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Measurement of Protease Release by a
Fluorogenic Casein Assay in Human Cells
Exposed *In Vitro* to Sulfur Mustard

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13. ABSTRACT (Maximum 200 words) Sulfur mustard (HD) exposure causes severe ocular, respiratory, and blistering injuries to skin. The mechanism(s) of injury has eluded identification, although a protease(s) may be involved in the dermal-epidermal separation seen in blister formation. Recent research using <i>in vitro</i> systems has demonstrated that protease release occurs in these models as a function of HD exposure. A number of protease inhibitors have been proposed as candidate anti-vesicant medical countermeasures and need to be screened in a rapid and cost-effective <i>in vitro</i> assay to determine efficacy before being transitioned to an <i>in vivo</i> model for further testing. New fluorogenic casein substrates that are sensitive to a number of proteases have been developed by Molecular Probes and may be helpful in developing a rapid assay for protease analysis. This report describes the use of these substrates in assays for screening compounds to develop medical countermeasures against this vesicant agent. The data suggest that these substrates are not appropriate to the development of a rapid protease assay.				
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Introduction

The chemical warfare agent sulfur mustard (2,2'-dichlorodiethyl sulfide, mustard gas, HD) produces serious injuries to the eyes, respiratory tract, and skin in unprotected personnel. The specific biochemical mechanism(s) involved in creating these injuries has not yet been elucidated but has been hypothesized as a cascade of events that may lead to vesication (1). Fundamental to this theory is the postulated action of a secreted protease that would cleave the anchoring filaments at the epidermal/dermal junction and result in the formation of a blister. Research performed during the late 1940's indicated that some type of proteolysis was involved in corneal loosening following HD exposure to the eye (2). Rat skin exposed to HD showed the transient appearance of a protease followed by its rapid disappearance (3). If a protease were involved in the blister formation, anti-protease therapeutic measures could then be taken to mitigate the sulfur mustard injury.

Recent research has shown that mustard exposure of a number of relevant *in vitro* models leads to the appearance of a protease. A number of reports have shown increased serine protease activity in human peripheral blood lymphocytes (PBL), human epidermal keratinocytes (HEK), Living Skin Equivalent cultures, and hairless guinea pig skin biopsies (4 -7). An enzyme found in sulfur mustard-exposed human epidermal keratinocytes has been purified and partially characterized (8) and appears to be a serine protease of approximately 80 kDa. These studies have shown that these *in vitro* models can be used to investigate hypothesized biochemical mechanisms of HD lesion formation. These *in vitro* systems may also be used to rapidly search for therapeutic compounds that can interfere with proteases presumably involved in the pathogenesis of the debilitating and slowly healing mustard lesion.

The development of medical countermeasures against sulfur mustard requires rapid and convenient assays to measure biological endpoints of HD exposure. Since the number of candidate anti-protease compounds is large, testing these compounds under *in vivo* conditions is too expensive. A rapid *in vitro* test is necessary for efficient screening of compounds and can be accomplished by assays that measure protease action using 96-well tissue culture plates containing either PBL or HEK. New fluorogenic substrates for detecting serine, acid, metallo- and sulfhydryl protease activity have been developed by Molecular Probes; these substrates may allow the measurement of broad spectrum protease activity using a microplate reader capable of fluorescence detection. This study examines the possibility of using these substrates for a rapid and sensitive protease assay in human cells that have been grown *in vitro* and exposed to HD.

Materials and Methods

Materials. RPMI tissue culture media, gentamycin, trypsin, phosphate buffered saline, trypan blue, and other laboratory chemicals were purchased from Sigma Chemical Company (St. Louis, MO). Percoll was obtained from Pharmacia (Piscataway, NJ). Human Epidermal Keratinocytes (HEK), Keratinocyte Growth Media (KGM), and Trypsin-EDTA Epi-Packs were purchased from Clonetics Corporation (San Diego, CA). Tissue culture vessels were purchased from Corning Corporation (Corning, NY) or Falcon Corporation (Newark, NJ). EnzChek™ Protease Assay kits were purchased from Molecular Probes (Eugene, OR). Sulfur mustard (CAS registry # 505-60-2, 96.8% pure) was obtained from the Edgewood Chemical and Biological Center, Aberdeen Proving Ground, MD, USA.

