



U.S. ARMY MEDICAL RESEARCH INSTITUTE OF CHEMICAL DEFENSE

USAMRICD-TR-96-05

Assessment of Edemogenic Effects with Cutaneous Sulfur Mustard Using the Mouse Ear Model

Fred W. Stemler
Robert P. Casillas
Theresa M. Tezak-Reid
Robyn B. Lee
Lilia R. Castrejon

November 1996

19970422 055

Approved for public release; distribution unlimited

U.S. Army Medical Research
Institute of Chemical Defense
Aberdeen Proving Ground, MD 21010-5425

DISPOSITION INSTRUCTIONS:

Destroy this report when no longer needed. Do not return to the originator.

DISCLAIMERS:

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

In conducting the work described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," NIH Publication 86-23, revised in 1985.

The use of trade names does not constitute an official endorsement or approval of the use of such commercial hardware or software. This document may not be cited for purposes of advertisement.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE November 1996	3. REPORT TYPE AND DATES COVERED April 1995 - July 1995	
4. TITLE AND SUBTITLE Assessment of Edemogenic Effects with Cutaneous Sulfur Mustard Using the Mouse Ear Model			5. FUNDING NUMBERS 61102A 3M161102BS11	
6. AUTHOR(S) Stemler, F.W., Casillas, R.P., Tezak-Reid, T.M., Lee, R.B., and Castrejon, L.R.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research Institute of Chemical Defense ATTN: MCMR-UV-DB 3100 Ricketts Point Road Aberdeen Proving Ground, MD 21010-5425			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research Institute of Chemical Defense ATTN: MCMR-UV-RC 3100 Ricketts Point Road Aberdeen Proving Ground, MD 21010-5425			10. SPONSORING/MONITORING AGENCY REPORT NUMBER USAMRICD-TR-96-05	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) Mustard gas (bis(2-chloroethyl) sulfide, HD) is a potent vesicant that rapidly penetrates the skin causing gross and microscopic pathological changes in humans. No definitive animal model produces elevated fluid-filled blisters (bullae) like those seen in human skin. Five dosages of HD (0.08, 0.16, 0.32, 0.64, or 1.28 mg) in methylene chloride (MeCl ₂) were applied to the inner surface of the right ears of male CD 1 mice (n=10/dosage). Methylene chloride only was applied to the inner surface of the left ears (vehicle control). At 6, 12, 18, and 24 hours post-exposure, 8 mm dermal skin punch specimens were taken from the center of exposed and control ears and weighed to determine fluid accumulation (edema). Skin punches were then fixed in 10 % formalin, embedded in paraffin and stained with hematoxylin/eosin (H&E) for histopathological evaluation. A two way ANOVA on the percent of control edema weight found a significant dose by time interaction, implying that the responses to doses of HD differ with respect to time. Preliminary microscopic examination suggests that sulfur mustard produces tissue damage that is consistent with vesicant injury. Dose and time related edema increases in HD exposed mouse ear skin support the use of this model for studies involving medical countermeasures to cutaneous HD injury.				
14. SUBJECT TERMS Sulfur Mustard, Mouse Ear Model, Edema, Vesicant, Methylene Chloride			15. NUMBER OF PAGES 17	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT UNCLASSIFIED	18. SECURITY CLASSIFICATION OF THIS PAGE UNCLASSIFIED	19. SECURITY CLASSIFICATION OF ABSTRACT UNCLASSIFIED	20. LIMITATION OF ABSTRACT UNLIMITED	

PREFACE

The work reported herein was conducted under U.S. ARMY MEDICAL RESEARCH INSTITUTE OF CHEMICAL DEFENSE Protocol 1-21-95-000-A-712, entitled "Further Studies of the Mouse Ear Model as an *In Vivo* Bioassay for the Assessment of Topical Sulfur Mustard Injury." The data are recorded in U.S. Medical Research Institute of Chemical Defense notebook 014-95. The work was initiated in April 1995 and completed in July 1995. A portion of this work was presented in abstract and in a poster session (Assessment of cutaneous sulfur mustard injury in the mouse ear model by Casillas, R.P., Smith, K.J., Reid, T.M., Castrejon, L.R and Stemler, F.W.) at Society of Toxicology, Anaheim, CA, March 1996.

ACKNOWLEDGEMENTS

The authors would like to thank Ernest H. Braue, Ph.D., Ruth Ann Way and Catherine R. Nalls for the training sessions in working safely with sulfur mustard. The authors are also grateful to SPC Fairley R. Smith, Jr., SPC John E. Zallnick, and CPT Lisa M. Tatum for their assistance with experiments, and especially to Thomas P. Logan, Ph.D, for performing off-gassing studies to determine the minimum post-exposure time at which animals could be safely handled.

