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INTRODUCTION

Sulfur mustard (SM) is a potent vesicant employed as a chemical weapon in various conflicts during the 20th century (1, 2). It functions as a powerful alkylator and highly cytotoxic blisterogen in both humans and animals (1-9). Skin exposed to SM develops erythema within 30 minutes to several hours after exposure followed by edema, vesicle and blister formation, ulceration, necrosis and desquamation (2, 7, 8, 10). The severity of lesions observed after exposure to SM has emphasized the need for an efficient pharmacological antidote against its vesicating activity. The powerful alkylating activity of SM (11-13) results from its conversion, in aqueous solution, to the highly electrophilic ethylene episulfonium derivative (2) which can be neutralized by nucleophilic agents. Protection against SM might be achieved by glutathione derivatives (14, 15), cysteine esters (16) and a cysteine precursor (17). Despite having some beneficial effects, these agents were not efficacious enough to be used as antidotes. Additional agents, such as arginine analogs (18-22), a calcium channel blocker (23), niacinamide (24) and its combination with promethazine and indomethacin (25) exhibited weak therapeutic effects as post-exposure treatment against SM in experimental animals, although some of these agents were beneficial in *in vitro* and *in vivo* systems, particularly if treated prophylactically. Therefore, we undertook the development of efficacious topical antidote for treatment of mustard gas-exposed victims.

BODY

IODINE PROTECTS AGAINST SM

In an attempt to adopt a different approach for addressing this issue, we assumed that the divalent sulfur atom of SM [$S(CH_2CH_2Cl)_2$] can be oxidized to form its sulfoxide form [$SO(CH_2CH_2Cl)_2$], an inactive derivative of the vesicant (26). The most suitable oxidizing agent was iodine, a widely used topical antiseptic agent. We demonstrated that topical application of povidone-iodine 5 and 10 min after skin exposure to SM significantly reduced tissue damage as compared to the control sites treated with SM only (27-33). A lower degree of protection was observed at a 20 min interval between exposure and treatment. However, practically, 10 min is too short an interval for treatment of the soldier in the battlefield or of a civilian in case of gas attack. At that stage support by the USAMRMC began (Cooperative Agreement No. DAMD17-98-2-8009). Our main objective was ***to extend the time interval between exposure and treatment*** by improving the iodine formulation. Our experience showed that iodine is more efficacious than povidone-iodine, a polyvinylpyrrolidone-iodine complex (27-29, 34). The currently available iodine preparations contain sodium or potassium iodide, believed to form the water-soluble I_3^- ion. However, the efficacy of the new iodine formulation was significantly improved due to employment of tetraglycol as solvent without iodide addition; (a patent (34) was filed) which enables 50% water content without iodine precipitation. It is assumed that under these circumstances iodine retains its molecular form i.e. I_2, a more hydrophobic molecule than I_3^-, resulting in enhanced skin penetration and improved biological activity.

Indeed, ***post-exposure treatment with this new iodine preparation resulted in a significant reduction in skin ulceration area at time intervals between exposure and treatment of 30 min and less, and to a lesser extent at 45 min***. These findings were corroborated by histopathological examination of SM-exposed skin treated with iodine 30 min after intoxication; statistically significant reductions of 35, 67, 43, 39, and 45% were observed in subepidermal microblister formation, epidermal ulceration, dermal acute inflammation, hemorrhage and necrosis, respectively. These findings were recently published (34-41) and, together with being non-toxic and safe preparation, encouraged us to further improve the iodine preparation in order to extend the interval between exposure and treatment.

