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PRINCIPAL INVESTIGATOR: Shambhu D. Varma, Ph.D.

CONTRACTING ORGANIZATION: University of Maryland at Baltimore
Baltimore, Maryland 21201-1691

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Shambhu D. Varma, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Maryland at Baltimore
Baltimore, Maryland 21201-1691

E-Mail: svarma2384@aol.com

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Sulfur mustard (HD) is one of the most common chemical warfare agents. The immediate manifestation of exposure to this agent consists of a generalized irritation and intense itching all over the body. This is soon followed by development of dermal erythema and blisters in various regions of the body. This report summarizes the indicial findings using the water soluble components of the above mixture against damage to mouse skin under incubation in vitro. The specific objectives of these experiments were to determine (a) if the deterioration of the barrier function of the skin epidermis as measured by the loss of the restriction to the trans dermal movement of rubidium ions caused by CEES could be prevented and (b) to evaluate the effectiveness of such a mixture at histological level.

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Sulfur Mustard Damage: Preventive Studies.
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Investigators:
(PI) Shambhu Varma Ph.D.
Collaborators
Medhat Henein, DVM.
John Petrali, Ph.D.

Consultant: Brian Lukey Ph.D.

Introduction:

Sulfur mustard (HD) is one of the most common chemical warfare agents. The immediate manifestation of exposure to this agent consists of a generalized irritation and intense itching all over the body. This is soon followed by development of dermal erythema and blisters in various regions of the body.
The toxicity seems to be triggered primarily by its strong alkylating effects, modifying a host of structural and nonstructural but metabolically important cellular constituents. However, development of an effective therapy, prophylactic or post-exposure, against mustard toxicity has been difficult and none is available so far. We propose that such difficulty may be due to use of preparations containing single agents. The failure of treatment by use of such preparations can be linked to the inability of active compounds to exert a more generalized inhibition against alkylation of the diverse of the tissue constituents, at sites extending from the outer limiting cell membranes to the cytosolic, mitochondrial and intra-nuclear regions. Attention has also not been given to toxicity caused by a simultaneously general inhibition of cellular metabolism because of the alkylation of various –SH and –NH- groups of the enzymes and their cofactors, and consequent decreased energy production required to drive transport pumps and sustain biosynthetic and repair activities. We have, therefore, continued to study the biochemical and physiological mechanisms involved in mustard damage and examine if the physiological damage caused by exposure to this gas can be prevented by topical treatment with preparations consisting of compounds endowed with appropriate biochemical and physiological properties such as.

(1) Competitively inhibit alkylation at -SH and --NH sites of the cellular constituents,
(2) Cleave –SS-to- SH
(3) Scavenge reactive Oxygen species and minimize oxidative stress to the tissue
(4) Provide additional metabolic and regenerative support by supplying additional substrates and maintaining the status of tissue redox

(5) Offer protection against the diverse effects of invading inflammatory cells, including the oxidative stress caused by liberation of oxygen radicals
(6) Decrease prostaglandin synthesis
(7) Help in tissue regeneration.

We have devised such formulation that is effective in inhibiting half mustard-induced damage to the cornea. Preliminary results also demonstrate a potential effectiveness of such a formulation.

We are therefore in the process of developing a mixture of compounds possessing the above properties and examine the efficacy of such a mixture against Mustard erdnced damage to skin and other exposed organs.

The proposed mixture consists of the following compounds, purporting to perform the functions indicated.

Taurine (Membrane stabilization and inhibition of N alkylation, Anti-oxidant)
N-acetyl cysteine, Penicillamine (Reduce –SS- to –SH, Inhibit S-alkylation).
Alpha-keto-glutarate (Oxyradical scavenger)
Pyruvate (Oxyradical scavenger and metabolic support)
Glucose (Metabolic support)
Insulin (Promote glucose availability to tissue metabolism, mitosis and tissue regeneration)
Patothenate (Provide metabolic support via Coenzyme A)
Salicylate (Inhibit prostaglandin synthesis)
Citrate (Modulate invasion by inflammatory cells)
Indomethacin or Dexamethasone (Inhibit prostaglandin synthesis).
Retinol palmitate or other esters of retinol, (epithelial regeneration)
Free or esterified alpha-tochopherol (Prevent oxidation of ointment base and membrane lipids)

