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2,2′-DICHLORODIETHYL SULFIDE (SULFUR MUSTARD) DECREASES NAD^+ LEVELS IN HUMAN LEUKOCYTES

(Sulfur mustard; 2,2′-dichloroethyl sulfide; NAD^+; leukocytes, human)

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SUMMARY

2,2′-Dichlorodiethyl sulfide (sulfur mustard, HD) extensively alkylates DNA in a concentration-dependent manner in many cell types. We have proposed a biochemical hypothesis that explains HD-induced injury by linking DNA alkylation and DNA breaks with activation of poly(ADP-ribose) polymerase, resulting in depletion of cellular NAD^+. This hypothesis was tested by treating human leukocytes with HD to determine whether NAD^+ depletion occurred as predicted. These cells demonstrated a decrease in NAD^+ levels which was dependent on both concentration of HD and time after exposure. Inhibitors of poly(ADP-ribose) polymerase or substrates for NAD^+ synthesis were able to prevent the HD-induced NAD^+ decrease.

INTRODUCTION

2,2′-Dichlorodiethyl sulfide (HD) is a radiomimetic alkylating agent that has mutagenic [1–5], carcinogenic [3,4,6–10], and cytotoxic [3,4,11] properties. HD is also a powerful vesicant that produces incapacitating injuries at the site of exposure [12–18]. In spite of 60 years of intensive research, the mechanism of HD-induced injury is still not understood and no effective treatment for preventing HD-induced lesions is available.

We have proposed a new and unique biochemical hypothesis — a mechanism that

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Abbreviations: HD, 2,2′-dichlorodiethyl sulfide; LDH, lactate dehydrogenase; 3-MB, 3-methoxybenzamide.

links DNA damage, activation of poly(ADP-ribose) polymerase, alterations of metabolism, and protease release [19]. In order to validate the portions of this biochemical hypothesis linking HD exposure to a decrease in NAD^+ levels, the effect of HD on the NAD^+ content of mixed human leukocytes was studied.

Circulating human leukocytes appear to be an appropriate model because these cells are affected directly in acute systemic HD poisoning and indirectly by the inhibitory effects on HD on hematopoiesis [13,14]. The leukocytes allow a study of the effects of HD on several diverse cell types such as lymphocytes, which are capable of undergoing replication and contain poly(ADP-ribose) polymerase [20], and granulocytes, which are capable of secretion but do not contain poly(ADP-ribose) polymerase [20]. Granulocytes also represent a terminal stage of differentiation and are known to participate in inflammatory responses. Since individual cell types can be isolated from the mixture, a detailed biochemical study on any desired parameter would be feasible.

In this paper, we demonstrate that HD causes a concentration- and time-dependent decrease in the NAD^+ content of exposed human leukocytes. Inhibitors of poly(ADP-ribose) polymerase and precursors of NAD^+ synthesis appear to prevent this HD-induced NAD^+ loss. These studies are consistent with the initial steps of the hypothesis.

METHODS

Materials

The following reagents were purchased: Tris base, perchloric acid, and reagents for making routine buffers (Fisher Scientific Co., Pittsburgh, PA); NAD^+, NADH, MTT tetrazolium, phenazine ethosulfate, alcohol dehydrogenase, and iodoacetate (Sigma Chemical Co., St. Louis, MO). A Corning pH meter, a Yellowsprings conductivity meter, and a Beckman DU-7 spectrophotometer were used to determine the pH, conductivity, and optical density, respectively.

HD was obtained through the Chemical Research and Development Engineering Center, APG, MD and was assayed as > 99% purity.

Isolation of human leukocytes

100 ml of blood from normal human volunteers was drawn and sedimented in 3 tubes with 12 ml of a dextran-EDTA mixture containing dextrose [21]. After 1 h of sedimentation, the plasma layer (containing the leukocytes) was removed and centrifuged at 900 × *g* for 10 min. The supernatant was decanted, red cells in the cell pellet were lysed with distilled water, and leukocytes were washed with calcium-free and magnesium-free Tyrode's buffer. The lysing and washing steps were repeated before cells were resuspended in the appropriate volume of Tyrode's buffer, dispensed into 1.5 ml Eppendorf tubes, and placed on ice.

Exposure of human leukocytes, lymphocytes, and/or granulocytes to HD

HD (freshly prepared in ice-cold Tyrode's buffer to decrease hydrolysis [11]), was

added directly to the tubes containing cells at 0°C. HD was handled in a hood under the conditions specified for hazardous materials in USAMRICD regulations covering safety and surety of operating procedures. The samples were then transferred to a 37°C bath and incubation was continued for 4 h unless specified otherwise. After incubation, cells were centrifuged in an Eppendorf microfuge at 14 000 × *g* for 2 min. The cell pellets were extracted with 0.5 M $HClO_4$ at 4°C overnight, neutralized, and their NAD^+ levels were determined by an enzymatic cycling assay [22].

Effects of drugs on the NAD^+ content of HD-treated human leukocytes

The cells were preincubated with the drug of interest for 10 min at 37°C prior to the HD exposure. In kinetic experiments, all cells were preincubated for 60 min at 37°C before HD addition. Niacinamide was added at the indicated times. After incubation, cells were extracted and assayed for their NAD^+ content.