TABLE OF CONTENTS

INTRODUCTION	1
MATERIALS AND METHODS	1
RESULTS	2
DISCUSSION	8
REFERENCES	11
DISTRIBUTION LIST	13

INTRODUCTION

Mouse ear assays are useful models for studying cutaneous irritant and allergic inflammation (Bouclier et al., 1990; Carlson et al., 1985; Crummey et al., 1987; Patrick et al., 1987). These assays allow for the quantitative assessment of biological and biochemical processes following a chemically induced cutaneous response, and for evaluating pharmacological agents against skin damage. Development of the mouse ear model as a suitable model of cutaneous sulfur mustard injury with potential for screening antivesicants was initiated at the USAMRICD by Brinkley et al. (1989). This current study was undertaken to determine the time course of edema formation in the mouse ear over a broader range of HD dosages in order to complement the findings in the studies of Brinkley et al. (1989 and 1995) and to identify a dose response relationship for evaluating therapeutic compounds.

MATERIALS AND METHODS

Experimental Design and General Procedures: The effect of topical application of sulfur mustard to the inner surface of the right ear was evaluated in albino male mice (Charles River CD 1 Strain) weighing 25-35 grams. The mice were maintained under an American Association for Accreditation of Laboratory Animal Care (AAALAC) program. During quarantine the animals were housed 10 per polycarbonate cage on contact bedding which was changed twice weekly. The mice were provided commercial certified rodent ration as appropriate and tap water *ad libitum*. Animal holding rooms were maintained at $21^{\circ} \pm 2^{\circ}\text{C}$ with $50\% \pm 10\%$ relative humidity using at least 10 complete air changes per hour of 100% conditioned fresh air. All mice were on a 12-hour light/dark, full-spectrum lighting cycle with no twilight.

Standard solutions with 12.5, 25, 50, 100 and 200 $\mu\text{l/ml}$ of neat HD ($d = 1.27 \text{ g/ml}$; M W 159; purity 97.5%) in methylene chloride were prepared. The respective standard solutions were equivalent to 0.08 (0.5), 0.16 (1.0), 0.32 (2.0), 0.64 (4.0), and 1.28 (8.0) mg (μmoles) of HD/5 μl aliquot. A 5.0 μl aliquot of the respective solutions was applied topically to the right ear of animals (10 mice/group) with a digital microliter positive displacement pipette (Drummond Scientific Co., Catalog No. 510, Broomall, PA). Due to the volatility of methylene chloride the bottle containing HD solution was immediately recapped following removal of an aliquot. A 5.0 μl aliquot of methylene chloride only was placed on the left ear as control. For comparative purposes, a group of 12 mice received 5 μl of methylene chloride only (six on right ear and six on left) while the opposite ear received no fluid. The latter group was euthanized at the end of 24 hours and evaluated for effects of vehicle on edema.

The mice were weighed and anesthetized with ketamine (57.1 mg/kg) and xylazine (11.4 mg/kg) administered as a single injection intraperitoneally. The mice were placed in a dorsal recumbency on a 4" by 6" cardboard pad with strips of tape over the two front legs and the tail. A large cotton swab (Texwipe) beneath the head was rotated to position the cupped right ear in a horizontal plane. Following evaporation of applied solutions, two mice were housed together in a

a polycarbonate container in the hood. Each container, resting on a pad with circulating heated water, was covered with a plastic backed paper diaper to protect the animals from draft.

All animals were kept in the hood until euthanized in a chamber containing halothane at 6, 12, 18, or 24 hours post-exposure. Both ears were removed and a circular 8-mm punched specimen from each ear was placed in a tared vial and was weighed on an analytical balance (Mettler-Toledo Co., Model A0104, Switzerland).

Edema was expressed as percent of control according to the following equation:

$$\frac{\text{weight right ear (exposed)} - \text{weight left ear (control)}}{\text{weight left ear (control)}} \times 100$$

Statistical analysis.

A two way ANOVA was used to compare weights of left versus right ears and weights of untreated ears (no MeCl₂) versus MeCl₂ treated ears. A two way ANOVA was used to assess dose by time interactions. The amount of edema following each dose was compared using percent of control edema. Statistical significance was defined as $p < .05$. A significant dose by time interaction was observed. Therefore, one way ANOVAS were used to analyze dose differences at each observation time followed by Newman-Keuls tests to assess individual dose differences.

RESULTS

The weights of methylene chloride only treated ears (right or left) and contralateral untreated ears are shown in Figure 1. No significant differences ($p < .05$) in ear weights were observed for untreated (no MeCl₂) versus MeCl₂ treated ears and in left versus right ear.

The weights of control left (MeCl₂ only) and right ears at the five dosages of HD are shown in Figures 2, 3, 4, and 5. The percent of control was calculated for edema and used to compare the doses at each observation. At all time points, the three lower doses, 0.08, 0.16, 0.32 mg HD, had significantly lower percent of control edema ($p < .05$) than the 0.64 mg HD dose. At 6-, 12- and 24-hour time points, the three lower doses had significantly lower percent of control edema than the 1.28 mg HD dose, $p < .05$. In addition, at all time points, the 0.08 mg HD dose had a significantly lower edema effect than the 0.32 mg HD dose, $p < .05$.

The initial localized effect of HD, which begins as an erythema, was visibly obvious in all animals within 30 minutes. Subsequent observations of left and right ears revealed a progressive thickening and swelling of the right ear. Fluid accumulation as measured by progressive weight increases was observed to continue throughout the periods of measurement from six to 24 hours. Histological evaluation is ongoing and will be reported when completed.