The involvement of inflammatory mediators in SM-induced skin toxicity

The evolution of SM-induced skin lesion involves a variety of inflammatory processes and production of proinflammatory factors (42-48). The dermal infiltration of polymorphnuclear cells upon SM exposure (35, 47) further supports the involvement of inflammation in skin irritation induced by this vesicant. These findings stimulated the introduction of non-steroidal antiinflammatory drugs (NSAIDs) into preparations against SM-induced skin lesions. Buxton et al. (49), Babin et al. (50) and Zhang et al. (51) have all shown the protective activity of topical and parenteral treatments with NSAIDs, including indomethacin and olvanyl, against skin toxicity of SM. We have also shown that incorporation of anti-inflammatory agents into the iodine formulation improved the counter-irritating activity of the preparation in the haired guinea pig model. During the second year of project we

tested the effect of the combination of iodine and anti-inflammatory agents on hairless guinea pigs at both single and multiple treatments.

Despite the information about COX involvement in SM-indcued dermal toxicity, no information is available on the role of other inflammatory mediators such as interleukins and tumor necrosis factor-alpha TNFα in very early stages of exposure, and how iodine affects the levels of these inflammatory mediators. In order to address this issue we adopted the following approaches:

a) Testing the effect of combination of iodine and anti-inflammatory agents on hairless guinea pigs by single and multiple treatments.
b) Testing the effect of SM on pathological parameters of inflammation (neutrophil infiltration) at early stages of exposure (5 hours) in the haired guinea pig.
c) Testing the effect of iodine on SM-induced inflammation at early stages of exposure (5 hours) in the haired guinea pig.
d) Testing the effect of SM on levels of TNF-alpha and other inflammatory mediators in the mouse ear skin at very early (3 hours) and later (8 hours) stages after exposure.
e) Testing the effect of topical iodine treatment following SM exposure on TNF-alpha and other inflammatory mediators in the mouse ear skin at very early (3 hours) and later (8 hours) stages after exposure.
f) Testing the effect of anti TNF-alpha antibodies on SM-induced skin lesions in the mouse ear model.

EXPERIMENTAL PROCEDURES

Hairless guinea pigs

The animals were anesthetized by 30 mg/kg pentobarbital sodium ip. Backs were cleaned with wet soft white paper and let to dry out before the beginning of experiment. Six sites (three on each side) of each back were exposed to SM vapor for 16 min. Iodine formulation was applied on 3 exposure site of each animal while the other 3 SM-exposed sites of the animal were treated with iodine vehicle. Iodine was applied 10 min after SM exposure. Animals were evaluated 2 days after exposure.

Haired guinea pigs

Backs of haired guinea pigs were shaved 24 hours prior the experiment. The animals were anesthetized by 30 mg/kg pentobarbital sodium ip. Backs were cleaned with wet soft white paper and let to dry out before the beginning of experiment. Six sites (three on each side) of each back were exposed to 1 μl SM. Iodine formulation was applied 20 min after SM exposure. Five hours after exposure the animals were sacrificed by 100 mg/kg pentobarbital sodium ip. Skin samples were removed and fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 5-6 mm, and stained with hematoxylin and eosin (H&E) for histopathological evaluation

Mouse ear test

Male ICR mice (~25g) were anesthetized by sodium pentobarbital, 60mg/kg ip (0.4ml/100g BW of 1.5% solution), and placed on their abdomens. Anesthesia was maintained by administering 0.12ml/100g BW whenever needed. The outer side of the ear was exposed to 0.32 mg SM (5μl of 1:20 dilution in dichloromethane so that the active material was uniformly spread on the outer-ear surface. The iodine formulation (2% iodine in 40% tetraglycol, 25% ethyl alcohol, *q.s.* 100% with water) was applied 20

min after SM treatment. To expose the animals, 3 layers of gauze pad (approximately 0.7 X 0.7 cm) soaked in the iodine formulation or its vehicle (40% tetraglycol, 25% ethyl alcohol, *q.s.* to 100% with water) were placed on the ear so the entire site of exposure was covered with the liquid formulation. The pad was left on the skin for 2 h; during that time the iodine formulation was reapplied to the gauze pad 40 and 80 min after the first application. At the end of the procedure (2 h after iodine application), the pad was removed. Animals were sacrificed 3 and 8 hours after SM exposure using 100mg/kg pentobarbital. The ear specimens (n=6 for each group) were dissected, fixed in lysine-phosphate-paraformaldehyde containing sodium periodate for 24 h, further fixed in 70% ethanol until processing, embedded in paraffin, and sectioned at 5-6 μm; part of them were stained with hematoxylin and eosin (H&E) for histopathological evaluation, and the rest were immunostained for TNF-alpha, COX-1, COX-2, and IL-1beta.