Human peripheral blood lymphocyte isolation. Human peripheral blood lymphocytes (PBL) were obtained from freshly drawn whole blood from human volunteers under an approved human use protocol. The lymphocytes were isolated by Percoll (density 1.08) centrifugation as previously

described (9). Cells were counted and viability determinations were routinely performed by trypan blue dye exclusion and the use of a hemacytometer. Cells were then resuspended in RPMI 1640 + gentamycin (50 µg/mL), and 100 µL aliquots were dispensed into 96-well tissue culture plates at the appropriate density for experimentation.

Cell culture. Normal human epidermal keratinocytes (HEK) were purchased as second passage cells from Clonetics. They were cultured in Keratinocyte Growth Media (KGM) in T-150 flasks in a 5% CO₂ incubator at 37°C. These HEK were harvested by using Trypsin/EDTA, counted by using a hemacytometer and trypan blue, and subcultured into 24-well plates at densities ranging from 5-10,000 cells/well. The media was changed on alternate days, and cells were grown until confluencies of 80-90% were reached prior to experimentation.

Exposure of cells to sulfur mustard. A stock solution of 4 mM HD was diluted into either RPMI (for lymphocytes) or KGM (for keratinocytes) and added to the appropriate cell culture plates in a chemical surety hood to yield final HD concentrations varying from 10-500 µM. After one hour at ambient temperature to allow reaction and hydrolysis of agent, the tissue culture plates were transferred to a 37°C incubator under a humidified 5% CO₂ atmosphere for post-exposure incubation.

Fluorescence Assay using Trypsin and EnzChek™ Kits. The 20X digestion buffer (0.2 M Tris-HCl, pH 7.8 – 0.002 M NaN₃) supplied in the EnzChek™ kit was diluted to 1X by addition of 19 volumes of doubly distilled water (i.e., 2.5 mL to 50 mL). A vial containing 200 µg of casein-BODIPY-Fluorescein was reconstituted with 0.2 mL of doubly distilled water and mixed with 19.8 mL of 1X digestion buffer. According to product literature, this substrate is stable for up to 4 weeks at 4°C (12). Aliquots of 100 µL of Trypsin (1-10 µg/mL) were pipetted into the wells of a 96-well plate, and 100 µL of the casein-BODIPY-Fluorescein substrate (10 µg/mL) was added. The plates were incubated for 1, 4, and 24 hours in a 37°C incubator under a humidified 5% CO₂ atmosphere to determine the optimal conditions for running the assay. The plates were removed at the appropriate times and read by placing in CytoFluor® II microplate fluorescence reader at room temperature with excitation at 485 nm and emission measured at 530 nm.

Fluorescence Assay using Cells and EnzChek™ Kits. The 20X digestion buffer supplied in the EnzChek™ kit was diluted to 1X by addition of 19 volumes of doubly distilled water (i.e., 2.5 mL to 50 mL) as above. A vial containing 200 µg of casein-BODIPY-Fluorescein was reconstituted with 0.2 mL of doubly distilled water and placed into 19.8 mL of 1X digestion buffer as above. Alternatively, a vial containing 200 µg of casein-BODIPY-Texas Red was first reconstituted with 0.2 mL of NaHCO₃, pH 8.3 and then diluted in 19.8 mL of 1X digestion buffer.

The 96-well tissue culture plates containing PBL (± HD) were removed from the incubator at the appropriate times, and 100 µL of media was removed from each well. Aliquots of 100 µL of the casein-BODIPY-Texas Red substrate (10 µg/ml) or casein-BODIPY-FI substrate (10 µg/ml) were added to the PBL as well as to the appropriate blanks. The plates were mixed for 5 minutes by gentle swirling on a Belly Dancer shaker (Stovall, Greensboro, NC) and then incubated in the dark for 1 or 4 hours in a 37°C incubator under a humidified 5% CO₂ atmosphere. The plates were removed from the incubator at the designated time, and 100 µL of supernatant from each well was placed into the corresponding well in fresh 96-well plates. Samples were read at room temperature in a CytoFluor® II microplate fluorescence reader (Perceptive Biosystems, Framingham, MA) with excitation wavelength at 485 or 590 nm and emission wavelength measured at 530 or 645 nm depending on the substrate used.