NAD^+ extraction from leukocytes

1 ml of leukocytes was extracted overnight at 4°C with 0.1 ml of 5.5 M $HClO_4$. The extraction mixture was neutralized by addition of 0.275 ml of 2 M potassium hydroxide-0.66 M potassium phosphate buffer, pH 7.5 and then vortexed before placing on ice for 30 min. The samples were centrifuged at 14 000 × *g* for 1 min to remove insoluble $KClO_4$ and two 0.35 ml aliquots of supernatant were removed for NAD^+ determinations.

NADH extraction from leukocytes

1 ml of leukocytes was extracted overnight at 4°C with 0.1 ml of 2.5 M sodium hydroxide. The mixture was incubated at 60°C for 5 min and then neutralized to a pH between 7.5 and 8.5 by adding 0.5 ml of 0.37 M phosphoric acid. The NADH in the mixture was then oxidized to NAD^+ by adding 0.08 ml of 2 mM phenazine ethosulfate in the dark for 15 min [22].

NAD^+ determinations

The NAD^+ was determined using an enzymatic cycling assay [22]. This assay employs a cycling of NAD^+ to NADH and back to NAD^+ by alcohol dehydrogenase and phenazine ethosulfate. The release of electrons during this process results in the reduction of MTT tetrazolium to the blue formazan, whose absorbance is determined on a Beckman DU-7 spectrophotometer and compared to the color produced by known amounts of NAD^+.

Recovery of cofactor (NAD^+ and NADH)

NADH extracts from human leukocytes were assayed alone or with addition of known amounts of NADH for their total NADH content. The samples were oxidized to form NAD^+ and then assayed by the enzymatic cycling assay. NAD^+ extracts from human leukocytes were assayed alone or with the addition of known amounts of NAD^+ to determine the total NAD^+ content of the samples. The cofactor con-

tent of the cells was subtracted from the spiked samples and the difference was divided by the amount of the added cofactor and multiplied by 100 to obtain the percent recovery of NADH and NAD^+ respectively. It was found that the NADH recovery by this process was greater than 98% as NAD^+ and the NAD^+ level in spiked cell preparations was 85 + 3.8% over the linear range of the assay (0–100 pg/ml).

Histamine content

After incubation, cells were centrifuged at 14 000 × *g* for 1 min, and 0.8 ml of the supernatant was added to 0.2 ml of 10% perchloric acid. The histamine content of these samples was determined by the automated fluorometric method [23] in Dr. Lichtenstein's laboratory (Johns Hopkins School of Medicine, Baltimore, MD).

Lactate dehydrogenase (LDH) determinations

LDH determinations were performed by use of the Sigma reagent kit [24,25].

Isolation of human mononuclear lymphocyte populations and polymorphonuclear granulocyte populations

100 ml of diluted blood (10 ml blood/25 ml saline) was centrifuged at 400 × *g* on a Percoll (Pharmacia Fine Chemical, Piscataway, NJ) cushion (1 ml cushion/1 ml blood; density = 1.080 g/ml) for 30 min at 20°C. The interface containing the mononuclear cells (about 90% lymphocytes) was removed and washed twice with Tyrode's buffer before the cells were resuspended and added to the incubation mixture containing various concentrations of mustard. The red cell pellet under the Percoll cushion was lysed twice with cold distilled water for 30 s. The resulting polymorphonuclear leukocytes (99% granulocytes) were washed twice in Tyrode's buffer before they were added to reaction mixtures [26].

RESULTS

Mustard treatment of mixed leukocytes

Leukocytes from 7 Caucasian volunteers (6 females and 1 male) were isolated and exposed to 10^{-3} M HD in Tyrode's buffer at 37°C for 4 h. The NAD^+ levels of HD-exposed cells fell to 66.0 + 4.8% of control. To determine intraperson variability, leukocytes from 2 volunteers were isolated on 2 separate occasions and exposed to 10^{-3} M HD. They demonstrated a consistent drop in leukocyte NAD^+ levels (64.0 + 4.8% of control in volunteer 1 and 64.8 + 3.9% in volunteer 2) although they had different baseline levels.

Concentration-dependent decrease in NAD^+

Fig. 1 shows the concentration-dependent decrease in NAD^+ levels in HD-treated cells. NAD^+ levels began to decrease significantly at concentrations greater than 10^{-5} M HD and, at 10^{-3} M HD, dropped to 66% of control and remained at this level at higher concentrations of HD. The decrease in NAD^+ could not be explained by reduction of NAD^+ to NADH since the levels of NADH also decreased (approximately 25% decrease) over the same range, as shown in Fig. 2. The possibility that

