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Determination of ATP Levels in Sulfur Mustard-exposed Human Peripheral Blood Lymphocytes by a Chemiluminescent Assay

Marian R. Nelson
Susan A. Kelly
Clark L. Gross
William J. Smith

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U.S. Army Medical Research
Institute of Chemical Defense
Aberdeen Proving Ground, MD 21010-5400

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13. ABSTRACT (Maximum 200 words) Sulfur mustard (HD) is a powerful blistering agent whose mechanism is poorly understood. One of the effects of this chemical warfare agent is to lower NAD ⁺ levels through the activation of poly (ADP-ribose) polymerase; HD also depletes ATP, the major energy supply for biochemical reactions within the cell. ATP is a biological marker that can be measured in HD-exposed cells by using a chemiluminescent reaction based on the luciferin-luciferase reaction. This assay is conveniently measured by the use of a luminometer and exhibits a reproducible linear standard curve. Three prototypic inhibitors of the poly (ADP-ribose) polymerase have been studied to determine their effect on restoring or maintaining ATP levels in HD-exposed human peripheral blood lymphocytes (PBL). Two of the inhibitors, ICD 2250 (1 (2H)-isoquinolinone, 3,4-dihydro-5-[3-(methylamino)propoxy]-monohydrochloride) and ICD 2163 (3,4-dihydro-5-methyl-1(2H)-isoquinolinone), are very effective in maintaining ATP levels in HD-exposed (200 µM) PBL. With both drugs, a concentration of 100 µM restores ATP levels to nearly those found in unexposed cells with both drugs. The third inhibitor, ICD 967 (niacinamide), is not nearly as effective. This assay is now under development to test ATP levels in human epidermal keratinocytes, a more relevant <i>in vitro</i> model for vesication.				
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Introduction

Sulfur mustard (HD) is a chemical warfare agent that causes debilitating and slowly healing injuries to eyes, lung, and skin. The mechanism(s) for causing these injuries is not currently understood, and biological markers for the severity of mustard exposure are being evaluated. HD alkylates cellular targets such as DNA, RNA, and protein (1) and depletes cellular NAD (2,3) as well as ATP, whose loss leads to metabolic disruption and the potential death of the cell (4). Maintaining or restoring ATP levels within exposed cells may lead to the amelioration of the HD lesion.

The Drug Assessment Division of the United States Army Medical Research Institute of Chemical Defense has the responsibility of determining which compounds or formulations could be potentially efficacious as medical countermeasures against chemical warfare agents. The *in vitro* team of the Basic Assessment Branch uses various relevant human *in vitro* models to develop assays for measuring critical biological markers of sulfur mustard exposure. These assays are tested using certain drugs that have the desired pharmacological actions in either prevention or treatment of the vesicant injury. One of the hypothesized consequences of mustard exposure is the loss in ATP levels and disruption of metabolic activity. A sensitive assay to measure ATP levels has been developed using a commercial BioOrbit ATP measurement kit and a Dynatech ML 3000 luminometer. The effect of HD on ATP levels in human peripheral blood lymphocytes (PBL) has been measured, and three prototypic drugs have been tested and evaluated in protecting/restoring ATP levels in these cells. Maintenance of critical ATP levels may be effective in lessening the consequences of HD exposure in a metabolically active cell.

Materials and Methods

Reagents:

RPMI 1640 media, EDTA, and trypan blue were obtained from Sigma Chemical Co., St. Louis, MO. Percoll was purchased from Pharmacia, Piscataway, NJ. Bio Orbit Monitoring Reagent Kits were obtained from Wallac Inc., Gaithersburg, MD. Trichloroacetic acid (TCA) was obtained from Wallac Oy, Turku, Finland. Sulfur mustard (CAS Registry #505-60-2, 96.8% pure) was acquired from the Edgewood Chemical and Biological Center, Aberdeen Proving Ground, MD.

