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A mixture of physiologically compatible compounds with the properties of inhibiting alkylation reactions, providing metabolic support to the tissue and capable of preventing oxidative damage is being developed and tested for its effectiveness against mustard induced toxicity. A partial preventive effect of such a formulation using mouse skin as an animal model has been demonstrated for the first time.

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USARMY Progress Report 2003.

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Introduction:

Studies are in progress towards assessing the effectiveness of Varma Mixture (VM) (of the composition described in a previous report) against inflammatory and necrotic changes known to take place in the skin of persons exposed to sulfur mustard (H.D). The ingredients of the mixture have been selected on the basis of the following reactions and physiological processes.

1. Inhibition of –SH and –NH alkylations.
2. Protection against metabolic stress.
3. Protection against Oxidative stress
4. Stimulate tissue regeneration
5. Prevention of toxicity of the inflammatory cells
6. Inhibition of prostaglandin synthesis

Exposure to mustard is well known to have adverse effects on all of these physiological processes. We are of the view that prevention against tissue damage by mustard requires use of a formulation containing compounds that can effectively interrupt the progress of several of the above unwanted biochemical reactions and consequently attenuate the necrotic process involved in mustard induced tissue damage.

Methods of Study: CEES (2-Chloroethyl ethyl sulfide) has been used as model compound known to simulate the action of HD. The effect of VM ointment against the development of necrotic changes on skin caused by CEES was studied by its application on mouse skin pre-exposed to CEES and determining the extent of modulation of the pathological changes in this group (experimental) in comparison to the group where CEES exposure was not followed by treatment with VM (control). CD-1 mice weighing approximately 25 g. were anesthetized by intramuscular injection of a mixture of ketamine and xylazine (66mg of ketamine and 7mg of xylazine/ kg body weight). Immediately after the onset of anesthesia , a small area on the flank was clipped to remove hairs. 10 microliter of a freshly prepared solution of CEES in propylene glycol (20 microliter CEES per 100 microliter of PG) was painted on an area of skin covering 1.5 cm^2. After 10 minutes of this application, the sites were treated with the VM ointment. The treatment was repeated every hour till 3 hours. They were again treated at two hourly intervals till the end of the day. A total of 6 treatments were given on the first day. On subsequent days, four treatments were given at intervals of 1.5 hours. In these particular experiments, treatment was continued for four and five days. After the treatments, the animals were again anesthetized and skin sites excised and preserved in 10% buffered formalin, and then processed for histology.

Results and Discussion:

In the present period we have conducted experiments to examine the efficacy of VM against CEES induced damage to the tissue, using mouse as an experimental animal. Initially, the assessment of damage was made on the basis of development of superficial vesication (blister) like lesions on the site of CEES application. This was followed by assessment of the damage to structural integrity of the tissue, using histological techniques.

Figure 1 represents the external appearance of the CEES-exposed skin 5 days post exposure. In the skin painted with CEES , not followed by VM treatment , the lesion is noticeably necrotic and rougher than expected. The latter is attributable to a subsequent healing process as indicated by scab formation. In the skin where CEES application was followed by treatment with VM, signs of any blistering and subsequent healing are much less prominent in comparison to the group which did not receive VM treatment. Indeed, the apparent scarring, in this case is very minimal, if any. Addition of dexamethasone to VM was without any further advantage.

Figures 2 to 4 represent the histology of the skin samples in three groups of experiments. As expected, the epidermis in the normal groups is made up of an external layer of stratum corneum consisting of some denuding keratinocytes and free keratin lamellae derived from the dying or dead keratinocytes. The layer of the stratum corneum is followed internally by 2 to 3 layers of live

keratinocytes in different stages of differentiation. The inner most layer (stratum germinativum) of these epithelial cells rests on a basement membrane, separating the epidermis from dermis. The latter is made up of collagen and elastic fibers and contains, in addition, some fibroblasts and inflammatory cells. The dermis also contains normal glandular structures and hair follicles. As would be apparent from the figures 2 and 3, on application of CEES, the epidermis gets completely separated from the dermis giving rise to sub-epthelial space and accumulation of cellular debris and fluids culminating into blister formation. Therefore a structural and physiological damage to the epidermis and blister formation characteristic of HD damage is fully mimicked by CEES. Since this and HD have the ability to induce toxicity to the tissue by many diverse reactions at sites starting from the cell membrane to various cytosolic, mitochondrial and nuclear regions, it has been difficult so far to find a preventive agent against mustard toxicity. We have approached this difficulty by formulating a mixture (VM) which could possibly prevent tissue damage by reacting at varied biochemical and physiological loci.

