevolgen voor de DNA synthese en de daarmee samenhangende celdeling. Remming van deze processen werd dan ook als één van de eerste gevolgen van blootstelling aan zwavelmosterd beschreven. In het verleden is zwavelmosterd veelvuldig als modelstof gebruikt zowel voor onderzoek naar de mutagene en carcinogene werking van alkylerende agentia als voor de ontwikkeling van cytostatica. Desondanks is de hoge toxiciteit van de verbinding nog steeds niet begrepen en evenmin kan verklaard worden waarom de stof bij contact met de huid van de mens blaren vormt. Na een besmetting met zwavelmosterd duurt het enkele uren voordat de eerste blaren ontstaan. In principe kunnen bij blaarvorming twee aparte processen onderscheiden worden. Het ene proces leidt tot een verbreking van de hechting tusen epidermis en dermis en tijdens het tweede proces hoopt vocht vanuit de dermis zich op in de ontstane "holte".

Het onderzoek dat in dit proefschrift is beschreven had ten doel enig inzicht te verkrijgen in de toxische werking van zwavelmosterd op de basale cellen van de epidermis, die op de grens liggen met de dermis. Waarschijnlijk ligt in de aantasting door zwavelmosterd van deze cellen de oorzaak van de splitsing tussen epidermis en dermis. Bovendien werd de werkzaamheid van een mogelijk therapeuticum tegen blaarvorming getest. Aangezien bij dieren geen zichtbare blaren op de huid ontstaan zoals bij mensen, kan de blaarvorming niet in een proefdiermodel bestudeerd worden. Voor de hier beschreven experimenten werden twee *in vitro* modellen voor mensenhuid gebruikt, namelijk gekweekte basale epidermiscellen van de mens en kleine stukjes mensenhuid die enige tijd in een kweekmedium vitaal gehouden worden.

In het algemeen wordt aangenomen dat binding van zwavelmosterd aan DNA het belangrijkste effect is van blootstelling van weefsel aan zwavelmosterd. Enkele jaren geleden werd een hypothese gepubliceerd waarin wordt gesteld dat de door zwavelmosterd geïnduceerde beschadiging van DNA, via remming van de glycolyse

(een energieleverend proces in de cel) uiteindelijk leidt tot blaarvorming. De hypothese houdt in dat bij het herstel van de door zwavelmosterd veroorzaakte schade aan DNA het enzym poly(ADP-ribose)polymerase geactiveerd wordt, dat NAD^+ verbruikt als substraat. Daardoor neemt de concentratie NAD^+ in de cel af. Omdat NAD^+ een belangrijke co-factor is bij energieleverende processen zal bij een aanzienlijke DNA beschadiging de glycolyse geremd worden. Dit kan vervolgens leiden tot het vrijkomen van proteolytische enzymen die de verbinding tussen epidermis en dermis aantasten. De geldigheid van een aantal aspecten van deze hypothese is getoetst in dit proefschrift.

Na besmetting van stukjes mensenhuid met verschillende doses zwavelmosterd werd op lichtmicroscopisch niveau een splitsing van epidermis en dermis waargenomen bij die hoeveelheden zwavelmosterd die volgens literatuurgegevens op de intacte huid van de mens blaarvorming veroorzaken. Biochemische analyse van de huidstukjes liet zien dat na blootstelling aan deze doses zwavelmosterd het NAD^+ gehalte in de huid daalt. Dit resultaat steunde de bovenbeschreven hypothese dat NAD^+ daling een voorname rol in blaarvorming speelt. Een verdere bevestiging zou verkregen worden als door middel van het op peil houden van het NAD^+ gehalte een splitsing tussen epidermis en dermis voorkomen kon worden. Daartoe werd de werkzaamheid getest van een potentieel therapeuticum, nicotinamide. Deze stof remt het enzym poly(ADP-ribose)polymerase en voorkomt daarmee daling van het NAD^+ gehalte. Ondanks dat inderdaad een zekere bescherming van het NAD^+ gehalte werd bereikt door het toevoegen van nicotinamide aan het kweekmedium van besmette stukjes huid, werd in lichtmicroscopische preparaten toch nog een scheiding waargenomen tussen epidermis en dermis. Hieruit werd geconcludeerd dat daling van het NAD^+ gehalte door activering van poly(ADP-ribose)polymerase tijdens het herstel van DNA waarschijnlijk slechts één van de mechanismen is waardoor zwavelmosterd blaarvorming induceert.