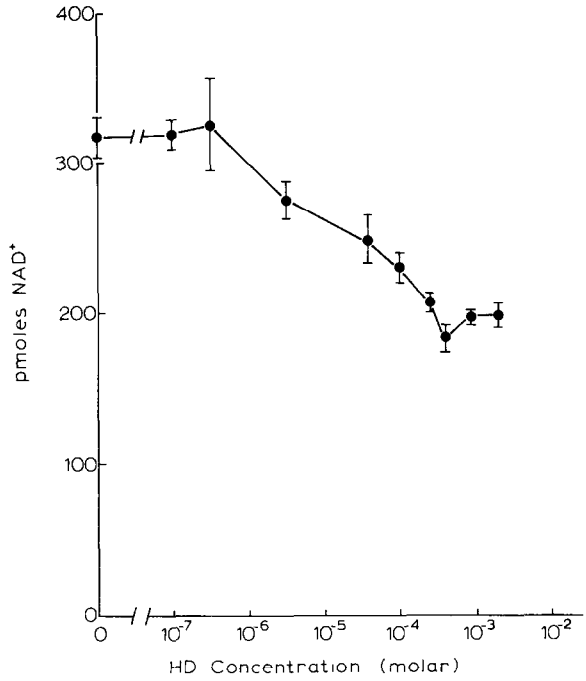

Fig. 1. The HD concentration-dependent decrease in the level of NAD^+ in human leukocytes. Each point is the average of 3 tubes whose NAD^+ levels were measured as pmol/sample and each sample contained the equivalent of leukocytes from 2 ml of dextran-sedimented blood. The NAD^+ level of each tube was determined in duplicate.

the drop in total NAD^+ could be the result of cell lysis was rendered unlikely by an inability to demonstrate the presence of measurable levels of lactic dehydrogenase (a cytoplasmic enzyme), NAD^+ (mainly a nuclear constituent), and histamine (a granular component of human basophils) in the cell-free supernatant (data not shown).

The time course of the drop in NAD^+ levels in HD-exposed cells

Leukocytes were exposed to 10^{-3} M HD and incubated at 37°C for various times (0, 15, 30, 60, 120, and 240 min) before the cells were centrifuged, decanted, acidified, and assayed for NAD^+. The level of NAD^+ in control leukocytes steadily increased during 4 h of incubation, indicating that there was a sufficiently large pool of substrates for continued NAD^+ synthesis. However, cells exposed to 10^{-3} M HD were able to maintain the increase in NAD^+ levels only during the first hour (Fig. 3). During the ensuing 3 h of incubation, these HD-exposed leukocytes demonstrated a consumption of NAD^+ when compared to the NAD^+ content of control leukocytes (Fig. 3). Since the fall in the NAD^+ levels does not occur immediately after exposure, there may be sufficient time to prevent NAD^+ depletion by therapeutic intervention.

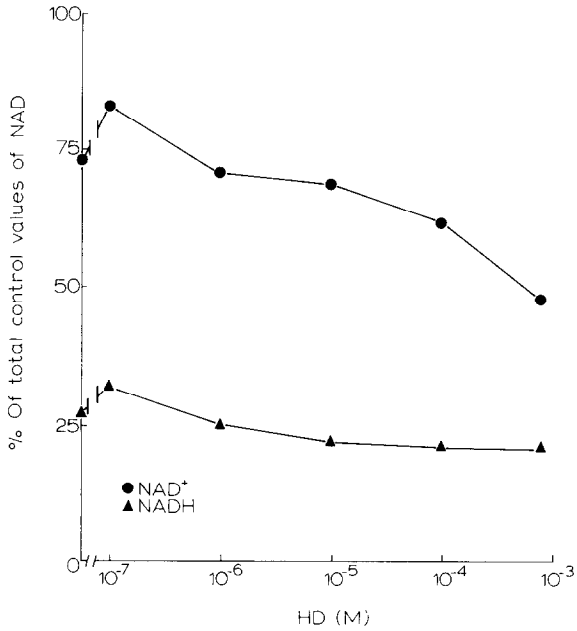

Fig. 2. The HD concentration-dependent decrease in the levels of both NAD^+ and NADH in human leukocytes. Each point is the average of 3 tubes whose NAD^+ levels were measured in duplicate. The NAD^+ and NADH levels are reported as a percentage of the sum of NAD^+ and NADH in unexposed control cells.

The effect of 3-methoxybenzamide on the NAD^+ levels in leukocytes

To study whether the drop in NAD^+ levels of leukocytes exposed to HD could be prevented by inhibiting poly(ADP-ribose) polymerase, leukocytes from normal individuals were preincubated in buffer for 10 min with 3-methoxybenzamide (3-MB), an inhibitor of poly(ADP-ribose) polymerase [25] but not a substrate for NAD^+ synthesis, before exposure to 10^{-3} M HD (Fig. 4). The maximum concentration of 3-MB used was 10^{-4} M because of its limited aqueous solubility. At this concentration of 3-methoxybenzamide, the level of NAD^+ in the HD-exposed leukocytes fell only 10% compared to the 35% drop in NAD^+ observed in the absence of 3-MB. Lower concentrations of 3-methoxybenzamide failed to offer any protection.

