

## **From Phage to PARP and Beyond: My Life in Mustard Research**

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### ***INTRODUCTION***

Major General Schoomaker, Colonel Lukey, distinguished colleagues from here and abroad, and my many friends, it is nice to be back after 20 years. It is a wonderful 80th birthday present. I am delighted and honored to have been asked to deliver the 3rd Biennial Clarence A. Broomfield address. Clarence and I go back many years and have enjoyed a very cordial, productive, and friendly relationship. Clarence is a distinguished and dedicated scientist who has made outstanding contributions in many areas of our medical chemical defense program.

The title of my presentation is, “From Phage to PARP and Beyond: My Life in Mustard Research”. Before I try to hit some highlights of our contributions spanning more than four decades, I would like to tell you how I got started.

After receiving my doctorate in Biochemistry from Johns Hopkins University in 1954 in the midst of the Korean War, I was drafted by the US Army and obtained a commission as a First Lieutenant, in the Medical Service Corps (MSC) with a likely assignment as an assistant battalion surgeon. My wife and I decided to go to the Pentagon to see if there might be an opportunity to do some biochemical research. There was, indeed, one vacancy at the Biomedical Research Laboratory in Edgewood Arsenal, MD. I took that assignment and was sent off to Ft. Sam Houston in San Antonio, Texas for basic training. I was perfect at target shooting; hitting nothing but bullseyes – unfortunately they were my neighbors’ target, not mine. I learned a lot during my five weeks at Ft. Sam Houston. I was in the Army now.

When I returned to Baltimore, I put on my uniform and went to work in the Biomedical Research Laboratory, carpooling with some big shots – Dr. Bernie Jandorf, Joe. Epstein, Dr. Sol Hormats, and Bernie Gerber – to name a few. Our car pool had daily “seminars” on every subject imaginable, and I learned how research at Edgewood is conducted and who the players were. On occasion, I was Officer of the Day, supervising the raising and lowering of the flag and performing my other duties.

At the Biomedical Research Laboratory, I was assigned to the Clinical Investigations Branch of which Dr. Gustave Freeman was the Chief. There I met Marge Filbert, Rudy Johnson, John Clements, and Brennie Hackley. Dr. Freeman informed me that I was to work on the effects of mustard gas (HD) on proteins. I asked to have 2 to 3 weeks to ponder my assignment. I consulted with Professor Roger M. Herriott,

Chief of Biochemistry at the Johns Hopkins School of Hygiene and Public Health, who had published extensively on the effects of HD on enzymes. Dr. Herriott was very much intrigued by the ability of HD to inhibit cell division in E-coli and the very high sensitivity of pneumococcal-transforming DNA to HD. Dr. Herriott also observed that HD-sterilized E-coli, while rendered incapable of synthesizing their own DNA, were able to synthesize viral DNA following infection with T2 bacteriophage. These findings suggested that the template DNA was the HD-sensitive target rather than the host energy-producing system and/or other host proteins. The efficacy of nitrogen mustards in the treatment of various cancers supported the idea that mustard-induced DNA damage prevents cell division.

Dr. Freeman went long with my desire to investigate the effects of HD on DNA. This was, indeed, an exciting time to get involved in fundamental DNA research – especially because of the recently elucidated nature of the double helical structure of the genetic material by Watson and Crick and the involvement of greatest array of scientists of the era. The bifunctionality of HD and its potential to produce crosslinks provided fascinating possibilities.

The bacterial virus systems, especially, T1 and T2 bacteriophages and their host, E-coli were our initial choices to investigate the chemical, biological, and genetic effects of HD on DNA. When asked why I chose bacteriophage, I am reminded of a quote by A.D. Hershey who said "...bacteriophages are all these things, and probably more to be discovered. To ask which is the correct view to ask what is the proper function of a window: to admit light, to let in air, to keep out wind, to exclude rain, or to pique the peeping Tom" [Harvey Lectures, 1957].

I believe that the information I will present confirms Hershey's vision, providing compelling evidence that the HD-induced alkylation of DNA has far-reaching consequences, which may ultimately be responsible for initiating and explaining the known cell-and tissue-damaging actions of this vesicant.

*Note from Dr. Margaret G. Filbert:* " Ben you once told me that when you were asked why you use E Coli for your studies, you said because what is true for E. Coli is true for elephants, only more so."

## **Introductory Comments on HD Chemistry and Effects of the Cellular Level**

The reactions of HD in aqueous media occur as a two-step process: (1) formation of a positively charged cyclic sulfonium intermediate – the rate-limiting step; and (2) a rapid reaction of the sulfonium intermediate with negatively charged groups, the extent of alkylation being determined by the concentration and avidity of the particular group. The reactions scheme is shown in figure 1– reactions of mustard gas with cellular targets, water and detoxicants.