24 hour post exposure control vs methylene chloride

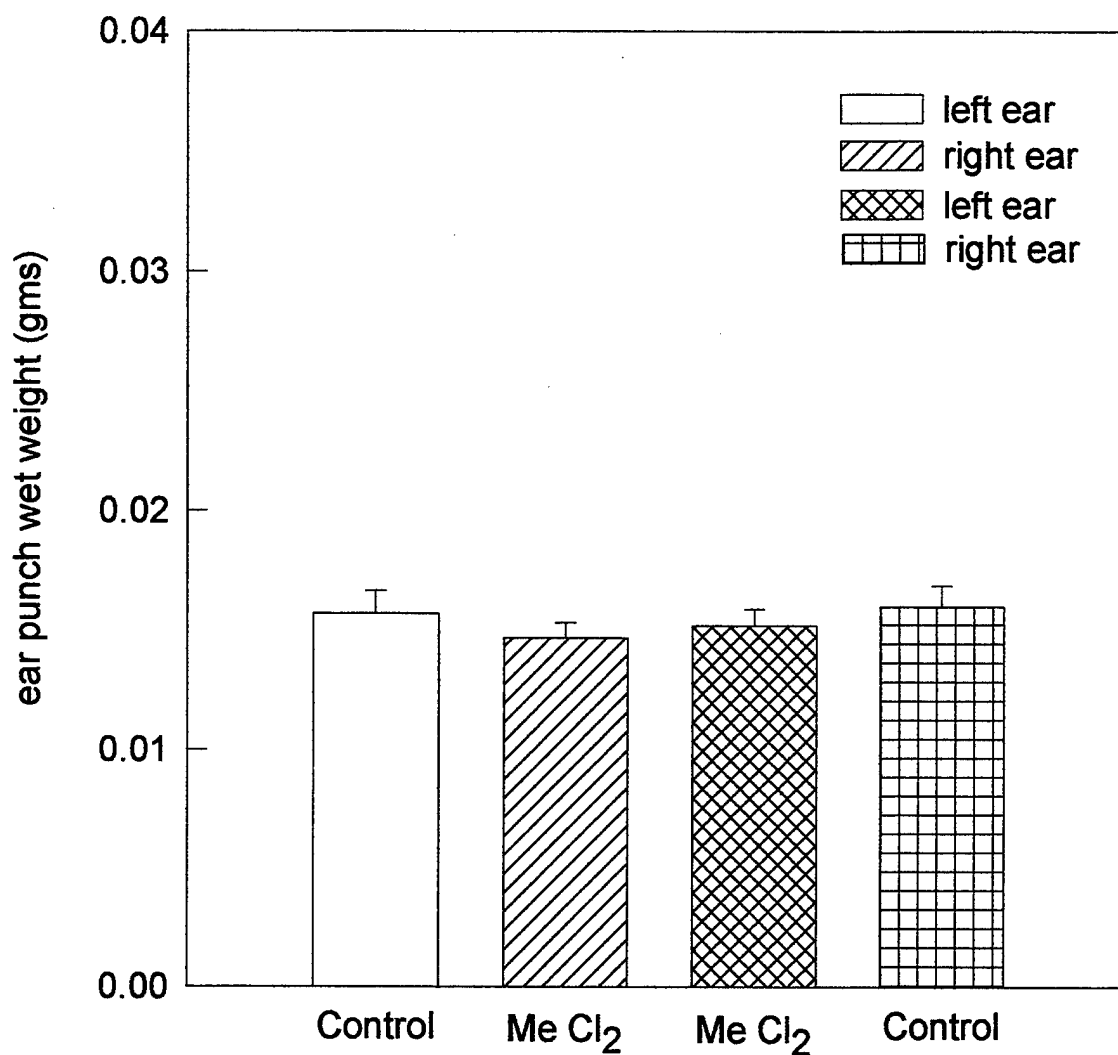


Figure 1 shows the mean weights of left and right ears of mice (N = 6/group) in which 5 μ l methylene chloride only was applied to one ear, while the opposing ear served as control. Error bars = \pm SEM. $p < .05$.