The effect of niacinamide on the decrease in NAD^+ levels induced by HD

Niacinamide, an inhibitor of poly(ADP-ribose) polymerase and a precursor of NAD^+ synthesis, was chosen to study its effect on NAD^+ levels in HD-treated leukocytes. Leukocytes were incubated in various combinations of buffer, HD, and niacinamide. Niacinamide added 10 min before HD treatment both increased the 4-h level of NAD^+ in the control cells and blocked the HD-induced fall of NAD^+ levels (Fig. 5). Maximal effects were observed at 10^{-4} M niacinamide, which caused a 196% increase in the amount of NAD^+ compared to HD-treated cells incubated in the absence of niacinamide. Niacinamide caused a 'superinduction' of NAD^+

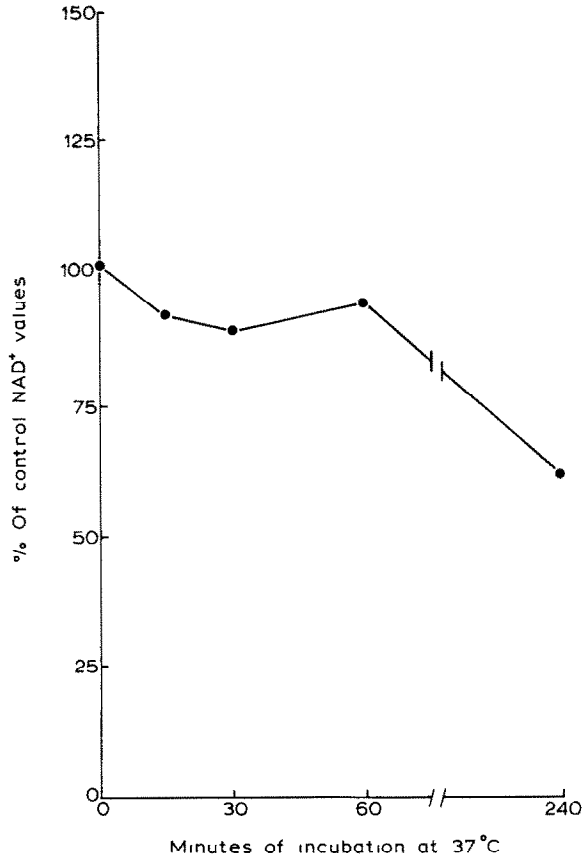

Fig. 3. The time course of the decrease in NAD^+ levels in mixed human leukocytes initiated by HD exposure. The data is shown as the percent of NAD^+ in the control cells which were incubated for the indicated time period. Each point is the average of 3 tubes whose NAD^+ levels were measured in duplicate.

synthesis in the HD-treated cells (278 and 370% increases above those seen in untreated and HD-treated cells that were incubated in the absence of niacinamide respectively).

The kinetics of niacinamide protection

The relationship between the time of niacinamide administration on its ability to prevent the HD-induced NAD^+ decrease was investigated. Cells were incubated with niacinamide for various times, both before and after exposure to HD (Fig. 6). All groups of cells were incubated at 37°C for a total of 5 h. After the first hour, HD was added to the appropriate cells. 10 μl of 10^{-2} M niacinamide in Tyrode's buffer was added at the indicated times to give a final concentration of 10^{-4} M. Niacinamide caused more than a doubling of NAD^+ levels in untreated cells even when added 2 h after the incubation started. When the cells were preincubated for 1 h with niacinamide, HD-treated cells demonstrated even higher levels of NAD^+ ('superinduction') which were approximately 2-fold above that seen in niacinamide-

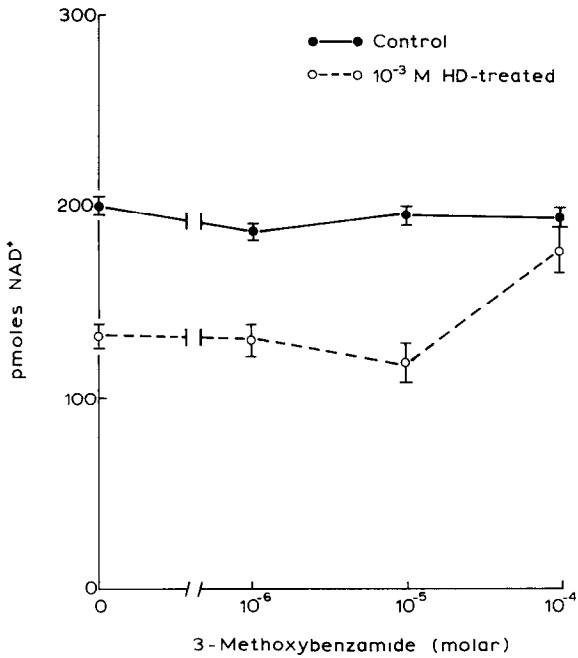

Fig. 4. The effect of the addition of 3-methoxybenzamide (3-MB) to the incubation mixture 10 min before the addition of HD on the level of cellular NAD^+ in mixed human leukocytes. Each point is the average of 3 tubes whose NAD^+ levels were measured as pmol/sample and each sample contained the equivalent of leukocytes from 2 ml of dextran-sedimented blood. The NAD^+ level of each tube was determined in duplicate.

incubated control cells. The 'superinduction' in NAD^+ levels in HD-treated leukocytes (about 200% of untreated cells incubated in the presence of niacinamide) occurred even when niacinamide was added 2 h after HD exposure.