Isolation of PBL:

Blood was obtained by venipuncture from volunteers under an approved human use protocol. The blood was diluted with a phosphate buffered saline (PBS) (0.003M KPO₄, 0.15 M NaCl, 0.1M EDTA, pH 7.4) solution (5). Thirty-five mL of diluted blood was then layered on 10 mL of Percoll (d= 1.080). The density gradient was then centrifuged at 425 xg, for 30 minutes at 20° C. The lymphocyte bands were aspirated and pooled into 50 mL centrifuge tubes and washed with Tyrode's free buffer (3). The lymphocytes were then centrifuged at 250 xg for 12 minutes at 20°C. Cell pellets were resuspended in Tyrode's free buffer, combined into one centrifuge tube, washed again in Tyrode's free

buffer and finally resuspended in RPMI 1640 buffer. The cells were counted using trypan blue on a hemacytometer and their concentration was adjusted to 2×10^7 cells per mL.

Exposure of lymphocytes to sulfur mustard:

Lymphocytes were plated at 1×10^6 cells per well in a 96-well plate. The desired pretreatment compounds were added to the appropriate wells at concentrations ranging from 200 to 2000 μM . One-half of the plate represented control samples and samples containing buffer and pretreatment compound at designated concentrations. The other half of the plate represented lymphocyte samples to be exposed to 200 μM HD and pretreatment compound at designated concentrations. A 10 mL stock (4000 μM) vial of HD was diluted 1:4 with RPMI 1640 to a working concentration of 1000 μM , and 40 μL of this solution was added to each well to give a final HD challenge dose of 200 μM . After samples had been exposed to HD, the plate remained in the surety hood for one hour to allow complete hydrolysis of HD and venting of volatile agent. The plate was then placed in the 5% CO_2 incubator for an additional three hours until ATP levels could be measured.

Luminescent ATP assay:

ATP standard solution (10 μM) is prepared by adding 10 mL of doubly distilled, deionized water to the vial of ATP supplied in the Bio Orbit ATP assay kit. After withdrawing enough ATP standard solution (1.0 mL) to perform a standard curve, the remaining reconstituted ATP standard is distributed into 1.0 mL aliquots in Eppendorf tubes and frozen at -20°C .

ATP Standard Curve Procedure

Sample	Tris-Acetate mL (pH 7.7)	mL 10 μM ATP	** mL 1 μM ATP	[ATP]
1	0.100	0.100	-----	5.0 μM
2	0.150	0.100	-----	4.0 μM
3	0.375	0.125	-----	2.5 μM
4	3.600	0.400	-----	1.0 μM **
5	0.250	-----	0.250	0.5 μM
6	0.225	-----	0.025	0.1 μM
7	0.190	-----	0.010	0.05 μM

Twenty-five μL of each of the above samples was transferred to a Micro-Lite 96-well plate, and each standard was run in triplicate.

The 96-well plate containing the HD-exposed PBL was removed from the 37°C incubator after three hours. Cells were solubilized by the addition of 10 μL of 20% TCA to each well, and the plate was incubated for 15 minutes at room temperature. The samples (210 μL) were removed from the wells and placed in tubes containing 790 μL of 0.2 M Tris-

acetate, pH 7.75. Aliquots of 25 μL from each sample were pipetted (in quadruplicate) into a Micro Lite 96-well plate containing 0.125 μL of 0.1M Tris buffer, and the plate was placed in a ML 3000 Luminometer. ATP Monitoring Reagent was reconstituted by adding 10 mL of doubly distilled deionized water to the reagent vial. This assay was performed at room temperature as recommended in the product literature (6). The luminometer was set up to inject 36 μL of ATP Monitoring Reagent and 7 μL of ATP standard (spike) into each well prior to reading samples.

Data Analysis:

Data was analyzed by One Way Analysis of Variance (ANOVA), and the level of significance was $p < 0.001$ (*) and $p < 0.05$ (**)

Results

A typical standard curve of ATP is shown below in Figure 1. The curve was linear between 5 nM and 5 μM ATP and is reproducible. The r^2 value was very consistent at the 0.99 level using this chemiluminescent assay.