In an additional series of experiments, antiTNF-alpha antibodies were diluted appropriately with 0.9% NaCl and iv-injected (1 and 2 μg in 0.25ml/30g BW) into mice 30 min after SM exposure. The inner side of the ear was exposed to 0.08 mg SM (5μl of 1:80 dilution in dichloromethane) so that the active material was uniformly spread on the outer ear surface. Mouse-ear thickness was measured 48 h after exposure using a micrometer (Model PK-0505, Mitutoyo Corporation, Japan). Edema was assessed by the difference between ear thicknesses measured prior to and after exposure. Animals were sacrificed 48 h following exposure using 100 mg/kg sodium pentobarbital ip. Ear specimens were removed, fixed in 10% neutral-buffered formalin for 24 h then in 70% ethyl alcohol until processing, embedded in paraffin, sectioned at 5-6 μm, and stained with H&E for histopathological evaluation.

Immunostaining procedure.

For immunohistochemistry, tissues were fixed in periodate-lysine-paraformaldehyde, processed routinely and paraffin-embedded. Serial sections (6 μm) of mouse skin were stained for TNF-alpha, IL-1-beta, COX-1 and COX-2. Detailed Sections were

deparaffinized, hydrated and blocked for endogenous peroxidase staining. Heat-induced epitope retrieval was performed for all antibodies using a vegetable steamer.

Histopathological evaluation.

Each mouse-ear section was evaluated in a blinded manner, without the investigator's knowing the identity of the treatment group, and scored for histopathological changes. In the mouse ear the entire section, including inner and outer sides, was evaluated. The reactive and inflammatory changes in the epidermis and dermis were assigned a severity grade of 0-4 representing unremarkable, minimal, mild, moderate, and marked changes, respectively. Epidermal parameters included microblister formation, ulceration, and necrosis. Dermal parameters included acute and subacute inflammation, neutrophilia (increased number of neutrophils in the blood), hemorrhage, and necrosis.

Assessment of the immunohistochemical staining

Staining intensity of relevant tissue components (e.g. neutrophils, fibroblasts) was assessed by a board certified pathologist (A.N.), who was blinded to sample identity. Staining intensity was graded on scale of 4, where 0 – represents no staining; 1 – slight staining; 2 – moderate staining; 3 – marked staining. D – Diffuse; MF – multifocal; F – focal. The location and intensity of staining were described. Different grades of immunoreaction were restricted to particular cell types within the samples, and these specific reactions, when being different in localization and/or intensity, are crucial for the understanding of the potential role of these cytokines in the development of very early skin damage induced by sulfur mustard. The results of the immunohistochemical staining are reported as group assessment and not individually. Photographs of representative results are included.

Evaluation of cell proliferation

Formalin-fixed, paraffin-embedded skin sections were deparaffinized and hydrated using xylene and ethanol. Antigen retrieval was performed using 2N HCL and trypsin digestion. BRDU was detected using a Vector® (Burlingame, CA) mouse peroxidase kit according to the manufacturer's recommendation. The primary antibody (Dako® Corporation, Carpinteria, CA) concentration was 1:400. Visualization of the antibody complex was achieved using DAB Chromagen system (Dako).

To score the BRDU stain, a blind count was conducted at 200X magnification under a light microscope (Olympus® BX50). For each skin section, the number of total positive stained cells for BRDU was counted per unit length (mm). An average was calculated for each treatment group.