6 hour post-exposure

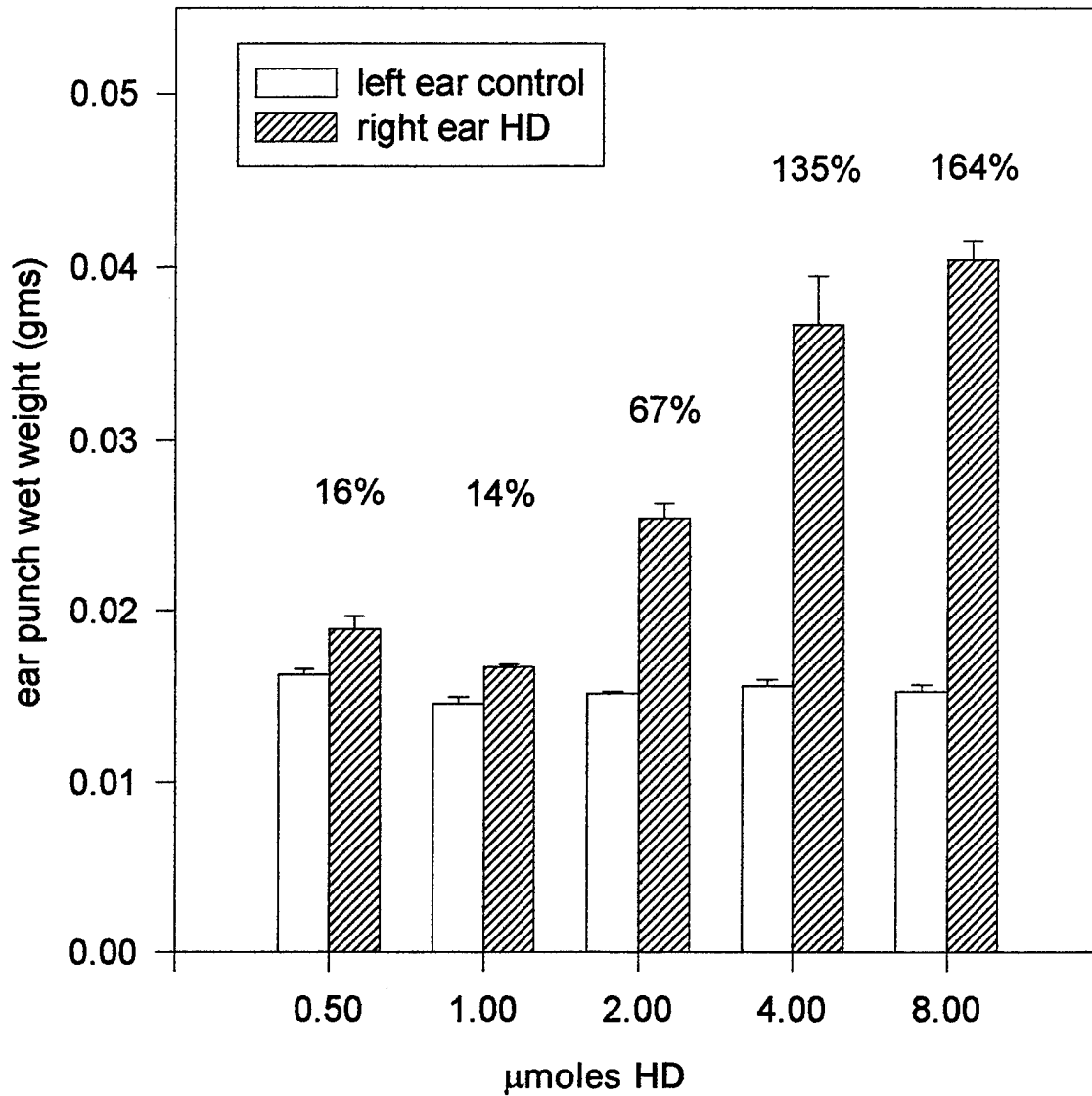


Figure 2 shows the mean weights at 6 hours post-exposure of left methylene chloride treated ears and after topical application of 5 μ l aliquots of HD on the right ears of mice (N = 10/group). Error bars = \pm SEM. $p < .05$. Numbers refer to % of control.