In PBL lysis experiments, 120 µL of media was aspirated and discarded from each well. Twenty µL aliquots of 10% Triton-X100 or RPMI media were added to the appropriate wells and

the plates were incubated for 20 min to facilitate lysis. Aliquots of 100 μ L of the casein-BODIPY-Texas Red substrate (10 μ g/ml) or casein-BODIPY-Fl substrate (10 μ g/ml) were added and mixed for 5 minutes by gentle swirling on a Belly Dancer shaker (Stovall, Greensboro, NC). Plates were incubated in the dark for 1 or 4 hours in a 37°C incubator under a humidified 5% CO₂ atmosphere and processed as above.

The 24-well tissue culture plates containing HEK (\pm HD) were removed from the incubator, and the media from each well was aspirated off. The wells were covered with 1.0 mL of 1X digestion buffer, followed by the addition of 1 mL of casein-BODIPY-Fl (10 μ g/ml) to each well. The plates were incubated in the dark for 1 and 4 hours in a 37°C incubator under a humidified 5% CO₂ atmosphere. The plates were removed from the incubator at the designated time, and 1.0 mL of supernatant from each well was placed into the corresponding well in fresh 24-well plates. Samples were read at room temperature in a CytoFluor® II microplate fluorescence reader with excitation wavelength at 485 nm and emission wavelength measured at 530 nm.

In experiments where lysis of HEK was attempted, media was removed and 0.9 mL of digestion buffer was added to each well followed by 100 μ L of 10% Triton X-100, and the plates were incubated for one hour at 37°C to encourage lysis. The fluorescent substrate was added and the plates were incubated and processed as above.

Data Analysis. Data was analyzed by One Way Analysis of Variance (ANOVA), and the levels of significance were $p < 0.05$ (*) and $p < 0.01$ (**).

Results

The optimal conditions to perform this assay for high through-put were investigated by using protease at various concentrations for two different times and two different temperatures.

The fluorescently labeled BODIPY-Fl casein substrate was exposed to a serine protease, trypsin, in a 96-well tissue culture plate as shown in Figure 1.

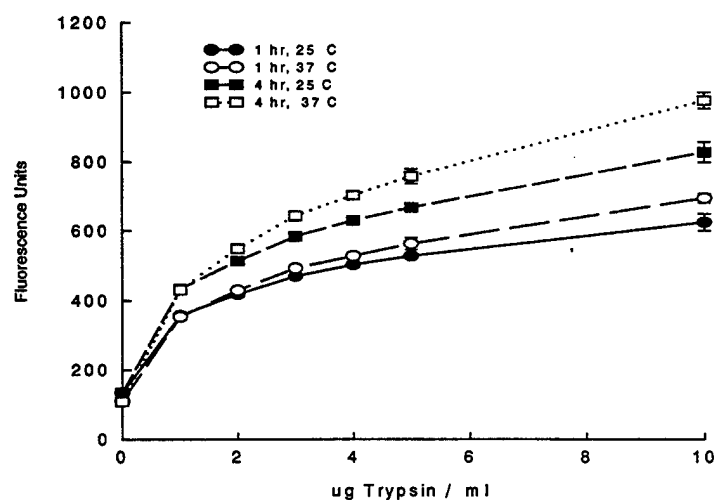


Figure 1. Trypsin (in a final volume of 100 μ L) was added to 96-well plates containing 100 μ L of casein-BODIPY-Fl and incubated for 1 hour or 4 hours at either 25 or 37°C and then read at 530 nm after excitation at 485 nm. Each point is the mean \pm s.e.m. of 4 separate determinations.

The optimal conditions for the fluorescence assay using the casein-BODIPY-FI assay appeared to be for 4 hours at 37°C, and these conditions were employed in all subsequent studies. Incubation times for up to 24 hours did not increase the sensitivity of the assay since the background also increased (data not shown).

The number of cells necessary to produce a measurable protease response was investigated by using cell concentrations ranging from 1×10^5 – 2×10^6 cells/well. These results are presented in Figure 2, and 2×10^6 cells/well gave the best signal/noise ratio, so this concentration was used in subsequent studies.