RESULTS

Effect of iodine formulation on SM-induced skin lesions

Effect of topical iodine against SM-induced skin lesions in the haired guinea pig 5 hours after exposure

histopathological findings indicated the epidermis from the control animals (either not treated or treated with the vehicle) and those of the iodine treated animals appeared to be of normal thickness, without any alterations in this layer or in the dermis. The skin samples from the animals treated either with the sulfur mustard (SM) and SM + Vehicle were characterized by epidermal thickening due to swelling and clearing of chromatin of

the nuclei. Dermal polymorphonuclear (PMNL) cell infiltration was noted. Histopathology indicates an acute inflammatory response to SM treated skin (Fig. 1).

Effect of topical iodine on cell proliferation (by BUDR) in guinea pig skin exposed to SM

The BRDU cell proliferation data (Fig. 2) indicated no statistical significant difference between SM + Iodine treated group and the SM+Vehicle ($p = 0.8664$) and Sulfur mustard ($p = 0.6268$) treated groups. However, we found a statistically significant difference between SM + Iodine group and Iodine ($p = 0.0397$) and between SM + Iodine group and the Vehicle control ($p < 0.0001$). Using Mann-Whitney-Wilcoxon test we found no significant difference between untreated and the vehicle control groups ($p > 0.10$). Proliferation does not return to untreated or vehicle treatment groups' level in the SM + Iodine treated skin.

Effect of SM followed by iodine treatment on levels of TNF-alpha and other inflammatory mediators in the mouse ear skin at short time intervals after exposure

SM-induced inflammation 3 and 8 h after exposure

Histological evaluation of mouse ear 3 h after SM exposure revealed neutrophilia consisting of acute inflammatory-cell aggregates in the vascular bed close to the irritant-exposed area (Fig. 3A) and their adherence to the endothelium and/or crossing through the blood vessel wall (Fig. 3B). This appearance reflects

the earliest stage of histomorphological changes following exposure to the irritant. Evaluation of the ear 8 h after SM exposure demonstrated the adherence of the neutrophils to the endothelium, and/or crossing through the vessel wall, and infiltrating the adjacent dermis (Fig. 3C, D).

SM-induced TNF-alpha immunostaining and effect of topical iodine

positive neutrophils within the lumen of the blood vessel adhered to the endothelium and was present within the dermis (Fig. 3F) ,while the control animal showed few neutrophils within the blood vessel, most of them TNF-alpha-negative (Fig. 3G). When skin was treated with iodine after SM exposure, however, no inflammatory cells in the blood vessel were seen in tissue sampled 3 h following exposure; 8 h after exposure, blood vessels contained only very few inflammatory cells that were TNF-alpha-positive (Fig. 3H, I). This occurrence contrasts with our findings in the case of SM exposure (Fig. 3F) or SM exposure followed by treatment with the iodine vehicle (Fig. 3J), in which blood vessels contained numerous inflammatory cells that were TNF-alpha-positive.

Sulfur mustard did not cause significant changes in other inflammatory mediators such as IL-1beta, COX-1, and COX-2 at time intervals of 3 and 8 h after SM exposure (Fig. 3K-R).

Effect of anti TNF-alpha antibodies on SM-induced skin lesions in the mouse ear model.

The involvement of TNF-alpha in the inflammatory process elicited by the irritant led us to test the effect of antiTNF-alpha antibodies against SM-induced skin lesions. Administration of 1μg and 2μg per 30g body weight of antiTNF-alpha antibodies significantly reduced ear edema by 49% and 30%, respectively (Fig. 4). These findings were corroborated by quantitative analysis of the histological findings (Table 1) showing a statistically significant reduction of 46% in acute inflammation and no signs of

subacute inflammation in the treated group, in contrast to the control group (SM only) (Table 1). Although not statistically significant, the other epidermal (microblister formation, ulceration, and epidermal necrosis, Table 1) and dermal (neutrophilia, hemorrhage, and dermal necrosis, Table 1) parameters also became markedly reduced in the antibodies-treated group, in comparison to the control group.