Since the bifunctional mustard has two reactive centers, it has the ability to crosslink target molecules; for example, the N7G-HD-N7G adduct, which covalently crosslinks the two strands of the double helical DNA molecule. Partially hydrolyzed

mustard produces the half-mustard, semi-HD, which also reacts with DNA producing monofunctional alkylations of guanine (N7G) and adenine (N3A) as well as a minor guanine alkylation product (R-O6G).

Although HD extensively alkylates nucleic acids, proteins, variety of metabolites, and other low molecular compounds, strong evidence implicates DNA as the most critical cellular target. DNA repair is characterized by the recognition and excision of the HD-induced crosslink, restoration of the native DNA structure, resumption of DNA replication, and cell division. E-coli mutant strains that are incapable of repairing HD-damaged DNA exhibit an irreversible inhibition of DNA replication and cell division and are more sensitive to HD than their repair-capable strains. The crosslink is, therefore, deemed the most lethal lesion produced by HD in dividing cells [Papirmeister, B., and Davison, C.L., 1964; Lawley, P.D., and Brookes, P., 1965; Kohn, K.W. et al., 1965].

Other strains of E-coli are unable to cope with apurinic sites, which are formed by release of unstable monofunctional DNA adducts, especially Na-alkyladenine (R-N3A). Endonuclease II-deficient strains are unable to make an incision at apurinic sites, and, therefore, cannot initiate excision-repair of this lesion. Such a variant is more sensitive to monofunctional sulfur mustards than is the wild type strain [Gilbert et al., 1974].

Mutagenesis by sulfur mustards is attributed to monofunctional alkylations. The minor alkylation product (R-O6G) is an especially powerful mutation producer by causing instant mispairing with cytosine and giving rise to GC to AT transitions during replication. R-O6G is however, susceptible to excision-repair. Other mutations result from error-prone repair of apurinic sites. (For a detailed discussion of mutagenesis by monofunctional sulfur mustard, consult Gilbert et al., 1974.)

A final point regarding figure 1 is that the reaction of HD with glutathione is designated as a potential mechanism for the detoxification of HD. Another view, however, might be that depletion of glutathione may actually be detrimental by removing a major defense against oxidative damage to cells and tissues. It is gratifying that this possibility is being actively investigated in more recent research. Perhaps, additional amplifying systems exist that could be exploited for improving our medical chemical defense against HD.

## **Effects of Mustard Gas on T2 Bacteriophage**

The mechanism of inhibition of T2 bacteriophage by HD involved a 6-year effort by a team of dedicated scientists and was described in great detail in a 200-page report, which was awarded First Prize at the CRDL-NDL Joint Science Conference in 1961 [Papirmeister, B., 1961].

Briefly, the T2 phage is a syringe-like viral structure consisting of a protein coat (the syringe) and its contents (mainly DNA). Following adsorption to a receptor on the surface of susceptible E-coli, the viral DNA is injected into the host cell while the proteinaceous syringe remains on the outside and can be sheared off in a Waring

blendor without interfering with viral reproduction. The injected DNA then utilizes the host energy system to initiate the synthesis of several new and specific enzymes required for the formation of phage DNA and phage proteins. After about 15 minutes, the host cell lyses and releases approximately 150 complete progeny phage particles into the medium, ready to infect more available hosts. When an agar-coated Petri disk seeded with susceptible bacteria is incubated with viable phages or phage-infected cells, each viable unit will give rise to a plaque. Counting plaques provides a rapid means for accurate quantitation.

Figure 2, summary of the effects of HD on properties of the T2 bacteriophage system, is a busy slide but an easy way to place the important effects on HD in a proper perspective. Detailed descriptions of methods and interpretations of each study can be found in reference by Papirmeister, 1961.

Analysis of T2 survival data following HD exposure leads to the following conclusions: Infectivity of T2 phage is reduced according to first-order kinetics indicating that at 37% survival, a specified reaction of HD with a single vulnerable locus (at lethal hit) suffices to inactivate the viability of the virus. Thus, the dose of HD is given either as a concentration of HD or as the number of phage lethal hits.

We have presumed that the lethal event is the formation of a DNA crosslink because single-armed sulfur mustards are about 50 to 100-fold less lethal to T2 phage than HD. Furthermore, the lethal event is due to an action on DNA rather than viral protein because intracellular phage is as sensitive to HD as is free phage. The most compelling evidence supporting crosslinking of DNA by HD as the lethal lesion is the identical dose-response for viability loss and the inhibition of viral DNA systems.