This report summarizes the indicial findings using the water soluble components of the above mixture against damage to mouse skin under incubation in vitro. The composition of the incubation the mixture was as follows:

Taurine 50 mM,
Sodium Pyruvate 12mM
Alpha-ketoglutarate (disodium) 60mM
Na-pantothenate 15mM
N-acetyl Cysteine 7.5mM
Sodium salicylate 7.5mM
Sodium citrate 3.8mM
EDTA 0.33mM

The specific specific objectives of these experiments were to determine:

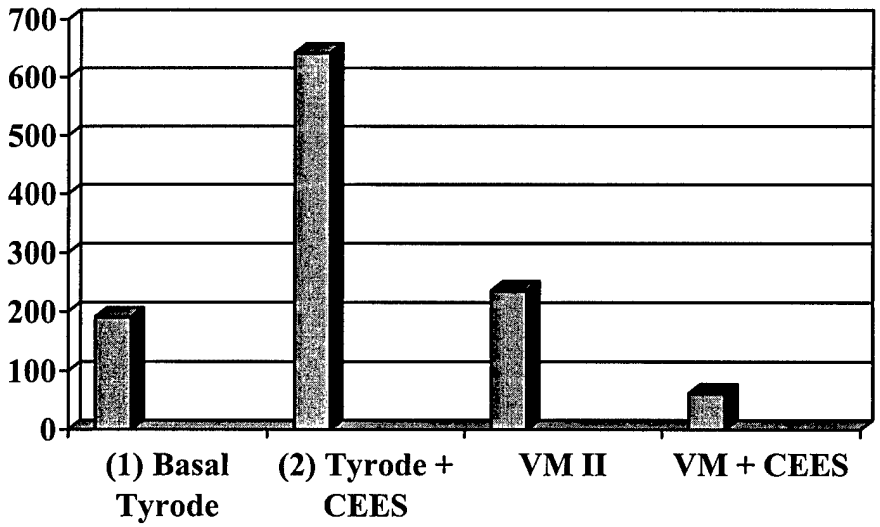
A. If the deterioration of the barrier function of the skin epidermis as measured by the loss of the restriction to the trans dermal movement of rubidium ions; cause by CEES could be prevented.

B. To evaluate the effectiveness of such a mixture at histological level .

Method of study: Mice of CD-1 strain weighing about 25 g. were used. They were anesthetized by intra-muscular injection of a mixture of ketamine and xylazine (66mg ketamine and 6.7 mg Xylazine/kg body weight). Immediately after inducing anesthesia a portion of abdominal skin was shaved, and skin dissected out atraumatically. The isolated skin sections were then kept moist by floating them upside down in normal saline for a few minutes. A rounded portion of this skin 'button' (about 2cm diameter) was then mounted in a home made Ussing cell. Four cells numbered as 1, 2, 3, and 4 were used in each experiment. The anterior and the posterior sides were marked so as to avoid any misidentification of the chambers created. A Tyrode based isotonic media (1.5 ml) labeled as control was added in both the chambers of cells labeled as # 1 and # 2.In cells # 3 and # 4 both chambers were filled with 1.5 ml of a Tyrode modified to contain the above compounds. The additions to Tyrode were done by iso-osmotic replacement of sodium chloride. in the basal Tyrode. The osmolarity of both the media were similar. The pH was 6.8. The cells were then allowed to equilibrate at 37'C in an incubator gassed with 95:5 air: C02 mixture. After the equilibrium, 10 microliters of radioactive ^{86} RbCl were added on the anterior side of all the four cells. Three microliters of CEES were also added on the anterior side of cells # 2 and # 4. Immediately after the addition of RbCl, 50 microliters of the fluids were taken out from all the chambers for initial radioactivity determination. After 3 hours, an additional 5 microliters of CEES were added on the anterior side of cells # 2 and # 4 and incubation continued for overnight period of 1 8 hours. At the end of the experiment, 50 microliters of the samples were again taken out and their radioactivity determined by liquid scintillation counting Immediately after recording the radioactivity, skin buttons were taken out and fixed in 10% buffered formalin for histology.