Effect of single and multiple topical treatments of hairless guinea pigs with iodine preparation

Fig. 5 shows that the iodine formulation reduces SM vapor-induced erythema in the hairless guinea pigs. Multiple treatments had no beneficial effect over the single treatment.

Protective effect of H2A histone fragment agasint SM-induced skin lesions

In vivo studies

Protective effect of multiple peptide treatments on sulfur mustard (SM)-induced skin lesions in the haired guinea pig model.

The protective effect of multiple injections of peptide III in the guinea pig skin model is demonstrated in Fig. 6. Interestingly, the mouse sequence IIIM1, did not protect the guinea pig skin against SM. Peptides (III, IIIM1 or vehicle—saline) were administered (iv) into guinea

pigs at a dose of 1mg/kg. The animals were injected 8, 6, 4, 2 days and 20 min before exposure to SM. Six sites on each shaved back were exposed to 1µl (1.27 mg) neat SM. The protective effect of peptide III in the guinea pig skin model is demonstrated whereas the mouse sequence IIIM1, did not protect the guinea pig skin against SM.

Quantification of the ulceration area in the different treatment revealed that peptide III significantly reduced the size of ulceration area as compared to the vehicle (saline)-treated group (Fig. 7).

Peptide III: H-Lys-Gly-Asn-Tyr-Ala-Glu-Arg-Ileu-Ala-OH (KGNYAERIA)

Peptide IIIM1: H-Lys-Gly-His-Tyr-Ala-Glu-Arg-Val-Gly-OH (KGHYAERVG)

Protective effect of a single peptide treatment on sulfur mustard (SM)-induced skin lesions in the guinea pig model.

Fig. 8 shows that pretreatments of guinea pigs with peptide III resulted in statistically significant protection (*$p<0.05$, Mann-Whitney test) while IIIH and IIIU3 show trend of protective effect against SM-induced skin lesions.

Peptide III: H-Lys-Gly-Asn-Tyr-Ala-Glu-Arg-Ileu-Ala-OH (KGNYAERIA)

Peptide IIIH: H-Lys-Gly-Asn-Tyr-Ala-Glu-Arg-Val-Gly-OH (KGNYAERVG)

Peptide IIIU3: H-Lys-Ala-Asn-Tyr-Ala-Glu-Arg-Val-Gly-OH (KANYAERVG)

N-methylated analogs of peptide 3 did not protect against SM.

Peptide 4 and its N-methylated analogs (mono- and di- N-methyl) – did not protect against SM.

In vitro studies

Methods

In these experiments HaCat cells were transfected with peptide MIIIM1 which contains the sequence of IIM1 and additional moiety of methionine to give the following sequence: H-Met-Lys-Gly-His-Tyr-Ala-Glu-Arg-Val-Gly-OH.

Transfection protocol

Construction of plasmid: The cDNA sequence 5' ATG GCC AAC GCG CTC GGC GTA GTG GCC CTT 3' was inserted into the pTet- peptide plasmid (Gene Bio- application Ltd.) under the transcriptional control of the tet promoter. Down stream of the sequence, polyadenilation sequence from simian virus 40 was inserted. The plasmid contained a gene for neomycin resistance. pTet plasmid was used as a control and was the same as above but did not contain the cDNA sequence Another plasmid, the pCMV-tTa (Gene Bio- application Ltd.) contained the gene for the tetracycline transactivator under the transcriptional control of the CMV promoter).