12 hour post-exposure

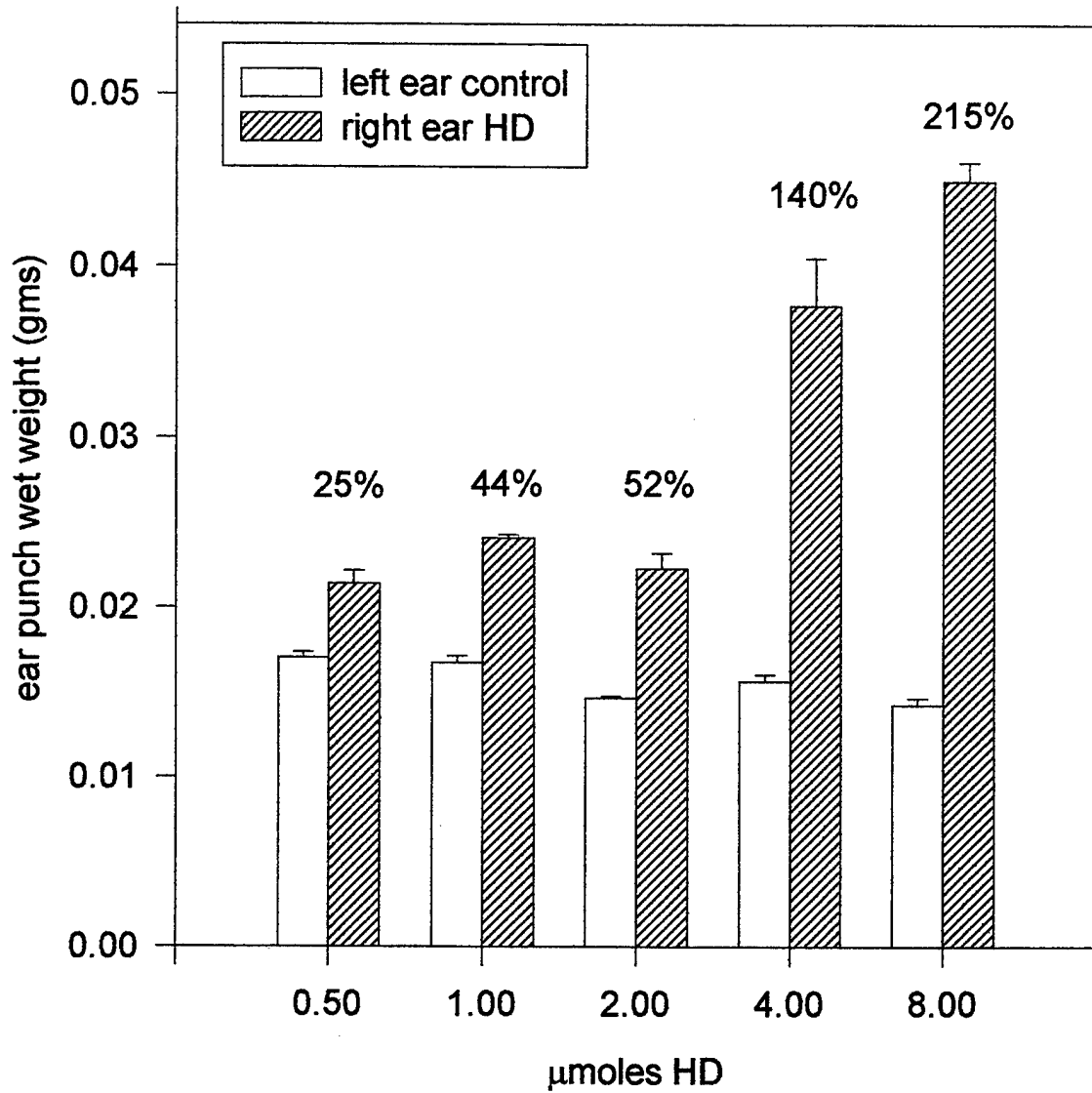


Figure 3 shows the mean weights at 12 hours post-exposure of left methylene chloride treated ears and after topical application of 5 μ l aliquots of HD on the right ears of mice (N = 10/group). Error bars = \pm SEM. $p < .05$. Numbers refer to % of control.