The effect of niacin (nicotinic acid) on the NAD^+ level in leukocytes

The ability of niacin, a substrate for NAD^+ synthesis, but not an inhibitor of poly(ADP-ribose) polymerase, to affect the HD-induced NAD^+ depletion was investigated. Leukocytes from normal individuals were preincubated in buffer with niacin for 10 min before exposure to 10^{-3} M HD and their NAD^+ levels determined after a 4-h incubation (Fig. 7). Niacin caused a concentration-dependent increase in NAD^+ synthesis and prevented the loss of NAD^+ due to HD exposure. Maximum levels of niacin-induced synthesis and protection occurred at 10^{-5} M, with both untreated and HD-treated cells showing NAD^+ levels from 250 to 370% higher than those found in the absence of niacin.

The effect of HD on the different subpopulations of leukocytes

The relationship of the HD-induced decrease in NAD^+ levels to poly(ADP-ribose) polymerase was further explored by dividing the leukocytes into a population of cells which do not contain poly(ADP-ribose) polymerase such as polymor-

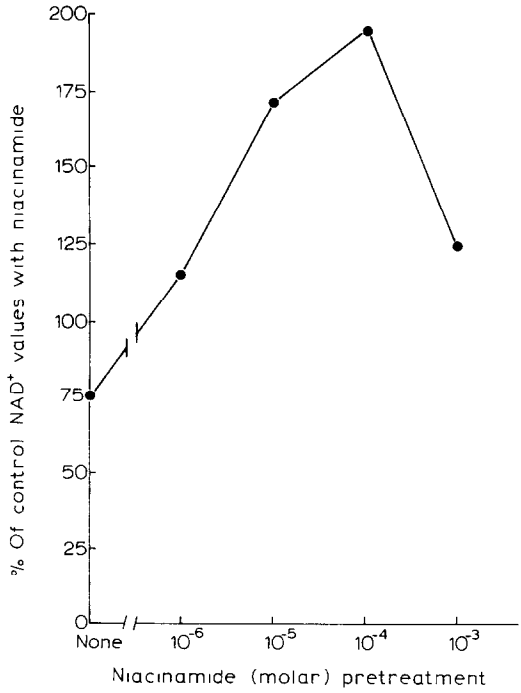

Fig. 5. The 'superinduction' of cellular NAD^+ levels in HD-treated leukocytes by the addition of niacinamide to the incubation mixture 10 min before the addition of HD. The data is expressed as the level of NAD^+ in the HD-treated leukocytes preincubated with the indicated concentration of niacinamide divided by the NAD^+ levels of untreated leukocytes preincubated with the same concentration of niacinamide multiplied by 100. Each point is the average of 3 tubes whose NAD^+ levels were measured in duplicate.

phonuclear granulocytes, and a population of cells in which most of the cells contain poly(ADP-ribose) polymerase such as mononuclear lymphocytes. As shown in Fig. 8, there is a HD concentration-dependent decrease in the NAD^+ levels of lymphocytes which occurs when they are exposed to HD. The decrease in NAD^+ levels of the lymphocytes begins at HD concentrations as low as 10^{-5} M. However, there is no significant change in granulocyte NAD levels even when they are exposed to concentrations of HD as high as 3×10^{-4} M.

DISCUSSION

HD, first synthesized by Guthrie in 1859 [27], was used extensively during World War I, causing incapacitating injuries in greater than 300 000 casualties. Since that time, extensive research has been conducted to elucidate the mechanism by which HD exposure of human skin causes the large blisters that occur between the epidermis and the basement membrane [12–17], and to develop therapeutic approaches to prevent incapacitation. However, in spite of this research, neither the mechanism by which HD induces injury nor a treatment for the resulting injury has been found.

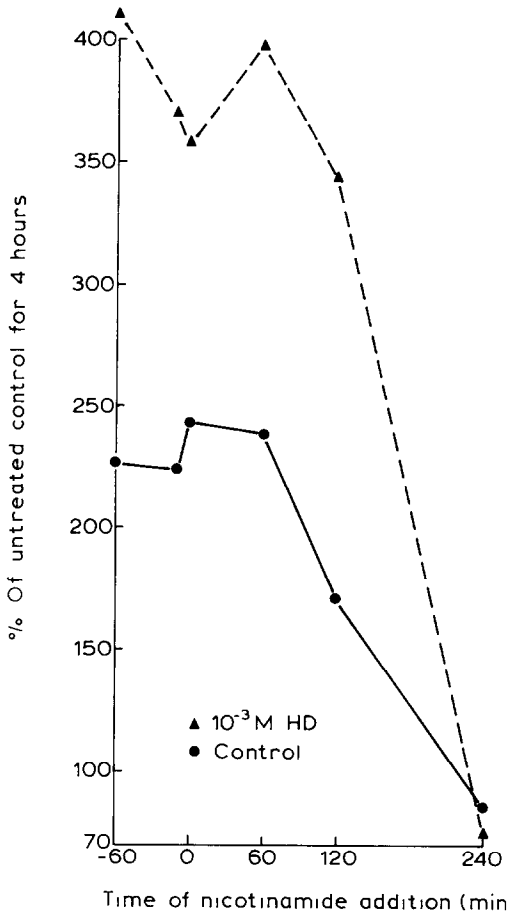

Fig. 6. The time course of the addition of niacinamide to offer protection against the HD-induced decrease of cellular NAD^+ in human leukocytes was studied. Niacinamide was added to the leukocytes 60 min before HD exposure (−60), at the time of HD exposure (0); or 5, 60, 120, and 240 min after HD exposure. Data was plotted as the percent of NAD^+ levels of leukocytes which were incubated for 4 h at 37°C free of either niacinamide or HD exposure. Control cells were incubated with niacinamide for the indicated times while HD-treated cells were incubated with HD for 4 h and niacinamide added at the indicated times during the incubation. Each point is the average of 3 tubes whose NAD^+ levels were measured in duplicate. Note that niacinamide (nicotinamide) could be added as late as 2 h post HD exposure and still cause a 'superinduction' of NAD^+ levels.