Transfection of cells: One day before transfection, cells were seeded in a 24 well plate at a concentration of $2*10^5$ cells/well in a volume of 500 µl in order to reach 90-95% confluency on the day of transfection. The following DNA- lipofectamine (invitrogen) complexes were prepared as follows: 2 µl of lipofectamine was mixed with 50 µl of the appropriate serum free medium and incubated for 5 minutes at room temperature; 0.8 µg of pTet- peptide/ pTet/ pCMV- tTA were mixed with 50 µl of the appropriate serum free medium and combined with the lipofectamine complex. After gentle mixing, the complex

was incubated for 20 minutes at RT to allow the DNA- lipofectamine complex to form. To each well were added the pTet- peptide- lipofectamine / pTet- lipofectamine complex and the pCMV-tTa- lipofectamine complex. The cells were incubated at 37^0C in a humidified incubator supplemented with 94% air/ 6% CO_2. 2 days after transfection the cells were removed to a 25 diameter flask with fresh medium. Twenty four hours later, 200 μg/ml of G418 (Gibco-BRL) and 1 μg/ml Doxycycline were added to the medium. The G418 concentration was increased in the following two weeks and reached a final concentration of 1000 μg/ml.

Cell culture

Along these experiments we have used two types of HaCaT cells: a) the ***peptide-transfected cells*** which contained the plasmid to whom the peptide was inserted; the cells expressed the peptide, b) ***control cells*** that were transfected with the plasmid only without peptide sequence; the cells did not express the peptide, but are appropriate controls for the peptide-transfected cells.

Each of the HaCaT cell lines was grown in Dulbecco's modified Eagle's medium/F-12 containing 10% fetal calf serum, 4mM Glutamine, 100 units/μl penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B at 37^0C in a humidified incubator supplemented with 94% air/ 6% CO_2. Cells were grown in a 25 diameter flask in a volume of 3 ml. Splitting was performed every 3-4 days by removal of the medium and the addition of trypsin. Once the cells were trypsinated, conditioning with the same volume of medium was performed followed by centrifugation at 4000 rpm for 7 minutes.

The trypsin-medium was removed, the cells were resuspended and transferred to a fresh medium so the final concentration of cells was diluted to 1/10 of the initial value. After splitting cells were grown in serum-free medium for 24 hours.

Experimental approach

Exposure of HaCaT cells to lysophosphatidic acid (LPA) results in production of hydrogen peroxide that can be monitored by conversion to hydroxyl radicals by horseradish peroxidase. The formed radical can be monitored by luminescence in the presence of luminol.

Luminescence assay

The reaction mixture contained 0.2ml cells (10^6/ml) suspended in Hank`s balanced salt solution (HBSS), luminol (100μM) and horseradish peroxidase (1.0 unit/ml), fatty acid free bovine serum albumin (0.2%). Reaction was started by lysophosphatidic acid (LPA) at the indicated concentrations. The reaction was performed in a white 96-well plate, using Tecan spectrofluoro Plus for luminescence measurement. Each point represents 3 different measurements taken at the peak response of lumuinescence.

Cytotoxicity assay

The cells were pre-incubated with the tested peptide for 4 hours then exposed to SM (1μM) or hydrogen peroxide (1mM) for 24 hours. Cell Viability was determined by the

MTT assay, based on production of blue crystals of formazan. The intensity of the blue color (absorbance at 405 nm determined by ELISA reader) is proportional to the number of viable cells.

Results

LPA-induced hydrogen peroxide production in transfected cells

Fig. 9 clearly shows that the peptide-transfected cells showed significantly lower luminescence than the control cells, probably due to their high content of peptide which scavenged the radicals formed by the LPA-induced hydrogen peroxide.

Effect of hydrogen peroxide on peptide-transfected cells

Fig. 10 shows that the control cells are more vulnerable to hydrogen peroxide, namely, reduction of 71% in viability of the control cells whereas the peptide-transfected cells were more resistant and showed reduction of only 38% upon exposure to hydrogen peroxide.

Effect of sulfur mustard (SM) on peptide-transfected cells and control HaCaT cells.

Fig. 11 shows that the control cells are more vulnerable to SM, namely, reduction of 59% in viability of the control cells whereas the peptide-transfected cells were more resistant and showed reduction of only 87% upon exposure to SM.