18 hour post-exposure

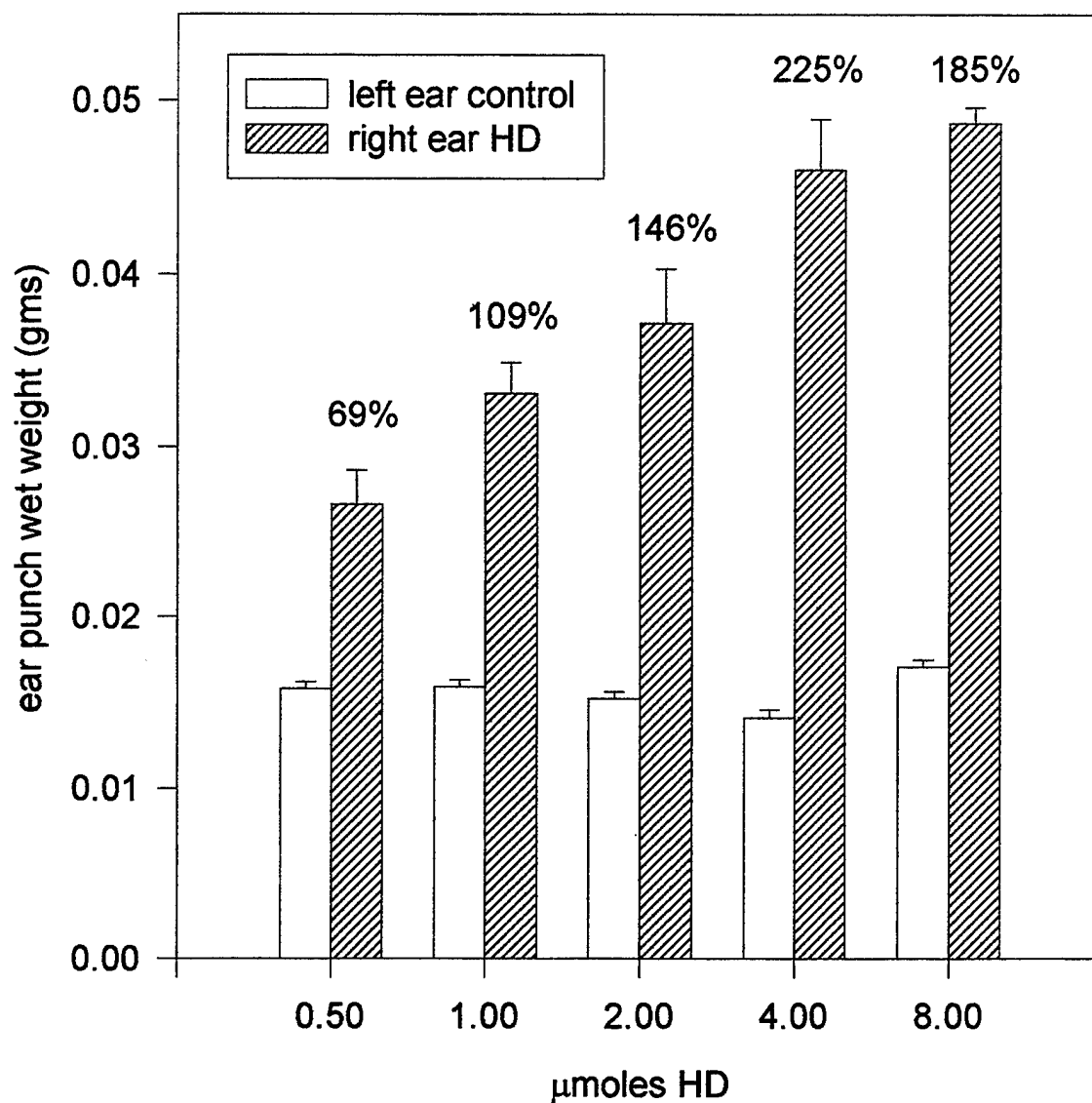


Figure 4 shows the mean weights at 18 hours post-exposure of left methylene chloride treated ears and after topical application of 5 μ l aliquots of HD on the right ears of mice (N = 10/group). Error bars = \pm SEM. $p < .05$. Numbers refer to % of control.

24 hour post-exposure

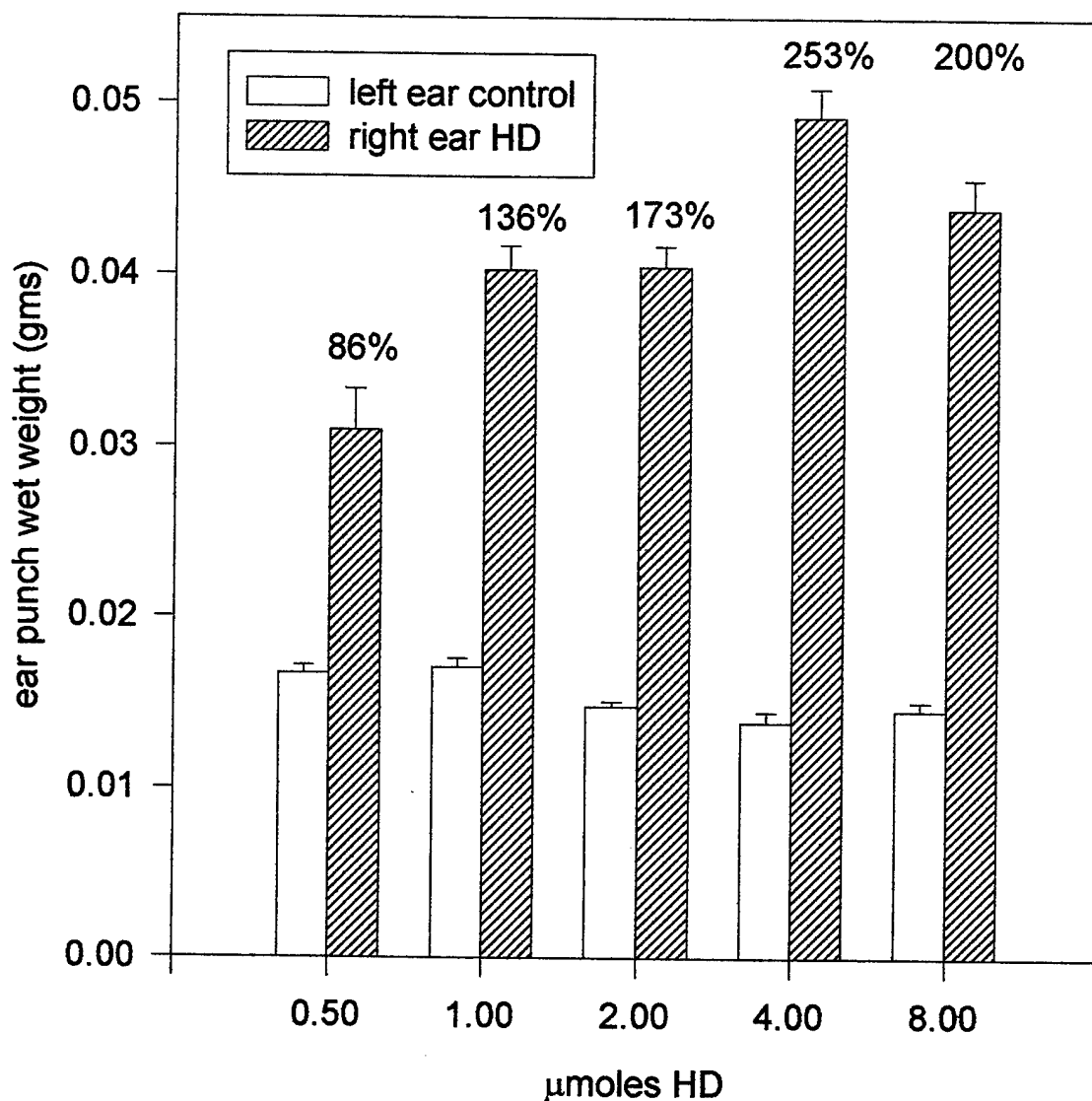


Figure 5 shows the mean weights at 24 hours post-exposure of left methylene chloride treated ears and after topical application of 5 μ l aliquots of HD on the right ears of mice (N = 10/group). Error bars = \pm SEM. $p < .05$. Numbers refer to % of control.