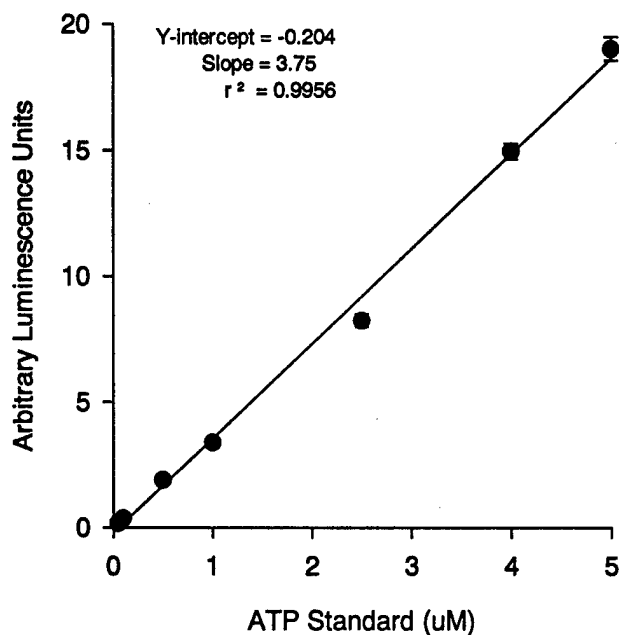


Figure 1. A standard curve of ATP concentrations between 50 nM and 5 μM using the chemiluminescent ATP assay. Each point is the mean \pm s.d. value of quadruplicate determinations.

When various concentrations of HD were added to PBL, intracellular ATP levels decreased in a dose-dependent manner (Figure 2). This reduction appeared to be in two phases: a rapid initial ATP loss between 0 and 50 μM HD, and a slower decrease that seemed to reach an approximate maximum of 50% loss at 250 μM HD.

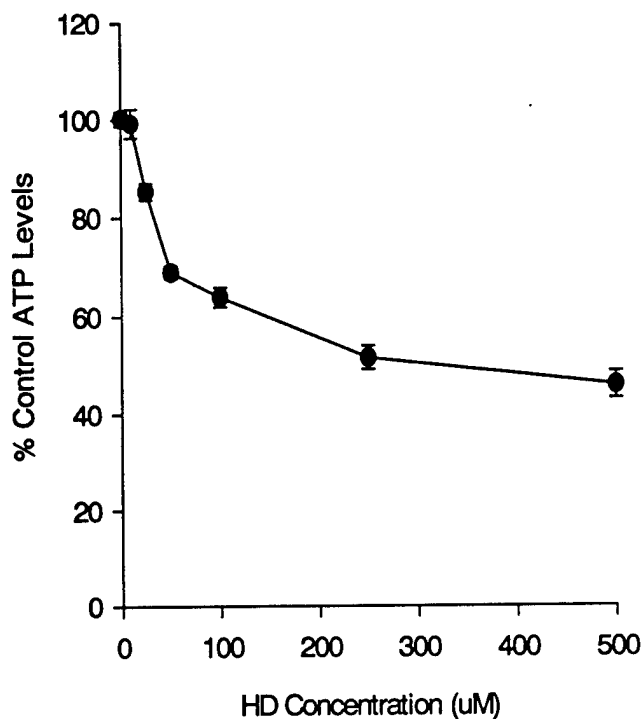


Figure 2. Profile of ATP levels in freshly isolated human PBL exposed *in vitro* to varying concentrations of HD. At 3 hours postexposure, ATP levels were measured using the chemiluminescence test kit (as described in Materials and Methods). Each point represents the mean value (\pm s.e.m) of three separate experiments.

When HD concentration was increased to 500 μM , there was no further loss in ATP levels beyond that seen at 250 μM . The challenge dose of 200 μM HD was used in subsequent experiments since it appears from data shown in Figure 2 that the maximum effect occurs at this dose.

Inhibitors of poly (ADP-ribose) polymerase were investigated to determine whether the ATP levels of lymphocytes would be spared or restored by the addition of these compounds to the cells before they were exposed to HD. Three prototypic compounds were selected from known inhibitors of this enzyme, and the results from these studies appear below. The poly ADP-ribose polymerase (PARP) inhibitor ICD 2250 (Fig. 3) was added to the cells immediately prior to HD exposure, and ATP levels were determined at 3 hr postexposure.