As apparent , application of this formulation has a significant protective effect against CEES induced damage to tissue structure and physiology. The separation of epithelium caused by CEES is nearly fully prevented. The cell cytology remains fairly normal. The disposition of epithelium along the hair shaft also remains normal. The number of inflammatory cells is also less in this case. As apparent from the histological sections application of VM while healing the tissue faster, also minimizes scab. This is more apparent from figure 4. formation. Hence treatment leads to a cosmetically a better maintenance of the lesion during healing. This is also likely to minimize unwanted vascularization of the burn.
In previous studies we have shown that compounds known to inhibit various sulfur mustard based alkylation reactions, and capable of acting as antioxidants (via their ability to scavenge various reactive species of oxygen such as super oxide, hydrogen peroxide and hydroxyl radicals) are effective in preventing CEES induced damage to cornea. Visual disability, initiated by damage to corneal epithelium is one of the earliest disabling manifestations of exposure to this gas. It is strongly believed that the mechanism of sulfur mustard induced damage in the war field is indeed similar to that of the CEES induced damage to animals observed under the laboratory conditions. Hence studies conducted with this agent are likely to be found useful in developing treatment against tissue damage caused by actual mustard compound (HD) as well.

Although we have found VM to be effective against CEES induced damage to skin, verification of its effectiveness against HD remains to be accomplished. The applicability of the finding to other species also remains to be determined. In addition, mechanistic studies are considered essential. Such studies are likely to yield information in developing simpler and more effective formulations.

Key Research Accomplishments:

A formulation of ingredients stated in the previous report and a study of the effect of such formulations against CEES induced damage to mouse skin is in development. These initial studies suggest that a prophylactic or post-exposure treatment against mustard induced toxicity can be more effective by using a mixture of compounds known to be effective in inhibiting alkylation as well as oxidative stress. It will also be useful to include compounds which can potentially supplement the tissue bio-energetic and repair systems.

Reportable Outcome:

The study will become reportable after the efficacy of VM is proven with HD models.

Conclusions and Summary:

A mixture of physiologically compatible compounds with the properties of inhibiting alkylation reactions, providing metabolic support to the tissue and capable of preventing oxidative damage is being developed and tested for its effectiveness against mustard induced toxicity. A partial preventive effect of such a formulation using mouse skin as an animal model has been demonstrated for the first time.

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5 Days Experiment

Mouse Skin
(Dorsal)

Figure 1

CEES

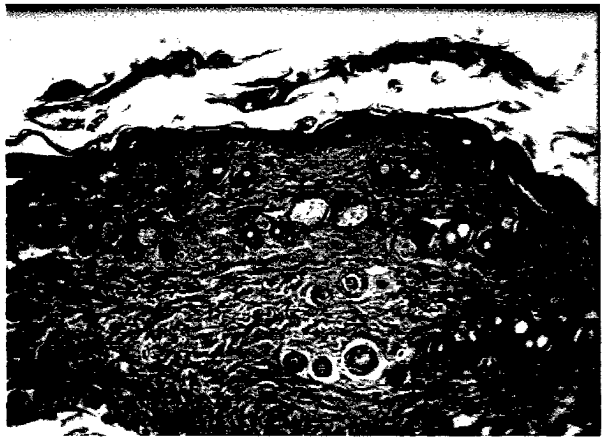
CEES + VM

CEES + VM + DEX

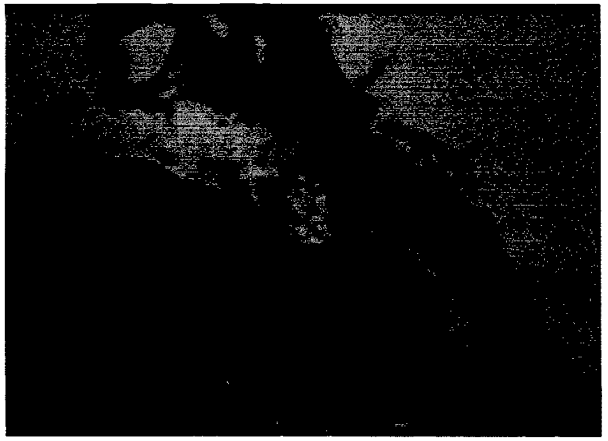
5 Days Experiment

Mouse Skin
(Dorsal)

Figure 2



Normal/Mice Skin



CEES/Mice Skin

CEES + VM/Mice Skin

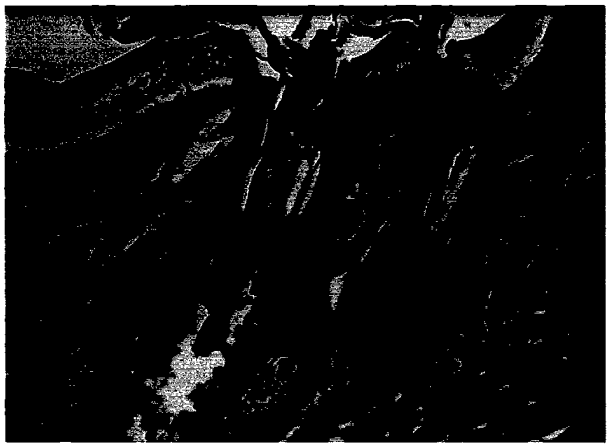
5 Days Experiment

Mouse Skin
(Dorsal)

Figure 3

Normal/Mice Skin

CEES/ Mice Skin



CEES + VM/Mice Skin

Figure 4

Mice Skin CEES VM+CEES-DEX CEES+VM+DEX