Protein-related activities of T2 phage are much less sensitive to HD than is infectivity. This includes the ability of phage protein ghosts to lyse host bacteria and the abilities of HD-treated phage to kill host bacteria, exclude other phages, inhibit bacterial RNA synthesis, and inject phage DNA. However, the ability of HD-treated phage to adsorb to host bacteria is not impaired.

The effect of HD on the synthesis of phage proteins (i.e., transcription/translation) is about 6 to 8 percent as sensitive for a phage-specific enzyme and a host range marker, respectively, as is viability. (The values shown in the graph were corrected for host bacterial capacity).

When singly infected cells are treated with HD, the intracellular phage DNA remained sensitive up to approximately 9 minutes and then, became more resistant to HD. At this time, multiple progeny DNA had been produced. The "recovery" is due to the recombination of functional DNA fragments derived from a number of inactive particles to reconstitute one or more viable phage genomes. Similarly, if a bacterial host cell is initially infected with several T2 phages (multicomplexes) and then HD-treated, the phage survival is greatly enhanced compared to that for HD-treated

monocomplexes, limited only by the bacterial capacity. The reconstitution process is called multiplicity reactivation.

The capacity of the bacterium to support T2 phage reproduction and to synthesize viral DNA is not especially sensitive to HD, thus showing that the agent does not seriously impair the enzymic machinery of the host. Furthermore, the result implies that the phage DNA is the HD-sensitive target in infected cells.

The bottom line of these investigations is that HD produces crosslinks in DNA that inhibit DNA synthesis. The inhibition of DNA synthesis by HD-treatment of the template was confirmed using purified E-coli polymerase in vitro. This is shown in the table of results that is figure 3. Figure 4 shows the sensitivity of extracellular T2 to various mustards, semi-mustards or monofunctional and polyfunctional mustards, i.e., those with various substitutions on each side of the molecule. The fraction of survivors in T2 phage system, a measure of the DNA dysfunction, was higher for the monofunctional adducts.

### **DNA Damage is Causally Related to HD-induced Skin Injury. Zeroing in on the Apurinic Site**

Although the DNA crosslinks could account for the damaging effects of HD on the rapidly dividing cells of the basal epidermal layer, we were unable to explain the ineffectiveness of semi-HD or CEES in the T2 phage system. After all, these monofunctional sulfur mustards had been shown to be potent vesicants [Renshaw, B. 1946]. We asked if vesication might also be due to an initial attack on DNA or to another mechanism. Several studies performed during the 1960s and 1970s shed some light on this problem by identifying the importance and relevance of unstable monofunctional adenine adducts in HD-damaged DNA and their subsequent conversion to apurinic lesions.

In one study, inhibitors of DNA repair in HD-treated E-coli – caffeine acriflavine, and proflavine – were able to intensify the severity of HD-induced lesions in exposed rabbit skin [Papirmeister, B., et al 1969]. The synergism of proflavine and HD is quite evident in figure 5, which shows that in the presence of the repair inhibitor, proflavine, skin injury caused by HD is greatly enhanced in a dose-dependent fashion. We obtained biochemical evidence for the presence of DNA repair and its suppression by proflavine from skin biopsies. We concluded that DNA damage is causally related to the vesicant action of HD.

In another study, while investigating the effects of some monofunctional alkylating agents on T1 bacteriophage, we made the surprising observation that when the DNA repair inhibitor, acriflavine, was included in the post-treatment plating medium, a monofunctional sulfur mustard was as lethal to the virus as a bifunctional mustard. This is shown in figure 6, the effects of mustards on survival of the intracellular T1 bacteriophage. The fact that no crosslinks were involved prompted us to search for the lethal event. Using <sup>35</sup>S-labeled semi-HD, we calculated that at 37% survival (i.e., one

lethal hit) the virus had sustained one adenine alkylation and six guanine alkylations at the N3 and N7 positions, respectively. We also noted that a monofunctional nitrogen mustard, which alkylates guanine but not adenine [Price et al., 1968], had no effect on the virus. We concluded, therefore, that the alkylated adenine was the potentially lethal lesion produced by the monofunctional sulfur mustard [Papirmeister et al, 1970; Dorsey et al, 1972].