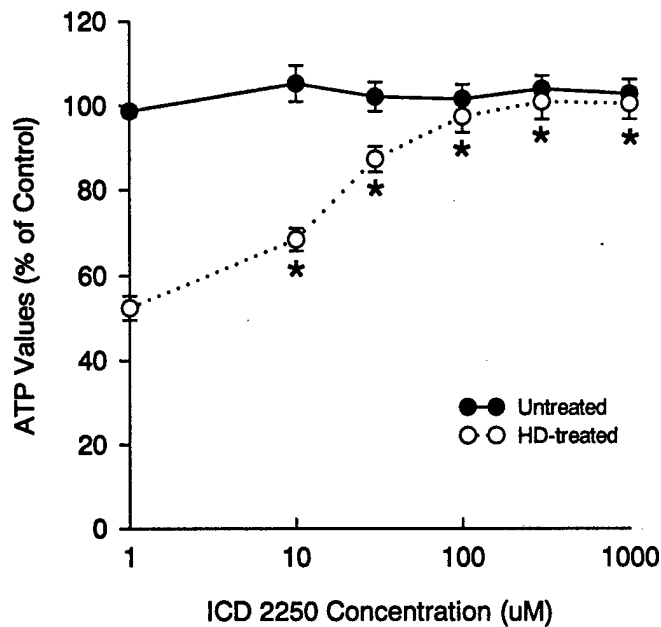


Figure 3. Percent of ATP values obtained when human PBL were exposed *in vitro* to 200 μM HD and assayed 3 hr postexposure with a chemiluminescence ATP test kit in the presence or absence of the poly ADP-ribose polymerase (PARP) inhibitor ICD 2250. Each point represents the mean \pm s.e.m value of six separate experiments. Level of significance was $p < 0.001$ (*) by ANOVA.

ATP levels were protected at ICD 2250 concentrations of 100 μM or greater. At ICD 2250 concentrations of 10 μM and 30 μM , there was incomplete but important protection of ATP levels, 34% and 71% ATP protection, respectively. ICD 2250 had no effect on control ATP levels in unexposed lymphocytes.

Figure 4 illustrates the ATP levels of PBL measured in the presence or absence of 200 μM HD when another PARP inhibitor, ICD 2163, was added. No effect on control ATP levels was observed in pretreated unexposed cells as shown below.

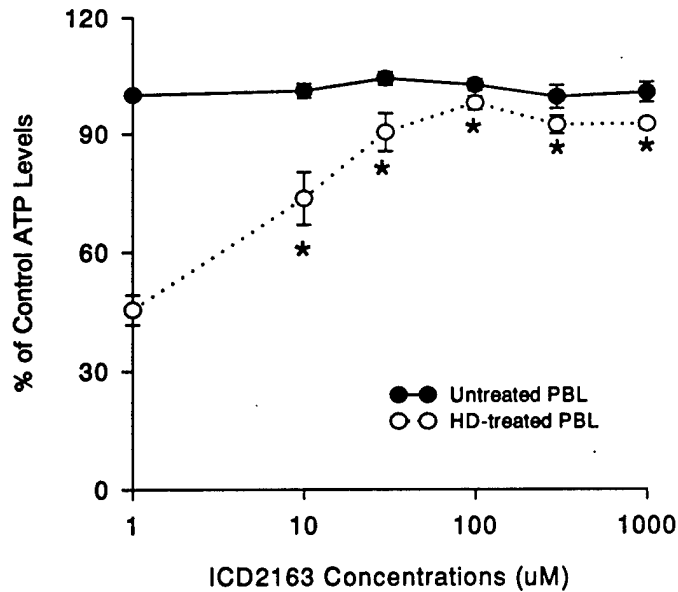


Figure 4. Human PBL exposed *in vitro* to 200 μ M HD were assayed 3 hr postexposure with a chemiluminescence ATP test kit in the presence or absence of the poly (ADP-ribose) polymerase (PARP) inhibitor ICD 2163. Each point represents the mean (\pm s.e.m.) value of three separate experiments. Level of significance was $p < 0.001$ (*) by ANOVA.

The results obtained with this PARP inhibitor were very similar to those of ICD 2250. At ICD 2163 concentrations of 10 μ M and 30 μ M, there was also incomplete protection of ATP levels, 51% and 81% ATP protection, respectively. No effect on control ATP levels with this inhibitor was observed, as in the case of ICD 2250.

However, the PARP inhibitor ICD 967 exhibited a different behavior, shown in Figure 5.