DISCUSSION

The prolonged time course of fluid accumulation observed following topical cutaneous application to HD on the mouse ear differs from the time course of edema seen with other irritants in this same model (Patrick et al., 1987). Inflammatory responses by measurements of ear weight or ear thickness were compared, and the responses showed that the time course varied considerably with the irritant. Maximal edema responses occurred in a range of minutes to hours. The prolonged duration of edema in response to HD in the mouse ear model contrasts sharply with the course of edema produced by two commonly used irritants, 12-*O*-tetradecanoylphorbol acetate (TPA) and arachidonic acid (AA) (Young et al., 1983). The application of TPA to the mouse ear produced a maximal edema response by 6 hours which was decreased substantially by 24 hours. With AA a maximal edema response was reported by 1 hour which decreased substantially by 6 hours. In contrast, fluid accumulation in HD exposed ears at three low dosages (0.08, 0.16 and 0.32 mg HD) progressed throughout the 24-hour period of observation. However, at two higher dosages (0.64 and 1.28 mg HD) fluid accumulation was accelerated and appeared to reach a maximal edema response at six hours. The skin from the mouse ear does develop initial erythema and edema followed by eventual separation of the epidermal-dermal junction to form microblisters which are consistent with HD blister formation in humans. HD differs also from the two above mentioned irritants with the delayed formation of microblisters.

Erythema is due to arteriolar vasodilation. However, the leakage of vascular protein and fluid into the extravascular tissue space is a more complex event involving the interaction of many physiologic factors. These factors include permeability changes in the vascular endothelial lining, mediator release, blood flow changes and/or accumulation of leukocytes (Patrick et al., 1985). The initial formation of tissue edema is due to leakage of proteins through postcapillary venules (Sedgwick and Willoughby, 1985).

It is unknown whether all chemical irritants produce inflammatory responses via the same mechanisms. A comparative study of reported tissue weight or ear thickness in response to several irritants applied to the mouse ear suggested that chemicals produce skin irritation by multiple mechanisms (Patrick et al., 1985). Microscopic examination of tissue lesions showed that each chemical differed in the time course of edema, vessel dilation, cellular infiltrates, permeability responses to dyes and ear temperatures. The latter is an indirect estimate of blood flow. Currently, there are several hypotheses which attempt to explain the mechanism(s) underlying irritant effects of HD (Papirmeister et al., 1991).

Despite a better basic understanding of the mechanisms of sulfur mustard injury, relatively few new therapeutic advances in treatment of HD injury have been made in the past fifty years. The rapidity of cutaneous HD penetration and resultant physiological damage have placed limitations on therapeutic options. Thus, the HD mouse ear model may be a valuable tool for evaluating and screening pretreatment pharmacologic compounds for anti-inflammatory effects, for

antiedemogenic activity, and/or possible inhibition of microvesication. Comparisons of the effectiveness of anti-inflammatory compounds against HD and other irritants in the mouse ear model may also provide insights into the mechanism(s) underlying inflammatory responses.

REFERENCES

- Brinkley, F.B., Mershon, M.M., Yaverbaum, S., Doxzon, B.F., and Wade, J.V. 1989. The Mouse Ear Model as an *In Vivo* Bioassay for the Assessment of Topical Mustard (HD) Injury. In Proceedings of the 1989 Medical Defense Bioscience Review, pp 595-602, U.S. Army Medical Research Institute of Chemical Defense, APG, MD. AD B139550.
- Brinkley, F.B., Maitland, G.F. and Hackley Jr., B.E. 1995. Validation of the Mouse Ear Model as an *In Vivo* Bio-assay for the Assessment of Topical Mustard (HD) Injury. USAMRICD-TR-95-04, U. S. Army Medical Research Institute of Chemical Defense, APG, MD. AD A296752.
- Bouclier, M., Luginbuhl, B., Shroot, B. and Hensby, C. N. 1990. Arachidonic Acid Induced Ear Oedema in Four Strains of Rats and Mice: A Comparative Study of Anti-inflammatory Drugs. Agents and Actions 29: 62-64.
- Carlson, R.P., O'Neill-Davis, L., Chang, J. and Lewis, A.J. 1985. Modulation of Mouse Ear Edema by Cyclooxygenase and Lipoxygenase Inhibitors and Other Pharmacologic Agents. Agents and Actions 17: 197-204.
- Crummey, A., Harper, G.P., Boyle, E.A. and Mangan, F.R. 1987. Inhibition of Arachidonic Acid-induced Ear Oedema as a Model for Assessing Topical Anti-inflammatory Compounds. Agents and Actions 20: 69-76.
- Papirmeister, B., Feister, A.J., Robinson, S.I. and Ford, R.D. 1991. Medical Defense against Mustard Gas: Toxic Mechanisms and Pharmacological Implications. CRC Press Inc. Boca Raton, Florida.
- Patrick, E., Maibach, H.I. and Burkhalter, A. 1985. Mechanisms of Chemically Induced Skin Irritation 1. Studies of Time Course, Dose Response, and Components of Inflammation in the Laboratory Mouse. Toxicol. Appl. Pharmacol. 81: 476-490.
- Patrick, E., Burkhalter, A. and Maibach, H. 1987. Mouse Ear Assays as Models for Studying Mechanisms of Chemically Induced Irritation. Models Dermatol. 3: 86-92. Models in Dermatology 1987 Eds. Maibach, H.I. and Lowe, N.J. Karger, London.
- Sedgwick, A.D. and Willoughby, D.A. 1985. Initiation of the inflammatory response and its prevention. Handbook of Inflammation Volume 5 Eds. Glynn, L.E., Houck, J.C. and Weissmann, G. The Pharmacology of Inflammation. Eds. Bonta, I.L., Bray, M.A. and Parnham, M.J. 27-47.
- Young, J.M., Wagner, B.M., and Spires, D.A. 1983. Tachyphylaxis in 12-O-Tetradecanoylphorbol Acetate- and Arachidonic Acid-Induced Ear Edema. J. Invest. Dermatol. 80:48-52.