Lawley et al reported that the hydrolysis of alkylated phage DNA could lead to inactivation, which could be attributed to the formation of apurinic lesions [Lawley et al., 1965]. We found, however, that the spontaneous rate of depurination was much too slow to account for the rapid loss of viability that was observed. It appeared reasonable to assume that an enzymatic process was responsible for the production of apurinic sites. This is precisely what we found. Treatment of DNA or synthetic adenine-containing polydeoxynucleotides with HD or monofunctional sulfur mustards sensitized these macromolecules to degradation by nucleases present in crude cellular extracts of E.coli (figure 7, degradation of alkylated [3H]-thymidine-labeled E.coli DNA by crude extracts of E.coli, figure 8, the sedimentation of [3H-T] poly[d(A-T)d(A-T)] on Alkaline sucrose gradients, and figure 9, the sedimentation of [32-P-G] poly[d(G).d(C)] on alkaline sucrose gradients. Using 35S-labeled semi-HD, we observed a rapid release of alkylated adenine adducts by the extract, whereas alkylated guanine adducts were not released. This is shown in figure 10, removal of alkylated purines from DNA treated with [35-S] – labeled semi HD by crude extracts of E.coli. Figure 10 also shows the slow spontaneous release rates of alkylated adenine and guanine in the absence of the extract, filled circles. The number of apurinic sites in DNA treated with chloroethyl ethyl sulfide (CEES, semi-HD) can be greatly increased by incubation for 48 hours at 37°C, which allows an enhanced non-enzymatic spontaneous release of both 3-alkyladenine adducts and the more prevalent N7-alkylguanine adduct. From figure 11, production of DNA breaks by treatment with half sulfur mustard, the alkylated/depurinated DNA has a much higher amount of acid soluble DNA than the alkylated DNA. The alkylated/depurinated DNA is a much better substrate than freshly-alkylated DNA when incubated with purified apurinic endonuclease from E-coli, endonuclease IV/exonuclease III.

The dose-response relationship for sensitization of HD alkylated/depurinated DNA to degradation by purified apurinic endonuclease VI/exonuclease III of E-coli is shown in figure 12. The extents of degradation were determined by incubating samples for 120 minutes, and the rate data were acquired after 30-minute incubations. The following results were noted: (a) Only double-stranded DNA was degraded; (b) The rate of degradation was linearly related to the dose of HD; (c) The extent of degradation was also dose-related, but never exceeded 40-45%. The remaining DNA was single-stranded and subject to further degradation by addition of **exonuclease I** and enzyme specific for hydrolyzing single-stranded DNA.

## Rate and Repair of the Apurinic Sites and Vesication

Since the loss of T1 bacteriophage viability by the monofunctional sulfur mustard occurred only when the DNA repair inhibitor was present, we hypothesized that the lethal lesion was readily repairable. Subsequent research by Lindahl identified three enzymes involved in producing apurinic sites and promoting repair of these lesions: The first enzyme, which is present in bacteria and a number of mammalian cells, is N3-alkyladenine glycosylase. This enzyme removes the alkylated purine from DNA and produces an apurinic site. The second enzyme is an endonuclease that is capable of making an incision at the apurinic site. The third enzyme is an exonuclease that removes neighboring nucleotides in a stepwise manner to produce a gap. The gap is then filled by DNA polymerase, which inserts the correct bases as dictated by the complementary strand. The excision-repair is then completed by DNA ligase that restores the intact DNA structure. [Lindahl, 1979]

Apurinic sites may be more difficult to repair if HD-induced inter-strand or intra-strand crosslinks are present. While inter-strand crosslinks can delay or prevent replication, depending on the efficiency of a cell's repair capability, the intra-strand crosslink may interface with the repair of apurinic sites. Faulty or delayed repair of either type would result in the accumulation of additional DNA breaks due to continuing formation of apurinic lesions, especially those from alkylated guanine. When a critical level of DNA breaks is reached, vesication results by depletion of NAD<sup>+</sup> levels. Such a scenario could explain the long latent period for blister formation in mustard exposed skin.

### **The Poly (ADP-ribose) polymerase (PARP) Hypothesis of Sulfur Mustard Cytotoxicity and Vesication.**

Our study with HD and semi-HD-treated T1 phage, DNA, and synthetic polydeoxynucleotides led to the following conclusions:

- (a) Most DNA breaks are not produced directly by the alkylating agent, but are the result of sensitization to enzymatic breakage by apurinic endonucleases.
- (b) Apurinic sites are formed primarily by enzyme-induced and/or spontaneous depurination of the major mono-adduct N3-alkyladenine and N7 alkylguanine;
- (c) DNA crosslinks, per se, make at best only a minor contribution to sensitization by apurinic endonucleases, but by retarding the repair of existing apurinic sites, these crosslinks might favor the formation of additional apurinic lesions as a consequence of spontaneous hydrolysis of the prevalent N7-alkylguanine adducts;
- (d) apurinic endonuclease quantitatively converts apurinic sites to DNA breaks, creating good substrates for further degradation by exonucleases.

The ability of monofunctional sulfur mustards to sensitize DNA to enzymatic breakage was of great interest, since these alkylating agents are also potent vesicants [Renshaw, 1946].

Editor's Note: Poly (ADP-ribose) polymerase (PARP) are proteins involved in a number of cellular processes involving mainly DNA repair and programmed cell death. A link to more information is <http://parplink.u-strasbg.fr>.