Because of recent advances in DNA damage and repair research, we have been able to develop a hypothesis to explain the mechanism by which HD induces cytotoxic effects and generates tissue injury.

This hypothesis [19] is based on the assumption that the initiating events in HD injury are the purine adducts in DNA [28–30] which would undergo both spontaneous and enzyme-mediated depurination at HD concentrations resulting in pathology. Incisions at apurinic sites by repair endonucleases [31–33] result in DNA breakage and the activation of the chromosomal enzyme poly(ADP-ribose)

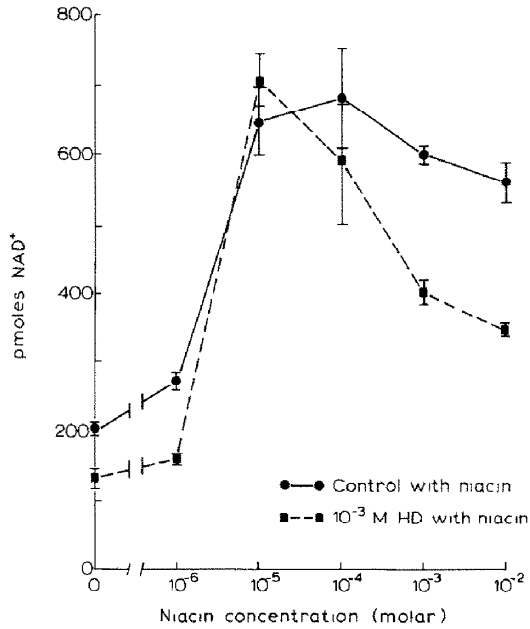

Fig. 7. The effect of the addition of niacin (nicotinic acid) 10 min before addition of HD on the level of cellular NAD^+ in mixed human leukocytes. Each point is the average of 3 tubes whose NAD^+ levels were measured as pmol/sample and each sample contained the equivalent of leukocytes from 2 ml of dextran-sedimented blood. The NAD^+ level of each tube was determined in duplicate.

polymerase [20,34,35]. Poly(ADP-ribose) polymerase, using NAD^+ as a substrate, polymerizes the ADP moieties from NAD^+ on a variety of protein receptors [20]. The activity of the poly(ADP-ribose) polymerase also appears to be required for the ligation step in the repair of DNA breaks [20]. An excessive activation of this enzyme, however, could deplete cells of the cofactor NAD^+ and thus inhibit energy production by glycolysis in the affected cells [20,34,35]. The inhibition of glycolysis, which was reported to be associated with the cytotoxic and vesicant actions of mustards [3,36–39], is postulated to cause stimulation of the hexose monophosphate shunt and result in enhanced secretion of active proteolytic enzymes [40]. By using the leukocyte model, portions of this hypothesis are being validated and therapeutic interventions are being designed and evaluated.

As predicted by the hypothesis described in this paper, HD caused a decrease in leukocyte NAD^+, which is both concentration and time dependent. The maximum decrease was between 30 and 40% of the NAD^+ content of untreated leukocytes (Fig. 1). The decrease in the NAD^+ could result from either total depletion of NAD^+ in 30–40% of cells or partial depletion of NAD^+ in all cells. Experiments using isolated populations of granulocytes and lymphocytes indicated that there is no change in the NAD^+ levels of granulocytes which contain no poly(ADP-ribose) polymerase but in lymphocytes which contain poly(ADP-ribose) polymerase, the NAD^+ content decreased to 25% of the control cells at 10^{-4} M or higher HD. These findings agreed with the former possibility and further supported the

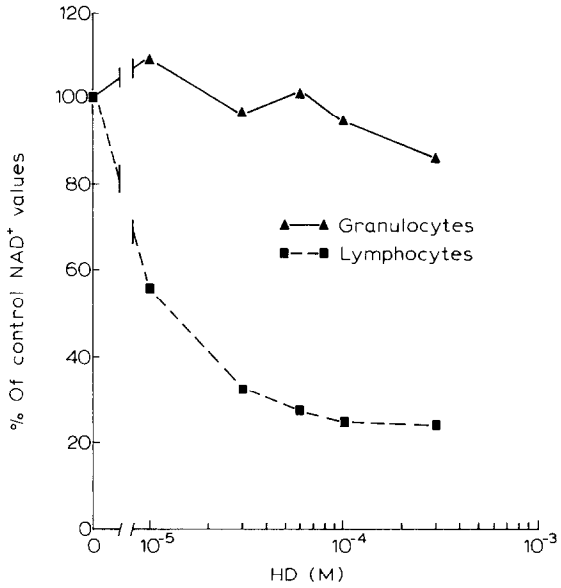

Fig. 8. The HD concentration-dependent decrease in the level of NAD^+ in human lymphocytes but not in human granulocytes. Each point is the average of 3 tubes whose NAD^+ levels were measured as pmol/sample and each sample contained the equivalent of lymphocytes or granulocytes from 2 ml of dextran-sedimented blood. The NAD^+ level of each tube was determined in duplicate

hypothesis since lymphocytes, which comprise between 30 and 40% of the mixed leukocyte population, are the only known leukocytes that contain the enzyme poly(ADP-ribose) polymerase [20].