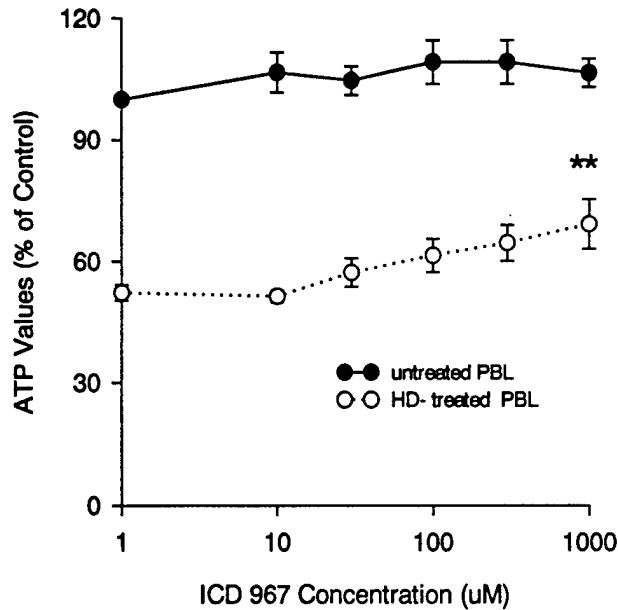


Figure 5. Typical representation of ATP response when human PBL were exposed *in vitro* to 200 μ M HD and assayed 3 hr postexposure with a chemiluminescence ATP test kit in the presence or absence of the poly(ADP-ribose)polymerase (PARP) inhibitor ICD 967. Each point represents the mean \pm s.e.m. of three separate experiments. Level of significance was $p < 0.05$ (**) by ANOVA.

Maximum ATP protection of approximately 40% occurred only at 1000 μM ICD 967, five times the concentration of the challenge dose of HD. It is unknown at this time whether concentrations of ICD 967 would protect ATP levels of lymphocytes challenged with lower doses of HD.

Conclusions

A chemiluminescent assay has been developed for ATP levels in HD-exposed human peripheral blood lymphocytes. This assay was linear within the concentrations of 50 nM and 5 μM ATP, was very reproducible, and has a consistent r^2 value of 0.99. The assay was easy to perform and can be learned in a short time by laboratory personnel.

ATP levels in PBL decrease in a dose-dependent manner when analyzed 3 hours after HD exposure. Two phases of ATP loss occur; a rapid initial phase between 0 and 50 μM HD and a slower loss at higher concentrations of HD up to 500 μM . This ATP loss appears to reach a plateau at approximately 50% and does not decline further with increasing HD concentration.

Breaks in DNA caused by alkylation activate the poly (ADP-ribose) polymerase that depletes the ATP levels in PBL. ATP loss and disruption of metabolic activity appears to be a very sensitive step in the injury and potential death of a cell following mustard exposure. Since ATP is involved in a myriad of metabolic activities, many critical biochemical pathways could be affected by this loss. Restoration of ATP levels and the resulting metabolic improvement may be helpful in reversing the cellular damage caused by this agent followed by recovery of the damaged cell.

With this goal in mind, we have investigated drugs that inhibit poly (ADP-ribose) polymerase. Three of the prototypic drugs that we investigated had varying effects on the ATP levels. ICD 967 showed a favorable effect only at very high levels.

However, ICD 2250 and ICD 2163 show beneficial effects in concentrations as low as 10 μM and approach ATP levels of unexposed cells when 100 μM concentration of drugs were used. Increasing the drug level to 300 or 1000 μM did not appear to have any extra advantage. The results of this study give some hope to finding a medical countermeasure for the vesicant agent HD. The *in vitro* findings in PBL will be further investigated in human epidermal keratinocytes, another *in vitro* model that appears to be a more relevant target of mustard exposure. If these ATP results can be confirmed in this model, the drugs will be passed on for further testing in an *in vivo* model currently available at this institute.

This assay was very accurate but had some technical aspects that must be considered. Temperature control was critical since the temperature optimum of the luciferin-luciferase assay is 25°C. Early results were dependent on the position of the assay wells in the plate, since the assay was slowly denatured before completion of plate reading when the heating platform was at 37°C. The assay is now performed at room temperature using temperature-equilibrated reagents. We also noticed that splashing of the reagents

was occurring inside the luminometer and may have been due to using reagents that were cold and not thoroughly dissolved. This problem was solved by pre-priming injector probes with room temperature reagents (25°C) and siliconizing the probe tips.

Another problem encountered was artifactual data results from a high background thought to be due to exogenous light activation. Wrapping the ATP Monitoring Reagent syringe in aluminum foil to preclude light activation solved the problem.

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