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The PARP hypothesis proposes a biochemical mechanism whereby HD-induced DNA damage serves as a primary cause of alterations of energy metabolism leading to the production of pathology. The hypothesis includes not only events associated with cytotoxicity, but also speculation about a larger series of later processes that might ultimately be responsible for the development of macroscopic skin lesions [Papirmeister, B. et al, 1985; Papirmeister, B. et al, 1983; Meier, H.L., 1984]. The proposed sequence of events leading to vesication is shown in figure 13. At vesicant doses of HD, pathogenesis is proposed to be initiated by rapid alkylation of DNA leading to enzymatic and/or spontaneous depurination, and in turn formation of a large number of apurinic sites, which are cleared by constitutive apurinic endonucleases to yield DNA breaks. Accumulation of DNA breaks is proposed to cause activation of the chromosomal enzymes, PARP, which uses nicotinamide adeninedinucleotide (NAD<sup>+</sup>) as a substrate to ADP-ribosylate a variety of nuclear proteins, resulting in severe reduction of cellular NAD<sup>+</sup> [Hayaishi, L. and Veda, K. 1982]. Depletion of NAD<sup>+</sup> results in the inhibition of glycolysis. Stimulation of the nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>) - dependent hexose monophosphate (HMS) shunt pathway follows as a result of an accumulation of glucose-6-phosphate, a common precursor for both glycolysis and the HMS. Stimulation of induction and secretion of proteinases (e.g., plasminogen activator), possibly as a result of enhanced HMS activity, is suggested. Proteolysis may lead to pathology characterized by basal cell necrosis, breakage of anchoring filaments of hemidesmosomes, an increase in the colloid osmotic pressure at the dermoepidermal junction, and accumulation of edema fluid within the cavity. This process culminates in the formation of subepidermal microvesicles and, following coalescence, production of the characteristics HD blister. Steps leading up to, and including inhibition of glycolysis, are the essential biochemical precursors of cell necrosis and are relevant to cytotoxicity occurring in a variety of tissues. The remaining steps constitute events that may lead to vesicle formation in skin.

### **Attempts To Validate The PARP Hypothesis Leading To Skin Injury**

We confirmed a major tenet of the PARP hypothesis – the depletion of NAD<sup>+</sup> in HD-exposed human skin. This could be attributed to the activation of PARP [Gross et al, 1985, Papirmeister, et al, 1985]. Figure 14 shows that HD treatment of human skin grafted to athymic nude mice caused a dose-dependent decrease in NAD<sup>+</sup> levels. At vesicant doses of HD, the low level of NAD<sup>+</sup> did not recover. That activation of PARP was responsible for the NAD<sup>+</sup> loss was shown by the prevention of NAD<sup>+</sup> loss by injecting mice with 3-methoxybenzamide, an inhibitor of PARP (figure 15). We next inquired whether PARP inhibitors would be effective at erythematous, vesicating, and necrotizing levels of HD. Figure 16 shows the results. Using <sup>14</sup>C-labeled HD on human skin grafts to determine the level of HD fixation, we found that pretreatment,

followed by a 90-minute post treatment of mice with 3-aminobenzamide, a powerful inhibitor of PARP, was effective in providing significant protection against subsequent HD-induced depression of NAD<sup>+</sup> in human skin at mild, moderate, and even severe injury levels. The amount on HD-fixed/cm<sup>2</sup> of human skin graft was correlated with the severity of skin damage previously described in man [Renshaw, 1946].

### **Attempts to validate the PARP hypothesis leading to Cytotoxicity**

Mixed human leukocytes were employed to extend the PARP hypothesis to the cellular level [Meier, et al, 1984a; Meier et al, 1984 b]. It was shown that exposure to HD caused dose- and time- dependent decreases in the NAD<sup>+</sup> content, which the PARP inhibitor, 3-methoxybenzamide, prevented. The maximum depression in mixed human leukocytes was only approximately 40%, whereas the maximum NAD<sup>+</sup> decrease in purified lymphocytes was greater than 90%. This finding is not surprising since lymphocytes, which comprise about 40% of the mixed leukocyte population, are the only cells known to contain PARP, whereas terminally differentiated granulocytes do not contain PARP [Hayaishi and Ueda, 1982]. Additional support for the PARP hypothesis is provided by the observation that granulocytes are also insensitive to the cytotoxic effects of HD.