Further supporting evidence for the involvement of poly(ADP-ribose) polymerase in the lowering of cellular NAD^+ levels is derived from results of experiments using the specific poly(ADP-ribose) polymerase inhibitor, 3-methoxybenzamide [25]. This inhibitor at 10^{-4} M prevented over 66% of the HD-initiated NAD^+ decrease (Fig. 4). Niacinamide, both an inhibitor of poly(ADP-ribose) polymerase and a substrate for NAD^+ synthesis, affected cellular NAD^+ levels in a more complicated fashion than did 3-methoxybenzamide. The difference appeared to be due to an HD-induced stimulation of NAD^+ synthesis in the presence of niacinamide. A possible explanation for the 'superinduction' of NAD^+ by HD exposure in the presence of niacinamide is that DNA damage or DNA repair mechanisms stimulate NAD^+ synthesis. The cellular concentration of NAD^+ increased while NAD^+ consumption due to excessive poly(ADP-ribose) polymerase activity was prevented. These processes resulted in the 'superinduction' of NAD^+ levels that was observed when HD-treated cells were preincubated with niacinamide (Fig. 5).

Since the enzymatic activity of poly(ADP-ribose) polymerase appears to be required for normal DNA repair [20–23], other compounds involved in NAD^+ synthesis were investigated. The effect of niacin, a substrate for NAD^+ synthesis but not an inhibitor of poly(ADP-ribose) polymerase, was studied to determine whether it would also prevent the HD-dependent NAD^+ decrease. Niacin caused a

concentration-dependent increase in the cellular levels of NAD^+ and was able to maintain this increase even in cells exposed to HD (Fig. 7). Thus, niacin was able to prevent the HD-induced decrease in NAD^+ levels without inhibiting poly(ADP-ribose) polymerase activity. This approach should maintain the cells' energy supply, permit it to continue repairing its HD-damaged DNA and prevent other deleterious metabolic effects leading to cell death. Furthermore, the ability to prevent the NAD^+ loss even 2 h after HD exposure may have therapeutic implications. The elucidation of responsible mechanisms must wait until definitive studies are carried out in isolated cell populations.

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REFERENCES

1 C. Auerbach and J.M. Robson, Chemical production of mutations, Nature, 157 (1946) 302.
2 C. Auerbach, D.S. Falconer and J.M. Robson, The production of mutations by chemical substances, Proc. R. Soc. Edin. B., 62 (1947) 271–283.
3 G.P. Wheeler, Studies related to the mechanism of action of cytotoxic alkylating agents, Canc. Rev., 22 (1962) 651–688.
4 M. Fox and D. Scott, The genetic toxicology of nitrogen and sulphur mustards, Mutat. Res., 75 (1980) 131–168.
5 R.M. Gilbert, S. Rowland, C.L. Davison and B. Papirmeister, Involvement of separate pathways in the repair of mutational and lethal lesions induced by a monofunctional mustard, Mutat. Res., 28 (1975) 257–275.
6 W.E. Heston, Carcinogenic action of mustards, J. Natl. Canc. Inst., 11 (1950) 415–423.
7 W.E. Heston, Occurrence of tumours in mice injected subcutaneously with sulphur mustard and nitrogen mustard, J. Natl. Canc. Inst., 14 (1953) 131–140.
8 W.E. Heston and W.D. Levillian, Pulmonary tumours in strain A mice exposed to mustard gas, Proc. Soc. Exp. Biol. Med., 82 (1953) 457–460.
9 IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man, Vol. 9, Some Aziridines, N-S and O Mustards, and Selenium, I.A.R.C., Lyon, 1975, pp. 181–203.
10 S. Wada, Y. Nishimoto, M. Miyanishi, S. Kambe and R.M. Miller, Mustard gas as a cause of respiratory neoplasia in man, Lancet, i (1968) 1161–1163.
11 B. Papirmeister and C.L. Davison, Unbalanced growth and latent killing of *Escherichia coli* following exposure to sulfur mustard, Biochim. Biophys. Acta, 103 (1965) 70–92.
12 S.W. Warthin and C.V. Weller, The Medical Aspects of Mustard Gas Poisoning, Mosby, St. Louis, MO, 1919.
13 W.P. Anslow and C.R. Houck, Systemic pharmacology and pathology of sulfur and nitrogen mustards, in NRDC Report, Chemical Warfare Agents and Related Chemical Problems, Division 9, Washington, DC, 1946, pp. 440–479.
14 B. Renshaw, Mechanisms in production of cutaneous injuries by sulfur and nitrogen mustards, in NRDC Report, Chemical Warfare Agents and Related Chemical Problems, Division 9, Washington, DC, 1946, pp. 479–512.
15 H. Collumbine, Medical aspects of mustard gas poisoning, Nature, 159 (1947) 151–153.
16 B. Papirmeister, C.L. Gross, J.P. Petrali and C.J. Hixson, Pathology produced by sulfur mustard in human skin grafts on athymic nude mice. I. Gross and light microscopic changes, J. Toxicol. Cutaneous Ocul. Toxicol., 3 (1984) 371–393.

