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**Estimation of Neutrophil Infiltration into Hairless Guinea Pig
Skin Treated with 2,2'-Dichlorodiethyl Sulfide**

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ABSTRACT

Despite growing acceptance of the hairless guinea pig (HGP) for evaluating sulfur mustard (2,2'-dichlorodiethylsulfide, HD) skin injury, there are presently few antivesicant drug assessment endpoints validated in vivo for this model. We measured the activity of myeloperoxidase (MPO) to characterize the dose- and time-dependence of polymorphonuclear leukocyte (PMN) infiltration during development of the HD lesion. Biopsies were obtained from the dorsal thoracic-lumbar area of HGPs at successive 3 hr time intervals for up to 24 hrs following controlled exposure to either 5, 7, 8 or 10 min HD vapor. The presence of PMNs, as judged by MPO levels, peaked at 9 hrs irrespective of total HD vapor dose. The maximum response was a 20-fold increase compared to unexposed control sites at 9 hrs following 10 min HD vapor. This time period coincides with epidermal detachment characterized previously by electron microscopy in the HGP. By 24 hrs post-exposure, the MPO levels subsided markedly (2-fold compared to controls). These results suggest that PMNs participate in the HGP cutaneous inflammatory response following exposure to HD and that MPO may be a useful biological marker for evaluating putative antivesicants.

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INTRODUCTION

Since the first qualitative observations of leukocytic infiltration and accumulation at the site of HD cutaneous applications in controlled experimental animal studies¹, the relationship of leukocyte infiltration to pathological changes have not been systematically studied².

Exposure of selected skin areas to HD causes the production of lesions varying from mild erythema to vesication and tissue necrosis. When HD is administered intravenously or subcutaneously in chronic levels, it can lead to the destruction of the bone marrow, causing severe reduction of neutrophils and lymphocytes (leukopenia)³.

In a series of studies, Cullumbine et al¹, showed that HD applications to the skin caused an increase in capillary permeability within ten minutes. At thirty minutes post application, the changes were mild, and the nuclei of the cells of the epidermis were hyperchromatic with commencement of hydropic degeneration of the cells. At this time, leukocytic accumulation could be seen beneath the epidermis. After one hour, the changes were even more pronounced with the cells being swollen and white cells migrating from the small blood vessels. In the dermis, small areas of edema were beginning to appear between the collagen bundles. At three hours post application, the leukocytic infiltration around the hair follicles, ducts of glands, and below the epidermis was evident and definitive. This cascade of events may contribute to vesication, full blisters in humans and microblisters in some studied animal species¹. When HD is applied to the skin in sufficient levels it leads to tissue necrosis. The pathological changes have been described in detail elsewhere^{2,4,5}.

It is apparent that HD causes an inflammatory response. Flammagens such as arachidonic acid (AA)⁶, 12-O-tetradecanoyl-phorbol-13-acetate (phorbol ester, TPA)⁷, dinitrochlorobenzene (DNCB)⁸, and HD⁹ induce skin inflammation, which is characterized by a rapid onset of erythema, edema, and PMN infiltration after a single topical dose. This study unambiguously addresses the PMN infiltrating into the hairless guinea pig (HGP) skin at various times with a changing dose of HD. The measurement of myeloperoxidase (MPO) in the tissue was established to detect neutrophils¹⁰. In addition to MPO, tissue weights were also measured to assess edema.

MATERIALS AND METHODS

Animal Use and Care. Male [Cr1:1af/HA (hr/hr)BR VAF/PLUS^{U/J}] euthymic, hairless guinea pigs weighing 250-400 g from the Newfield, NJ breeding facility were used. Upon arrival, they were quarantined and screened for evidence of disease before use. They were maintained under an AAALAC accredited animal care and use program. The general

procedures used in this study have been described^{3,4,5}.

HD Exposure - Cutaneous application (exposure) to HD vapor were accomplished using previously described methods². Briefly, a quantity of neat HD was pipetted onto filter paper discs (Whatman # 2, 14mm dia.) attached to the inside top surface of plastic disposable vial caps. The quantity of HD was sufficient to completely saturate the filter disc without run-off. Following an equilibration period, the caps were adhered sequentially to the adhesive assembly over the exposure sites (holes in tape assembly). Forceps were used to apply or remove caps (ending exposures) and to remove tape assemblies from the skin. After vapor exposures, the guinea pigs were maintained in the respective polycarbonate cages for specific time intervals (see correlation Studies). Afterwards, the guinea pigs were euthanized with an inhalation overdose of halothane (or similar anesthetic) in a bell-jar. When histopathology was required, the skin over the dorsal thoracic-lumbar area was removed. Dermal punch (8 mm) specimens were immediately taken from the center of all exposure sites of each animal.