Results on the trans-permeability of the rubidium ions through the skin are summarized in the following diagram (fig1). Following the 18 hours of incubation, relatively little rubidium (less than 1%) appeared on the inner side, either through the skin bathed with Tyrode or VM. If CEES was added to Tyrode, the permeability increased about three times demonstrating a physiological injury and loss of the barrier function of the epidermal epithelia. In the case of VM however, CEES failed to increase the permeability. It may appear that VM when combined with CEES protects the permeability functions somewhat better than VM alone. A definite conclusion however is reserved till further examination. At the present time, however, it is obvious that CEES has an adverse effect on the function of the epithelium in preventing penetration of unwanted substances and that this toxicity is substantially annulled by the VM components.

Figure 1



The ordinate indicates the radioactivity of the medium in terms of disintegrations per minute

That the excessive permeability of the rubidium in the presence of CEES is indeed related to the damage to epithelium of the epidermis was proven by histological examination of the tissue incubated with Tyrode and VM in the absence and presence of CEES. Fig 2 A represents the HE staining of the button incubated in Tyrode. As expected, the epithelial layers in the skin are well-delineated continuous layers, adherent on a basement membrane abutting the dermal layer. The layers remain physically intact despite 18 hours of incubation. However, as shown in 2 B, there is significant structural disorganization in the tissue incubated with CEES. The nuclei in the epithelial cells are now abnormal looking. There is a substantial separation of the epithelial layer from the dermis, showing blister formation along the anchoring points along the hair follicle. The entire tissue is edematous as apparent from swelling. There is also a significant increase in the number of inflammatory cells. As shown in 2C, the tissue incubated in VM alone is much more organized and healthier even in comparison to the basal Tyrode control (2A). The epithelial is layer is contiguous and well abutted with basement membrane. The inflammatory cells are also fewer in number in comparison to the Tyrode control. Fig 2D represents the structure of the tissue incubated with VM and CEES together. The structural organization in the epidermis is well maintained. The effect of CEES is in this case is apparently not as striking as in the case of CEES added to the Tyrode. The epithelium is continuous without detachment from the dermis, hydration is much less, blister formation has not taken place and various nuclei have normal appearance. Neutrophils are even less in number than the basal Tyrode. The protection of skin against CEES induced damage by the VM components as used is hence apparently due to its ability to protect against physiological damage to the permeability function, as well as by its ability to maintain normal tissue structure as apparent histologically.
Further studies are in progress