Vervolgens werd onderzocht welke andere essentiële celfuncties aangetast worden door zwavelmosterd door een dosimetrie studie uit te voeren in gekweekte humane epidermiscellen. Celvermeerdering en DNA synthese bleken het meest gevoelig te zijn voor zwavelmosterd. De inactivering van DNA is het gevolg van het feit dat reactie van zwavelmosterd met DNA mono- en diadducten oplevert die de DNA replicatie verhinderen. In het algemeen wordt aangenomen dat de cytotoxiciteit van een alkylerende stof direct gerelateerd is aan de aanwezigheid van interstrand crosslinks (diadducten die een kruisverbinding vormen tussen de twee strengen van het DNA molecuul). Om die reden zijn de vorming en het herstel van interstrand

crosslinks door zwavelmosterd onderzocht. Er werd een lineair verband gevonden tussen de vorming van interstrand crosslinks en de gebruikte concentraties zwavelmosterd. Na een herstelperiode van 24 uur bleken niet alle interstrand crosslinks verwijderd te zijn, zelfs niet in cellen die aan lage concentraties zwavelmosterd waren blootgesteld. Deze persistentie is er waarschijnlijk de oorzaak van dat DNA synthese en celvermeerdering 24 uur na blootstelling nog geremd zijn. Blootstelling van gekweekte epidermiscellen aan zwavelmosterd leidt pas bij hogere concentraties tot verstoring van andere belangrijke celfuncties zoals de eiwitsynthese en de energievoorziening. Effecten op het laatstgenoemde systeem manifesteerden zich in daling van het NAD^+ en ATP gehalte, afname van het glucoseverbruik en vermindering van de activiteit van het enzym lactaatdehydrogenase. Opvallend was dat zowel het glucose verbruik als de activiteit van lactaatdehydrogenase niet meer dan 50% geremd konden worden, zelfs niet bij gebruik van hoge concentraties zwavelmosterd. Net zoals voor de DNA synthese en de celreplicatie werd 24 uur na expositie geen herstel van de eiwitsynthese en de energievoorziening waargenomen.
Voor een zinvolle interpretatie van deze experimenten is het van belang te weten welke concentraties in culturen van humane epidermiscellen vergelijkbaar zijn met doses zwavelmosterd die blaarvorming veroorzaken in intacte huid. Gebaseerd op de waarneming dat microblaarvorming in stukjes mensenhuid in orgaancultuur gepaard gaat met een daling in het NAD^+ gehalte is aangenomen dat blootstelling van gekweekte keratinocyten aan concentraties zwavelmosterd die eenzelfde NAD^+ daling veroorzaken, dit is vanaf 250 μM, overeenkomt met expositie aan blaarvormende doses *in vivo*. De resultaten van de dosimetrie studie tonen aan dat na blootstelling van humane epidermiscellen aan concentraties zwavelmosterd hoger dan 50 μM er geen synthese van DNA en eiwit plaatsvindt en dat de energievoorziening stagneert. Hieruit kan worden afgeleid dat behalve remming van het energiesysteem ook remming van eiwitsynthese betrokken kan zijn bij het blaarvormingsproces. Het belang van eiwitsyntheseremming bij blaarvorming houdt verband met het feit dat de hechting tussen epidermis en dermis berust op interacties tussen een aantal specifieke eiwitten en glycoproteinen, die in de basale epidermiscellen gesynthetiseerd worden. Als men de hechting beschouwt als de resultante van een voortdurend proces van aanmaak en afbraak zal een langdurige remming van eiwitsynthese zoals door zwavelmosterd wordt geïnduceerd leiden tot een verzwakking van de hechting.
Het kan niet worden uitgesloten dat verstoring van andere celfuncties, die niet in dit onderzoek zijn inbegrepen, bijdragen aan het blaarvormingsproces. Zo is het heel