Cultured keratinocytes were also used to ascertain the validity of the PARP hypothesis in HD cytotoxicity [Mol et al, 1989]. In these studies, nicotinamide, an inhibitor of PARP, prevented the loss of NAD<sup>+</sup> at 4 hours, but had no effect on either the loss of viability or the development of cytopathology measured at 24 hours. These investigators concluded that PARP-dependent NAD<sup>+</sup> loss is not a critical event in the toxic mechanism in keratinocytes. In possible contradiction, Smith et al, [Smith,1990] reported that PARP inhibitors provided complete protection against HD-induced cell death at 24 hours, though these cultures subsequently failed to expand and the majority of cells apparently died by 72 hours.

Martens examined the mechanism of metabolic injury sustained due to HD exposure of human epidermal keratinocytes. [Martens, 1996] She observed that depletion of NAD<sup>+</sup> and inhibition of glucose metabolism are dose related, dependent on post-exposure time, and precede the loss of viability, as measured by dye exclusion (i.e. membrane integrity). Levels of NADP<sup>+</sup>, which is not a substrate for PARP, is unaffected by HD exposure. Thus, in agreement with the PARP hypothesis, NAD<sup>+</sup> depletion and inhibition of glycolysis play important roles in the subsequent development of HD-induced pathology. However, at higher doses of HD and longer post treatment times, she observed a shift in metabolism leading to an increased production of lactate, which is unrelated to NAD<sup>+</sup> depletion and, therefore, deviates from the PARP hypothesis as originally proposed. Increased lactate production, which could provide energy in the form of ATP, could be derived from amino acids and/or Krebs cycle intermediates or by the degradation of poly ADP-ribosylated nuclear proteins by poly (ADP-ribose) glycohydrolase and ADP-ribose pyrophosphorylase [Papirmeister et al, 1991]. The discrepancies noted above make it clear that the precise sequences leading to HD-induced metabolic injury are not yet fully understood and may need to be revised as more information becomes available.

## **Attempts To Validate Terminal Sequences Of The PARP Hypothesis And The Use Of Human Skin Grafts On Athymic Nude Mice To Investigate HD-Induced Pathology.**

The PARP hypothesis also proposed terminal sequences leading to HD-induced pathology, including the induction of proteases (e.g., plasminogen activator) by increased activity of the HMS shunt [Schnyder and Baggiolini, 1980], the formation of plasmin by interaction of plasminogen activator with plasminogen (which is concentrated at the epidermal-dermal junction) [Isseroff and Rivkin, 1983], and the formation of vesication by proteolytic breakage of anchoring filaments and accumulation of edema fluid in the lamina lucida. A similar mechanism had been implicated in bullous skin diseases such as bullous pemphigoid [Hashimoto et al, 1983]. A detailed discussion of the involvement of proteases and secondary inflammatory responses in HD-induced epithelial lesions can be found in Papirmeister, et al, 1991, Chapter 8.

To study the pathogenesis of the cutaneous HD injury we employed a new animal model for human skin - - human skin grafts in congenitally athymic nude mice. Injury produced in this skin model was similar to that reported in humans, including a latent period prior to formation of microblisters [Papirmeister, et al, 1984 a, b]. Consistent with the PARP hypothesis, light microscopic and ultrastructural evaluation showed that the earliest visible morphological changes appeared in the nucleus of the cells of the basal layer of the epidermis. Nuclear pathology was followed by cytoplasmic injury – swelling of the smooth and rough endoplasmic reticulum, formation of debris filled cytoplasmic vacuoles, loss of rosettes of polysomes, breakage of the plasma membrane which abuts the basal lamina, extrusion of intracellular debris into the lamina lucida, and loss of mitochondrial integrity. All of these necrotic changes in basal keratinocytes precede microblister formation.

Typical light microscopic changes 12 hours after exposure of a human facial skin graft to a vesicating dose of HD ( $635 \text{ ug/cm}^2$ ) are shown in figure 17A. Note the focal appearance of pyknotic nuclei in the basal cell layer. Figure 17B shows ultrastructural features of a basal keratinocyte undergoing pyknosis 24 hours after exposure of a neonatal foreskin graft to a subvesicating dose of HD ( $60 \text{ mg/cm}^2$ ). At this dose, basal cells with pyknotic nuclei coexist with neighboring basal cells containing normal appearing nuclei. The pyknotic nucleus was characterized by a preferential decrease of euchromatin (containing active DNA) and a condensation of heterochromatin (containing inactive DNA). Although the number of pyknotic basal cells increases with both dose of HD and post exposure time, nuclear pathology always preceded the appearance of cytoplasmic injury and microblister formation. These microblisters result from the separation of the graft epidermis from the basement membrane just above the epidermal-dermal junction beginning at 12 to 24 hours following exposure with formation of extra cellular vacuoles. These vacuoles become more numerous with time, fill with fluid and debris, coalesce, and cause widening of the cleft. Figure 18 depicts a full-fledged subepidermal blister 48 hours after exposure of a human facial skin graft to a vesicating dose of HD. The roof of the blister consists of necrotic basal cells, while the

base of the blister contains the basement membrane, which overlaid the slightly edematous, but otherwise intact, dermis.