17 B. Papirmeister, C.L. Gross, J.P. Petrali and H.L. Meier, Pathology produced by sulfur mustard in human skin grafts on athymic nude mice. II. Ultrastructural changes, J. Toxicol. Cutaneous Ocul. Toxicol., 3 (1984) 395–410.
18 R.F. Vogt, A.M. Dannenberg, Jr., B.H. Schofield, N.A. Hynes and B. Papirmeister, Pathogenesis of skin lesions caused by sulfur mustard, Fundam. Appl. Toxicol., 4(2) (1984) 571–583.
19 B. Papirmeister, C.L. Gross, H.L. Meier, J.P. Petrali and J.B. Johnson, Molecular basis for mustard induced vesication, Fundam. Appl. Toxicol., 5 (1985) S134–S149.
20 L. Hayaishi and K. Ueda, ADP-Ribosylation Reactions, Biology, and Medicine, Academic Press, New York, 1982.
21 L.M. Lichtenstein, The immediate allergic response: in vitro separation of antigen activation, decay, and histamine release, J. Immunol., 107 (1971) 1122–1126.
22 E.L. Jacobson and M.K. Jacobson, Pyridine nucleotide levels as a function of growth in normal and transformed 3T3 cells, Arch. Biochem. Biophys., 175 (1976) 627–634.
23 R.P. Siraganian, An automated continuous-flow system for the extraction and fluorometric analysis of histamine, Anal. Biochem., 57 (1974) 383.
24 L. Berger and D. Broida, The quantitative colorimetric determination of lactate dehydrogenase, Sigma Tech. Bull. 500, 1957.
25 M.R. Purnell and W.J.D. Whish, Novel inhibitors of poly(ADP-ribose) synthetase, Biochem. J., 185 (1980) 775–777.
26 D.W. MacGlashan, Jr. and L.M. Lichtenstein, The transition from specific to nonspecific desensitization in human basophils, J. Immunol., 127 (1981) 2410–2414.
27 F. Guthrie, Ueber einige Derivate der Kohlenwasserstoffe CnHn, Ann. Chem. Pharm., 113 (1860) 266.
28 P. Brookes and P.D. Lawley, The reaction of mustard gas with nucleic acids in vitro and in vivo, Biochem. J., 77 (1960) 478–484.
29 P. Brookes and P.D. Lawley, The reaction of mono- and di-functional alkylating agents with nucleic acids, Biochem. J., 80 (1961) 496–503.
30 P. Brookes and P.D. Lawley, Molecular mechanism of the cytotoxic action of difunctional alkylating agents and of resistance to this action, Nature, 206 (1965) 480–483.
31 T. Lindahl, DNA glycosylases, endonucleases for apurinic/apyrimidinic sites, and base excision-repair, Prog. Nucleic Acid Res. Mol. Biol., 22 (1979) 135–192.
32 B. Papirmeister, J.K. Dorsey, C.L. Davison and C.L. Gross, Sensitization of DNA to endonuclease by adenine alkylation and its biological significance, Fed. Proc., 29 (1970) 726.
33 D.B. Ludlum and B. Papirmeister, DNA modification by sulfur mustards and nitrosoureas and repair of these lesions, Int. Symp. on Mechanisms of DNA Damage and Repair, Gaithersburg, MD, 1985.
34 N.A. Berger, G.W. Sikorski, S.J. Petzold and K.K. Kurohara, Association of poly(adenosine diphosphoribose) synthesis with DNA damage and repair in normal human lymphocytes, J. Clin. Invest., 63 (1979) 1164–1171.
35 P.W. Rankin, M.K. Jacobson, V.A. Mitchell and D.L. Busbee, Reduction of nicotinamide adenine dinucleotide levels by ultimate carcinogens in human lymphocytes, Cancer Res., 40 (1980) 1803–1807.
36 M. Dixon and D.M. Needham, Biochemical research on chemical warfare agents, Nature, 158 (1946) 432–438.
37 I.M. Fraser, Effect of mechlorethamine on tumor glycolysis and diphosphopyridine nucleotide content, Fed. Proc., 19 (1960) 345.
38 I.M. Roitt, The inhibition of carbohydrate metabolism in ascites tumor cells by ethyleneimines, Biochem. J., 63 (1956) 300–307.
39 G.E.S. Barron, J. Meyer and Z.B. Miller, The metabolism of skin effects of vesicant agents, J. Invest. Dermatol., 11 (1948) 97.
40 J. Schnyder and M. Baggiolini, Induction of plasminogen activator secretion in macrophages by electrochemical stimulation of the hexose monophosphate shunt with methylene blue, Proc. Natl. Acad. Sci. U.S.A., 77 (1980) 414–417.