Myeloperoxidase Assay - Specimens were immediately placed into 15 mL conical plastic tubes and kept (one tube per animal) on ice during the specimens collection. Samples were weighted, 5 mL homogenate solution added, then homogenized in a polytron homogenizer at 12000 rpm for 1 minute. The homogenization buffer was 0.5% hexadecyltrimethyl-ammonium bromide in 50mM potassium phosphate, pH 6.0. It is important to note that the punches be drawn by the vortex action up and into the homogenization shaft. This explodes the tissue and drives it through the small vertical side openings. Homogenates were centrifuged for 20 min. at 14,000 x g. Afterwards, an aliquot of the supernatant was removed for the kinetic assay. To each microtiter well, 20 μ L of the skin homogenate was added. The plate was placed into the plate reader and 150 μ L of MPO reagent was added. The MPO reagent consisted of 0.167 mg/mL 3-3'-dimethoxybenzidine dihydrochloride, and 0.0005% hydrogen peroxide in 50 mM potassium phosphate pH 6.0. The plate was shaken prior to and during the assay. Total enzyme rate kinetics were optimized at 10 minutes. The assay was linear up to approximately 20.0 mOD/min. Output was in mOD Units (MPO)/punch¹⁰.

Correlation Studies - A total of six animals were utilized for each time point. Five animals were exposed for each time point. The sixth animal served as a naive (untreated) control. Each group of 4 animals were prepared for skin punches at the following time points: 3, 6, 9, 12, 15, 18, 21, and 24 hours. In addition to the exposed positions (8 sites per animal), two additional adjacent punches were utilized as internal controls for the MPO assay. The total exposure time was either 5, 7, 8, or 10 minutes per study group.

RESULTS AND DISCUSSION

Neutrophilic infiltration and edema are characteristic features of cutaneous inflammation and quantitative changes can be taken as a measure of the intensity of the injury process. As various factors act to stimulate neutrophil infiltration following HD exposure to the dermis, strict experimental conditions are required to demonstrate a relationship between dose level, time of exposure and post-treatment time accumulation.

In the present study, changes in tissue weights and MPO activity were measured to assess cutaneous inflammation, as a result of HD injury. A significant change in tissue weight was noted within 9 hours (18 % increase) after HD exposure. The maximum levels of edema were found at 15 hours post 5 and 7 min HD exposures, as evidenced by an 18 and 36 % weight increase, respectively (see Figure 1).

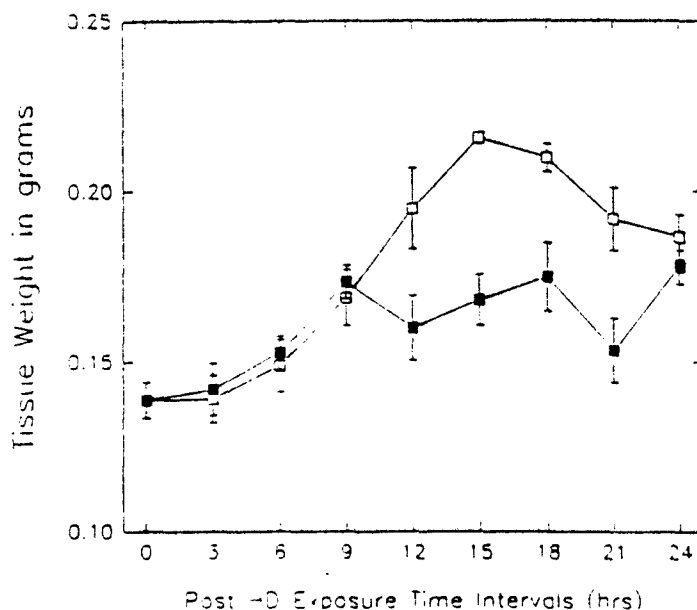


Figure 1. Time course for the development of edema after treatment with vapor mustard (HD). The two curves represent 5 min (■) and 7 min (□) vapor exposures. Groups of 5 hairless guinea pigs were treated with HD and then at 3, 6, 9, 12, 15, 18, 21 and 24 hrs groups were sacrificed, tissues were weighted as an indicator of edema formation. Time 0 represents naive (no treatment) controls.

These data agree with the clinical observations of mustard injury in man². The accumulation of neutrophils was measured at 3 hr intervals post 5 and 7 min HD exposures. There was a quantifiable change in the levels of neutrophils with maximum levels measured at 9 and 12 hrs for 7 and 5 min post exposures, respectively (see Figure 2).