Figure 18B shows the ultrastructural features of a typical microblister 48 hours after exposure to a vesicating dose of HD. An intact basal lamina formed the base of the blister cavity which contained debris and some degenerating cellular organelles which appeared to have been extruded through the highly damaged basal cell plasma membrane. Intact hemidesmosomes and remnants of the plasma membrane formed the roof of the blister. Anchoring filaments, which were attached to hemidesmosomes, were seen dangling into the cavity. Tonofilaments and anchoring fibrils appeared relatively undamaged.

The pathology results strongly support the PARP hypothesis by showing that skin injury by HD is characterized by early nuclear damage in proliferating keratinocytes in the basal layer of the epidermis, progressing to basal necrosis and vesication. Such early nuclear damage is also consistent with the data demonstrating the ability of HD to sensitize DNA to enzymatic breakage.

The role that apoptosis (programmed cell death) might play in sulfur mustard-induced injury was addressed in great detail in an article by Papirmeister in the Medical Chemical Defense Bulletin, Vol. 7, No. 1, 1-13, Sept., 1994 [Papirmeister 1994]. For this presentation, I will confine my remarks to what might happen to the apoptotic process when PARP inhibitors are added to HD-treated epidermal keratinocytes. Figure 19 presents a hypothetical model of the acute cutaneous sulfur mustard injury showing the relationships between the production of DNA damage, loss of cellular energy, blockage of the cell cycle, induction of apoptotic and/or necrotic cell death processes, and leakage of degradative enzymes. Note that the promotion apoptotic cell death sequences by PARP inhibitors delays the occurrence of necrotic cell death, reduces leakage of tissue destroying enzymes and attenuates the severity of the injury. It is also noted that erythemagenic doses of HD, which do not cause depletion of the energy supply, do not result in vesication.

Studies by Martens, Meier, Smith and Yourick, which were reported at the 1991 Medical Chemical Defense Bioscience Review, indicated that the inhibition of PARP following HD exposure appears to be more important for maintaining ATP levels, cellular viability, and prevention of tissue injury than the maintenance of NAD<sup>+</sup> levels. This deviation from the original PARP hypothesis was addressed in two publications by Papirmeister in the Medical Chemical Defense Bulletin, Current Controversies on the Poly(ADP-ribose) Polymerase Hypotheses for Sulfur Mustard-Induced Cytotoxicity, Vol. 4, No 2., 1-6, Aug 1991 and the PADPRP Hypothesis Reconsidered Vol. 4, No.2, 7-8, Aug 1991.

Figure 20 presents a modified PARP-dependent scheme for HD cytotoxicity. Two pathways are proposed: the first a cytoplasmic ATP-consuming turnover cycle that is stimulated by products of the PARP-mediated turnover cycle, which is stimulated by products of the PARP-mediated degradation of NAD<sup>+</sup> --nicotinamide and poly(ADP-

ribose); and, the second, a nuclear ATP-generating system. The nuclear ATP, which is NAD<sup>+</sup>-independent, is generated by the enzymatic breakdown of the poly (ADP-ribose) portion of poly (ADP-ribosylated) proteins by two nuclear enzymes — poly (ADP-ribose) glycohydrolase and ADP-ribose pyrophosphorylase. It is to be noted that nuclear ATP production and the operation of nuclear energy-requiring processes, such as DNA repair and enzyme induction, can be sustained for some time even in the absence of extranuclear ATP. Cell death caused by the cytoplasmic NAD<sup>+</sup> -turnover cycle could result from energy depletion, whereas cell death caused by nuclear ATP generation could result from the induction and functioning of degradative hydrolases. It is notable that many of these degradative nuclear enzymes, such as protease and phospholipase, are highly ADP-ribosylated, as is the Ca<sup>2+</sup> /Mg<sup>2+</sup> – dependent endonuclease which is causally involved in apoptosis, a programmed cell death process that bears similarities to HD-induced cell death.

## Conclusions

It has been known since the 1940s that the severity of the skin injury was directly related to the amount of HD fixed in the tissue. Careful examination of the data presented here reveals that vesication and acute tissue injury occur only at fixation levels much higher than those needed to produce genotoxic effects. Doses that injure tissues are, however, consistent with levels of alkylation that would cause metabolic disturbances (e.g., inhibition of glycolysis). Furthermore, cross-alkylation is not required to produce acute injury since, at comparable levels of alkylation, monofunctional sulfur mustards have been shown to equally effective as vesicants.