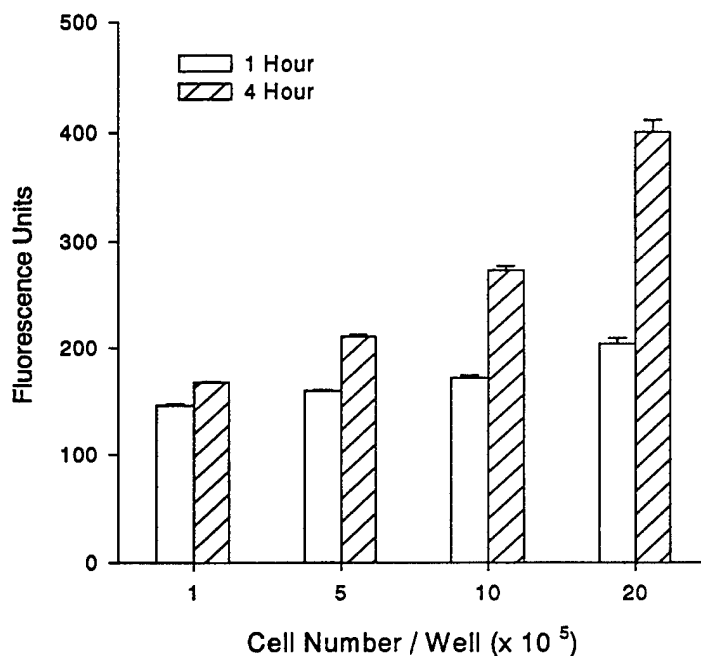


Figure 2. PBL at the indicated concentration/well of a 96-well tissue culture plate were incubated for both 1 hr and 4 hr at 37°C. The supernatants (100 μ L) were withdrawn and placed in a fresh 96-well plate and read at 645 nm after excitation at 590 nm. Each point is the mean \pm s.e.m. of 6 separate determinations.

The effect of HD on protease release from PBL was investigated by placing 2×10^6 PBL per well in a 96-well tissue culture plate and exposing them to various doses of HD (10-500 μ M) in a surety hood. After 1 hr to ensure complete reaction of HD, plates were removed from the surety hood and placed in a 5% CO₂ incubator at 37°C for the appropriate post-exposure incubation. One hundred μ L of supernatant was withdrawn from each well and replaced with 100 μ L of casein-BODIPY-FI substrate. The plates were incubated for 4 hr at 37°C in a humidified 5% CO₂ incubator. Aliquots of 100 μ L supernatant were then withdrawn, placed in fresh 96-well plates and read on a Cytofluor® II microplate spectrofluorometer at 530 nm after excitation at 485 nm. The results appear below in Figure 3.

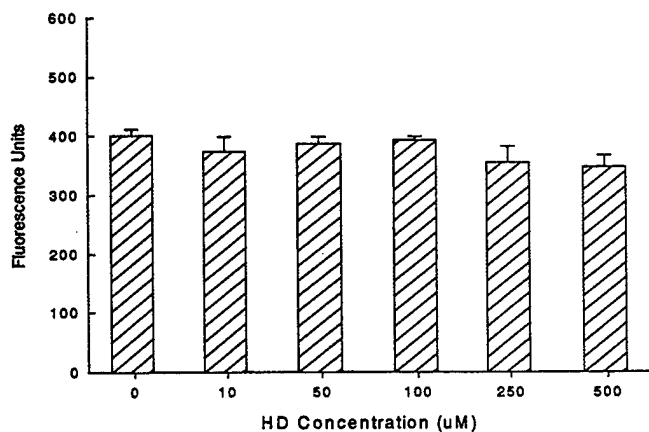


Figure 3. Effect of 5 different HD concentrations on protease release from PBL. Cells were treated with HD and assayed for protease as described. The supernatants (100 μ L) were withdrawn and placed in a fresh 96-well plate and read on a Cytofluor® II microplate spectrofluorometer at 530 nm after excitation at 485 nm. Each point is the mean \pm s.e.m. of 6 separate determinations.

The results from Figure 3 showed no activation of protease above that of control with the 5 different HD concentrations. Since the highly labeled casein substrate was not being hydrolyzed by protease, it is likely that the substrate was staying outside the cell because it was quite bulky. Non-lysed PBL and PBL lysed by exposure to 1% Triton X-100 were incubated with fluorescent substrate for 4 hours and the supernatants analyzed by the Cytofluor® II as described previously. The results are shown below in Figure 4.