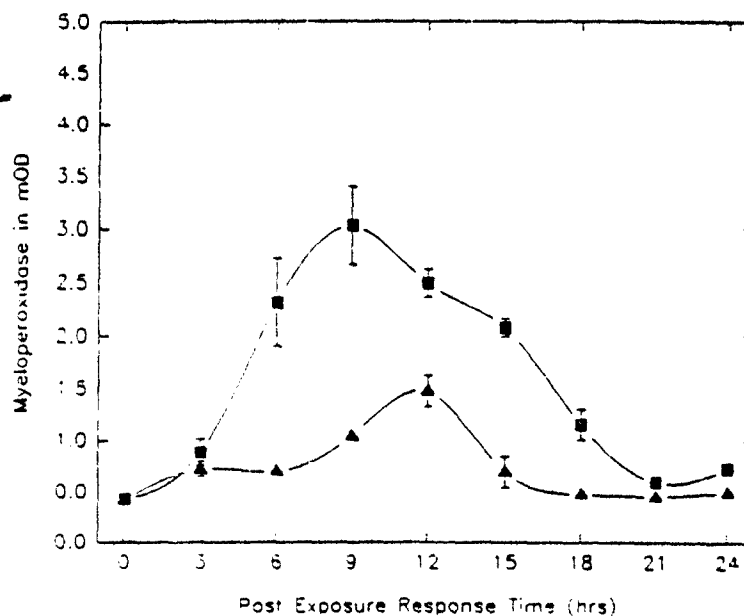


Figure 2. Time course for the appearance of neutrophils (myeloperoxidase-positive cells) after treatment with vapor mustard (HD). The two curves represent 5 min (▲) and 7 min (■) vapor exposures. Groups of 5 hairless guinea pigs were treated with HD and then at 3, 6, 9, 12, 15, 21, and 24 hrs groups were sacrificed and tissue taken for myeloperoxidase determination as described in Materials and Methods.

Initially it appeared that the shift in maximum aggregation was dose dependent. As a result neutrophilic infiltration measurements were redefined in a series of studies at 5, 7, 8, and 10 min HD exposures and MPO measured at 3, 6, 9, and 12 hrs. There was a definite 9 hrs maximum level for each exposure. It was also evident that HD induction of inflammatory cells did not yield a linear dose dependent curve.

It was found that 10 min exposures caused the most rapid increase at 3 hrs post exposure. Note that in table 1, the levels of MPO continuously increase with a maximum level at nine hours.

Table 1. Myeloperoxidase content of HD-treated HGP skin

Time (hrs)	HD (min)	MPO (mOD) mean $\pm$ sem	HD (min)	MPO (mOD) mean $\pm$ sem
3	7	1.0 $\pm$ 0.18	10	1.20 $\pm$ 0.30
6	7	2.31 $\pm$ 0.41	10	1.92 $\pm$ 0.14
9	7	3.02 $\pm$ 0.37	10	1.93 $\pm$ 0.54
12	7	2.53 $\pm$ 0.12	10	2.17 $\pm$ 0.05

There is a definite relationship between HD and leukocytic infiltration, but it appears that 7 to 8 min VC exposures may induce the maximum levels of neutrophils at the tissue. The overall trend is graphically represented (see Figure 3) to show that neutrophils systematically and quantitatively infiltrate the tissues.

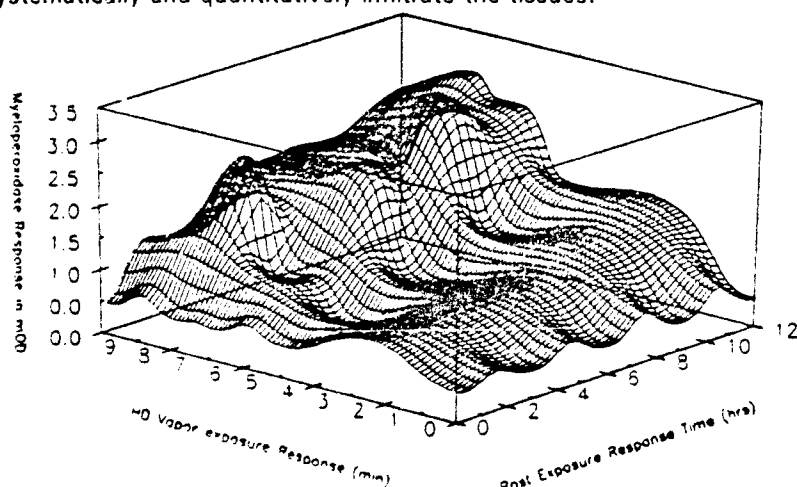


Figure 3. A three dimensional view of temporal infiltration of neutrophils (myeloperoxidase-positive cells) as a function of an increasing sulfur mustard (HD) level vs. time. Groups of 5 hairless guinea pigs were treated with HD and then at 3, 6, 9, and 12 hrs groups were sacrificed and tissue taken for MPO determination. Groups were exposed at 5, 7, 8, 10 min HD vapor exposures.

These results suggest that: (1) PMNs participate in the HGP cutaneous inflammatory response following exposure to HD, (2) MPO peak response is seen at 9 hrs and (3) MPO is a useful biomarker for evaluating putative antivesicants.

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