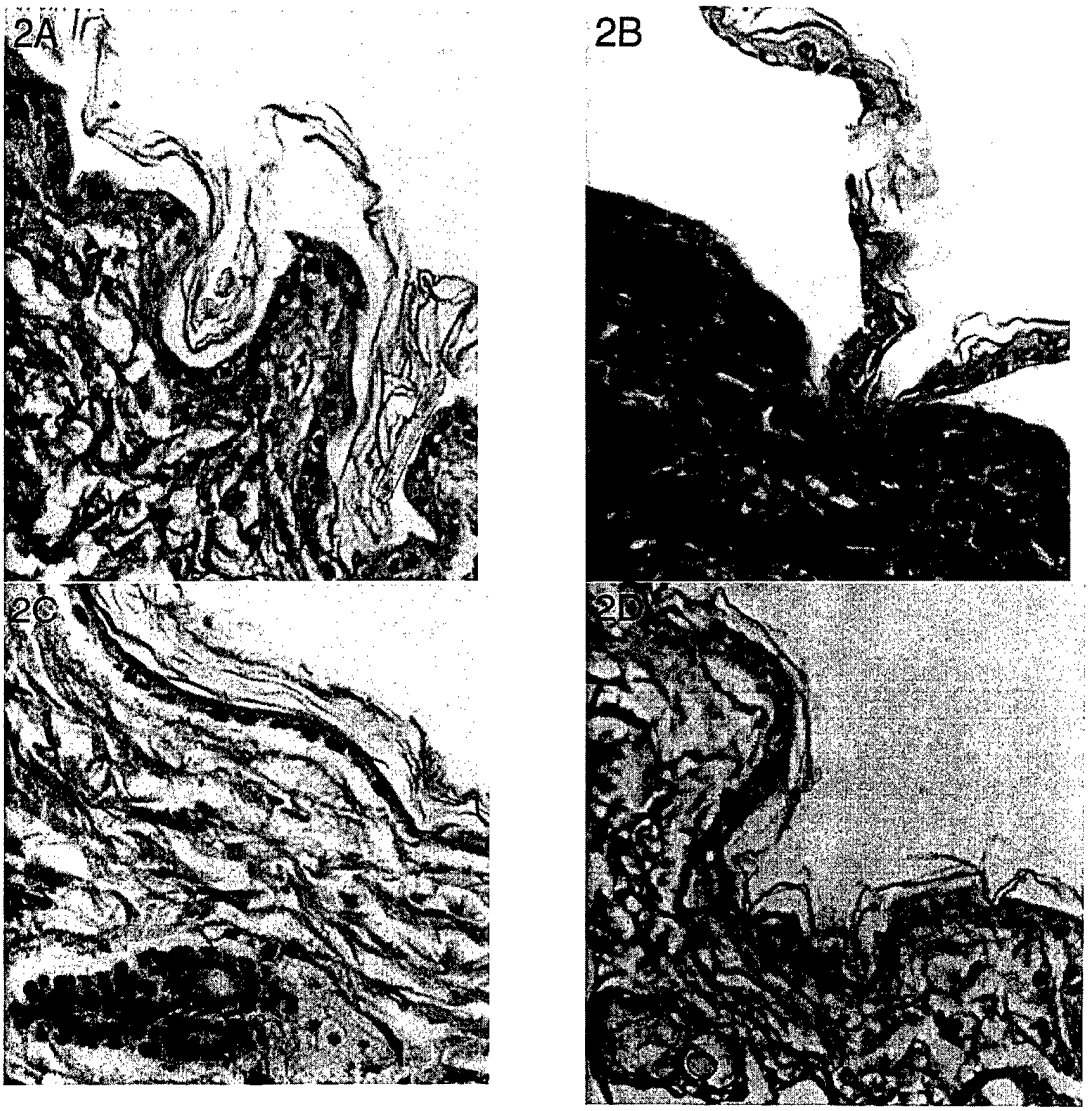


Figure 2

Figure 2A H:E staining of the section of the skin incubated in Ussing cell for 18 hour in Tyrode (Basal control)

Figure 2B.H:E staining of the section of the skin incubated in Ussing cell for 18 hour in Tyrode plus CEES.

Figure 2C. H:E staining of the section of the skin incubated in Ussing cell for 18 hour in VM medium.

Figure 2D. H:E staining of the section of the skin incubated in Ussing cell for 18 hour in VM plus CEES.

Conclusion and Summary.

Studies are in progress to understand the basic mechanism of damage to the skin on exposure to mustard gas and to develop preventive therapies. These studies have been done using mouse as experimental animal models and 2-Chloroethyl-ethyl sulfide (half mustard) as a model compound. It is hypothesized that the toxic effects of mustard are caused by reactions at multiple sites, including the sites located out side the cell membrane as well as inside. The manifestations are hence a consequence of derangements starting with the extra-cellular membrane and extending to the various intracellular sites, with adverse effects on the membrane permeability and transport activities, inhibition of multiple bio-energetic processes, and initiation of oxidative stress. We have hence felt desirable to develop a mixture of compounds capable of simultaneously antagonizing the various adverse biochemical reactions, and to further test the efficacy of such a mixture against tissue damage. As described in the report, we are in the process and developing such a mixture called VM. It has also been found to attenuate corneal damage caused by exposure of the tissue to half mustard. We now plan to extend this study using HD the real warfare agent.

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Legends to the Figures:

Figure 1: Effect of CEES on the transport of rubidium – 86 ions form the fluid bathing the outer side of the skin. The bathing media were Tyrode (T) or Varma mix (VM) with or without CEES. Freshly prepared abdominal mice skin buttons were mounted in Ussing cell. The chambers were then bathed with the respective basal media (T or VM). After equilibration for 30 minutes, 5 ul of CEES were added on the anterior (outer) side of the indicated cells. The penetration of the ion from outer to the inner side was then determined by radiation counting of the aliquot after 18 hours, the initial count being zero.

Figure 2: Physical separation of epidermis from the dermis caused by CEES: Attenuation by VM. Antero-posterior section of the skin buttons taken out after incubation in the Ussing cells as described under figure 1, were processed for H&E staining. 2A: Tissue incubated in basal Tyrode (T) 2B: Tissue incubated with Tyrode plus CEES. 2C: Tissue incubated in VM. 2D: Tissue incubated in VM plus CEES.