goed mogelijk dat zwavelmosterd een verhoging van het gehalte aan vrije calcium ionen in de cellen veroorzaakt, wat gevolgen heeft voor de hechting tussen epidermis en dermis. Waarschijnlijk zullen geen van de hier genoemde factoren op zichzelf de primaire oorzaak zijn van blaarvorming door zwavelmosterd en zal blaarvorming voortkomen uit de gelijktijdige verstoring van een aantal vitale celfuncties. In dit opzicht kan er een parallel getrokken worden tussen blaarvorming door warmte en zwavelmosterd. Op basis van de resultaten van de hier beschreven experimenten is de hypothese omtrent het mechanisme van blaarvorming in de huid ten gevolge van zwavelmosterdblootstelling herzien. Toekomstig onderzoek zal moeten uitwijzen of geremde synthese van eiwitcomponenten uit de hechtingszone tussen epidermis en dermis en een verhoogd cellulair calciumgehalte de voornaamste factoren zijn in door zwavelmosterd geïnduceerde blaarvorming.

Dit proefschrift bevat ook een hoofdstuk waarin een andere toepassing van gekweekte humane epidermiscellen wordt beschreven dan voor onderzoek naar de werkingsmechanismen van zwavelmosterd, namelijk toepassing in de kliniek. Het is onderzocht of gekweekte huidcellen een waardevolle bijdrage kunnen leveren aan de behandeling van uitgebreide, diepe huidwonden. Bij een aantal patiënten met slechthelende veneuze ulcera (open benen) werd een huidsubstituut aangebracht bestaande uit gekweekte epidermiscellen van de patient zelf en een collageen-gel met fibroblasten. Voor deze methode hoeven bij de patient slechts kleine stukjes donorhuid te worden weggenomen aangezien door het kweken een 40-voudige vergroting van het oppervlak van de afgenomen huid bereikt wordt. Het goede resultaat dat met deze methode werd behaald, geeft aan dat toepassing ook van belang kan zijn bij de behandeling van uitgebreide derdegraads verbrandingen van het lichaam.

CURRICULUM VITAE

Marijke Mol werd geboren op 29 januari 1955 te Rijswijk. De middelbare schoolopleiding aan het Lodewijk Makeblijde College te Rijswijk werd in 1973 afgesloten met het behalen van het diploma Gymnasium-β. Aansluitend daarop begon zij met een studie biologie aan de Rijksuniversiteit van Leiden. Het kandidaatsexamen B4 werd afgelegd in 1976. Voor het doctoraalexamen, dat behaald werd in oktober 1981, werd onderzoek verricht bij de afdeling Gastro-Enterologie van het Academisch Ziekenhuis te Leiden, de vakgroep Farmacognosie van de farmaceutische faculteit van de Rijksuniversiteit Leiden, de vakgroep Theoretische Biologie van de subfaculteit Biologie van de Rijksuniversiteit Leiden en bij de vakgroep Humane Voeding van de Landbouwuniversiteit Wageningen. Tevens behaalde zij de onderwijsbevoegdheid Biologie.
Gedurende het studiejaar '81/'82 werkte zij als hoofdassistent bij het Organisch Chemisch praktikum voor 1e jaarsstudenten aan de Landbouwuniversiteit Wageningen. In september 1982 trad zij in dienst van het Medisch Biologisch Laboratorium TNO te Rijswijk en is sindsdien als wetenschappelijk medewerker verbonden aan de afdeling Farmacologie en Toxicologie (hoofd dr. O.L. Wolthuis). Het onderzoek dat in dit proefschrift is beschreven werd uitgevoerd in opdracht van het Ministerie van Defensie.