DISTRIBUTION LIST

Addresses	Copies	Addresses	Copies
DEFENSE TECHNICAL INFORMATION CENTER ATTN DTIC OCP 8725 JOHN J KINGMAN RD STE 0944 FT BELVOIR VA 22060-6218	2	DIRECTOR ARMED FORCES MEDICAL INTELLIGENCE CENTER FORT DETRICK MD 21702-5004	1
COMMANDER US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND FORT DETRICK MD 21702-5012	2	COMMANDER US ARMY INSTITUTE OF DENTAL RESEARCH BUILDING 40 WASHINGTON DC 20307-5300	1
HQDA DASG HCD WASHINGTON DC 20310	1	COMMANDER US ARMY INSTITUTE OF SURGICAL RESEARCH BUILDING 2653 FORT SAM HOUSTON TX 78234-6200	1
DIRECTOR WALTER REED ARMY INSTITUTE OF RESEARCH BUILDING 40 WASHINGTON DC 20307-5100	1	COMMANDANT ACADEMY OF HEALTH SCIENCES US ARMY ATTN HSHA CDC FORT SAM HOUSTON TX 78234-6100	1
COMMANDER US ARMY AEROMEDICAL RESEARCH LABORATORY ATTN SCIENTIFIC INFORMATION CENTER PO BOX 577 FORT RUCKER AL 36362-5000	1	COMMANDANT ACADEMY OF HEALTH SCIENCES US ARMY ATTN HSHA CDM FORT SAM HOUSTON TX 78234-6100	1
COMMANDER US ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES BUILDING 1425 FORT DETRICK MD 21702-5011	1	DIRECTOR ENVIRONMENTAL AND LIFE SCIENCES OFFICE OF THE DEPUTY DIRECTOR FOR RESEARCH AND ENGINEERING ROOM 3D129 WASHINGTON DC 20301-2300	1
COMMANDER US ARMY RESEARCH INSTITUTE OF ENVIRONMENTAL MEDICINE BUILDING 42 NATICK MA 01760-5007	1	COMMANDER US ARMY TRAINING AND DOCTRINE COMMAND ATTN ATMD FORT MONROE VA 23651	1
COMMANDANT US ARMY CHEMICAL SCHOOL ATTN ATZN CM C FORT MCCLELLAN AL 36205	1		

COMMANDER US ARMY NUCLEAR AND CHEMICAL AGENCY 7500 BACKLICK ROAD BUILDING 2073 SPRINGFIELD VA 22150-3198	1	AFOSR/NL BUILDING RM A217 BOLLING AFB DC 20332	1
EXECUTIVE OFFICER NAVAL MEDICAL RESEARCH INSTITUTE NAVAL MEDICINE COMMAND NATIONAL CAPITAL REGION BETHESDA MD 20814	1	COMMANDER US ARMY CHEMICAL BIOLOGICAL DEFENSE AGENCY ATTN AMSCB CI ABERDEEN PROVING GROUND MD 21010-5423	1
USAF ARMSTRONG LABORATORY/CFTO SUSTAINED OPERATIONS BRANCH BROOKS AFB TX 78235-5000	1	LTC RICHARD R. STOTTS BATTELLE MEMORIAL INSTITUTE JM 3 505 KING AVENUE COLUMBUS OH 43201-2695	1
DEPARTMENT OF HEALTH AND HUMAN SERVICES NATIONAL INSTITUTES OF HEALTH THE NATIONAL LIBRARY OF MEDICINE SERIAL RECORDS SECTION 8600 ROCKVILLE PIKE BETHESDA MD 20894	1	COMMANDER US ARMY MEDICAL RESEARCH INSTITUTE OF CHEMICAL DEFENSE 3100 RICKETTS POINT ROAD ATTN MCMR UV ZA MCMR UV ZB MCMR UV ZS MCMR UV RC (5 copies) MCMR UV R (11 copies) MCMR UV AI W MCMR UV D MCMR UV P MCMR UV C ABERDEEN PROVING GROUND MD 21010-5425	23
STEMSON LIBRARY ACADEMY OF HEALTH SCIENCES BUILDING 2840 RM 106 FORT SAM HOUSTON TX 78234-6100	1		
US ARMY RESEARCH OFFICE ATTN CHEMICAL AND BIOLOGICAL SCIENCES DIVISION PO BOX 12211 RESEARCH TRIANGLE PARK NC 27709-2211	1		