These considerations, in conjunction with new awareness of emerging concepts of cell death processes, have led investigators to propose three hypotheses for the mechanism of HD injury which are discussed in Papirmeister et al [Papirmeister, 1991]. The PARP hypothesis presented today, proposes DNA as the initial target and an injury-producing pathway whereby DNA damage leads to metabolic disturbances and cell death. The thiol-Ca<sup>2+</sup> and lipid-peroxidation hypothesis propose depletion of glutathione as the initiating event, followed by the generation of endogenous oxidants. According to the thiol-Ca<sup>2+</sup> hypothesis, oxidative stress causes a loss of protein thiols, deregulation of Ca<sup>2+</sup> homeostasis, activation of autolytic enzymes, and cell death. According to the lipid peroxidation hypothesis, oxidative stress is followed by irreversible damage to cellular membranes. There may exist additional HD-induced injury mechanisms or interactions of several injury-producing pathways that merit attention.

One parting thought regarding the potential usefulness of the PARP hypothesis for generating either preventive and/or therapeutic measures against HD injury is in order. Does the PARP-mediated NAD<sup>+</sup> loss represent part of a suicide pathway to rid the organism of cells with highly damaged DNA or does it initiate an SOS pathway wherein metabolism is slowed down in order to prevent unbalance growth, promote more efficient repair, and bring about eventful recovery? Martens results [Martens 1996] favors the SOS pathway notion by her observation that at higher doses HD-treated human keratinocytes eventually shift their metabolic pattern away from NAD<sup>+</sup> dependence which provides energy by glycolysis to a lactate-mediated production of

ATP. On the other hand, the suicide pathway is favored by the finding that a great number of sulfur mustard induced mutations are caused by error-prone repair of apurinic sites in DNA [Gilbert et al, 1975; Capizzi et al, 1974]. Future research should be directed to a resolution of this significant discrepancy.

## **Addendum**

After 31 years at Edgewood, I left the laboratory, relaxed for a few months and then joined SAIC in Joppatowne on a part-time basis. For health reasons, Elly and I moved to Florida in 1996 and I continued to work for SAIC until my retirement in 2000. I figured that 45 years was enough. At SAIC, I started the Medical Chemical Defense Bulletin, which contained original articles of my choosing, reviews of extramural contracts, and open literature items of interest to the medical chemical defense community.

### **Publications during my years with SAIC included the following HD-related items:**

- Book by Bruno Papirmeister, Alan J. Feister, Sabrina I. Robinson, and Robert D. Ford, "Medical Defense Against Mustard Gas. Toxic Mechanisms and Pharmacological Implications" 1991. CRC Press, Boca Raton, FL
- Bruno Papirmeister, keynote address on vesicants, "Excitement in Vesicant Research – Yesterday, Today, and Tomorrow." 1993 Medical Defense Bioscience Review Proceedings, Vol 1, pp 1-14

### **Medical Chemical Defense Bulletin Articles:**

- Toxic Chemicals and Cell Death, Vol 2, No 1-2, pp 1-4 may 1988
- Scavengers: A Promising Approach to In Vivo Detoxification of Sulfur Mustard, Vol 4, No 1, pp 1-8, April 1991.
- Current Controversies on the Poly (ADP-Ribose) polymerase Hypothesis for Sulfur Mustard – Induced Cytotoxicity, Vol 4, No 2, pp 1-6, August 1991.
- The PADRP Hypothesis Reconsidered, Vol 4, No 2, pp 7-8, August 1991
- A Global Picture of Battlefield Vesicants: Part 1 A Comparison of Properties and Effects, Vol 5, No 1, pp 1-12, October 1992
- A Global Picture of Battlefield Vesicants: Part 2. Rationale for a Common Therapy, Vol 6, No 1, pp 1-10, May 1993
- Does Apoptosis (Programmed Cell Death) Play a Role in sulfur Mustard Injury? Vol 7, No 1, pp 1-11, September 1994

- Mustard gas (H) Blisters: A View from the Basement (Membrane), Vol 8, No 1, pp 1-12, February 1996.

## Acknowledgements and Final Thoughts

Appended is a list of people who participated in the research I presented. I could not have done without each one of you. If I left anyone out, I apologize. It was not intentional but an admission that I am now an octogenarian and my memory is the same age. I am proud that I was influential in getting some of you to take graduate courses and grooming you for a rewarding career in science and medicine. In order not to slight anyone, I will not mention individuals by name. I know that I have taken you for a ride and there may still be detours ahead before we reach the final destination. One of the detours may be skin replacement by use of epidermal stem cells, which has shown promise in treating thermal burns. Whatever it takes. I just hope that I helped you get on the right track. Keep me informed, and thanks for the invitation.

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