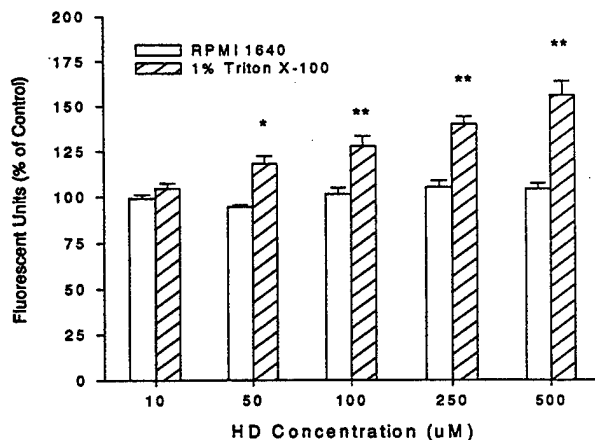


Figure 4. PBL were exposed to the indicated concentrations of HD and assayed for protease 20 hours later. Cells (\pm 1% Triton X-100) were incubated with fluorescent substrate for 4 hours as described in Materials and Methods and processed as described previously. Each point is the mean \pm s.e.m. of triplicate observations in 2 experiments. Level of significance by ANOVA was $p < 0.05$ (*) and $p < 0.01$ (**).

Unlysed PBL did not appear to release a protease with HD exposure, although cells lysed by addition of 1% Triton X-100 did release some type of protease in a HD dose-dependent fashion. Protease began to appear at 50 μ M HD and reached a maximum of approximately 50% above controls at 500 μ M. The addition of the 1% Triton X-100 also caused higher background fluorescence, presumably due to the unfolding of tertiary structure of the substrate in this detergent.

HEK exposed to HD were evaluated for protease with these fluorescent substrates by using cells grown on cover slips to 100% confluence (10). The data are shown in Figure 5 below.

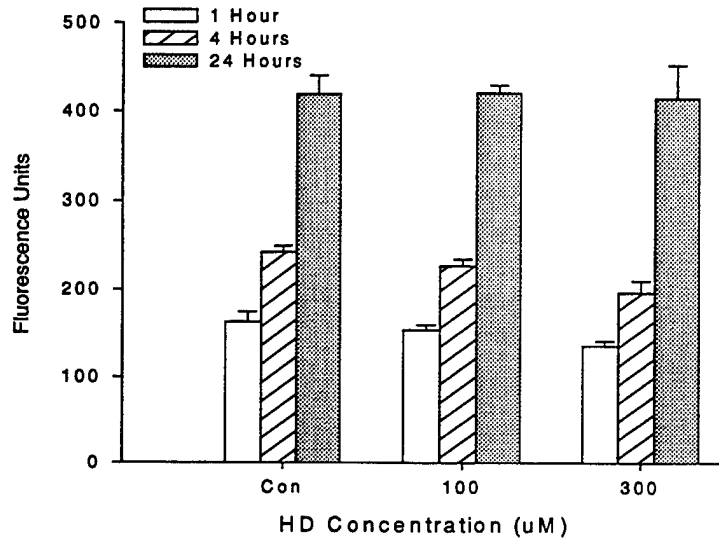


Figure 5- HEK grown on coverslips to 100% confluence were exposed to the two concentrations of HD and then incubated for 22 hours. The cells were processed as described and the supernatants were analyzed for protease activity. Each point represents the mean \pm s.e.m. in duplicate samples of 5 separate determinations.

When HEK grown on coverslips in 6-well tissue culture plates were exposed to 2 different concentrations of HD, there did not appear to be any HD-stimulated release of protease using these fluorescent substrates. Even though supernatants were withdrawn at 1, 4, and 24 hours for analysis, the substrate was not hydrolyzed to any measurable extent above untreated controls using HEK grown on coverslips. Cover slips were not exposed to Triton X-100 for lysis.

HEK grown on more conventional tissue culture support media were investigated for HD-stimulated protease release. In these experiments, HEK grown to confluencies of 80-100% in 24-well tissue culture plates were exposed to HD and analyzed 22 hours later. The data is shown below in Figure 6. Triton X-100 exposure of HEK did not influence protease release (data not shown).

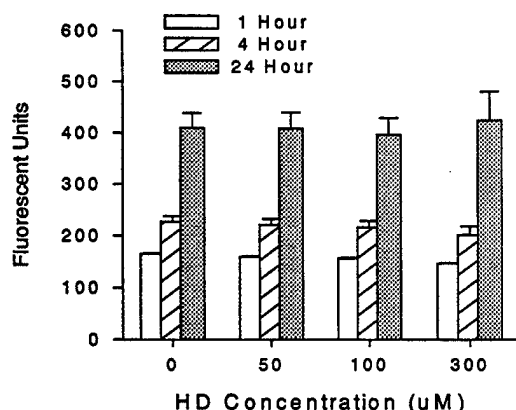


Figure 6. Twenty-four-well tissue culture plates of HEK were grown to confluencies of 80-100% and exposed to the indicated concentrations of HD. After 22 hours, fluorescent substrate was added and the supernatant was assayed for protease release at 1, 4, and 24 hours. Each point represents the mean \pm s.e.m. in 5 separate determinations.

At any of the times examined, protease release did not occur as a function of mustard treatment with HEK grown in 24-well tissue culture plates. Supernatants withdrawn at 1, 4, and 24 hours from HD-treated cells did not show any measurable substrate hydrolysis compared with non-exposed controls. A Triton X-100 lysis step did not influence release of protease.

Conclusions

Proteases have been implicated as responsible for the epidermal-dermal separation seen in the evolution of the frank blister from HD exposure (1). Some *in vitro* studies as well as a few *in vivo* studies appear to support this hypothesis. The inhibition of protease activity could be an important step in the treatment of the incapacitating and slowly healing blisters formed from HD exposure to skin. Anti-protease compounds have been identified as potential anti-vesicant medical countermeasures, but no convenient assay has been developed to assess their efficacies against protease activity.

A rapid and sensitive protease assay should be developed that uses substrates whose hydrolysis can be conveniently measured. Some of the protease assays use radioactive substrates whose hydrolysis products can be measured by scintillation counting, but disposal costs of radioactivity make these types of assays prohibitive. Some of the substrates have chromogenic leaving groups that can be measured spectrophotometrically, and our institute has investigated their possible use in some limited studies (11).

Recently, Molecular Probes has developed some fluorescent protease substrates that are sensitive to a variety of serine, cysteine, acidic, and metallo-proteases. These casein molecules are heavily labeled with either BODIPY-Fluorescein or BODIPY-Texas Red dyes that exist in a highly quenched form. Protease activity releases these dye-labeled peptides with an increase in fluorescence that is proportional to enzyme activity and can easily be measured by using a

microplate reader. These substrates were investigated by using two of our *in vitro* models, human PBL and HEK.

PBL have been used for studying effects of HD and do release proteases when using Chromozym TH from Boehringer Mannheim in selected donor PBL (11). According to this study, PBL from 7 out of 13 donors consistently displayed protease release following exposure to HD and appeared to be thrombin-like since the Chromozym-TH substrate was hydrolyzed. When the HD-exposed PBL were assayed for protease using the casein-BODIPY-substrates, none of the cells released protease as a function of HD exposure. When cells were lysed by the addition of 1% Triton X-100 and then incubated with the fluorescent substrates, there was a HD-dependent increase in protease activity. The fluorescently labeled substrates did not appear to be able to penetrate the cell, and lysis was necessary to distinguish any protease activity induced by the HD.

Prior studies with human epidermal keratinocytes (HEK) showed that there was protease activity between 100 and 300 μM HD with the chromogenic substrates Chromozym U and Chromozym t-PA. Using the fluorescently labeled casein-BODIPY substrates, no protease activity in HEK could be shown at HD concentrations ranging from 10-500 μM . HEK grown in 24-well tissue culture plates did not demonstrate protease activity even when 1% Triton was added (data not shown).

Fluorescently labeled casein substrates did not appear to be useful in devising a rapid 1-step assay for studying protease release in HD-exposed PBL or HEK. An intermediate Triton X-100 lysis step is mandatory if PBL are used and did not appear to work using HEK. The casein substrate appears to be hydrolyzed only if cells are lysed after exposure to HD.

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