Effect of IIIM1 on SM cytotoxicity in HaCaT cells

Fig. 12 shows that IIIM1 (30μg/ml) increases the number of viable cells exposed to SM (1μM) (in other words, IIIM1 reduces the cytotoxic effect of SM in HaCaT cells).

KEY RESEARCH ACCOMPLISHMENTS

- The protective effect of a single topical iodine treatment was shown in hairless guinea pigs. Multiple treatments did not improve the results.
- Di- and tri-N-methylation of the peptide resulted in inactivation of peptide III and peptide IV.
- The inflammatory response at very early stages of exposure was demonstrated.
- The protective effect of iodine at very early stages of exposure was demonstrated.
- The protective effect of anti TNF-alpha antibodies was demonstrated
- Di- and tri-N-methylation of the peptide resulted in inactivation of peptide III and peptide IV.
- The protective effect of prophylactic single and multiple treatments of non-modified peptides was demonstrated.
- The protective effect of the peptide was demonstrated in cultured keratinocytes.
- Peptide-transfected cells were more resistant than normal cells against SM cytotoxicity.

REPORTABLE OUTCOMES

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CONCLUSIONS

The optimized iodine preparation was efficacious in hairless guinea pigs at a single post-exposure treatment. The anti-inflammatory effect of topical iodine involved suppression of TNF-alpha induction. Anti TNF-alpha antibodies reduced the toxic effect of SM. Prophylactic treatment (7 days prior) with the peptide or its analogs reduced SM skin toxicity in guinea pigs. The peptide also reduced the cytotoxic effect of SM in cultured keratinocytes. In addition, cells transfected with the nucleotide sequence encoded the peptide were more resistant against SM than control cells.

So what: The present study confirmed the efficacy of iodine preparation in hairless guinea pigs and contributed to the understanding of the role of TNF-alpha in SM skin toxicity. The present study further demonstrated the protective effect of the peptides in cultured keratinocytes and by single and multiple prophylactic treatments.

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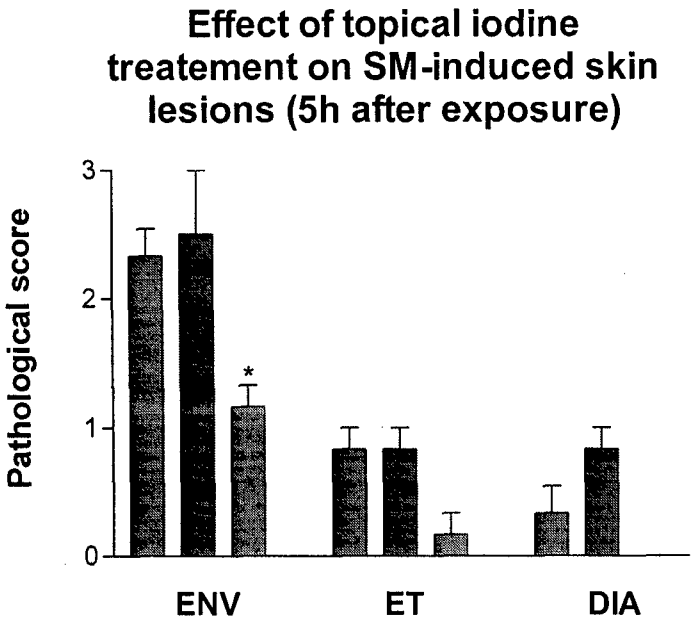
APPENDICES

Fig. 1:



ENV - epithelium-nuclear vaculotaion
ET - epidermis thickened
DIA - dermis-inflammation acute (note: SM+iodine - zero value)

purple - SM
blue - SM+vehicle
red - SM+iodine

*p<0.01 (comparison with SM)

p=0.0649 when SM+I was compared with S+Vehicle for ENV

p=0.0649 when SM+I was compared with SM or SM+vehicle for ET

Fig. 2: Cell proliferation in skin exposed to SM and topical ioidneiodine

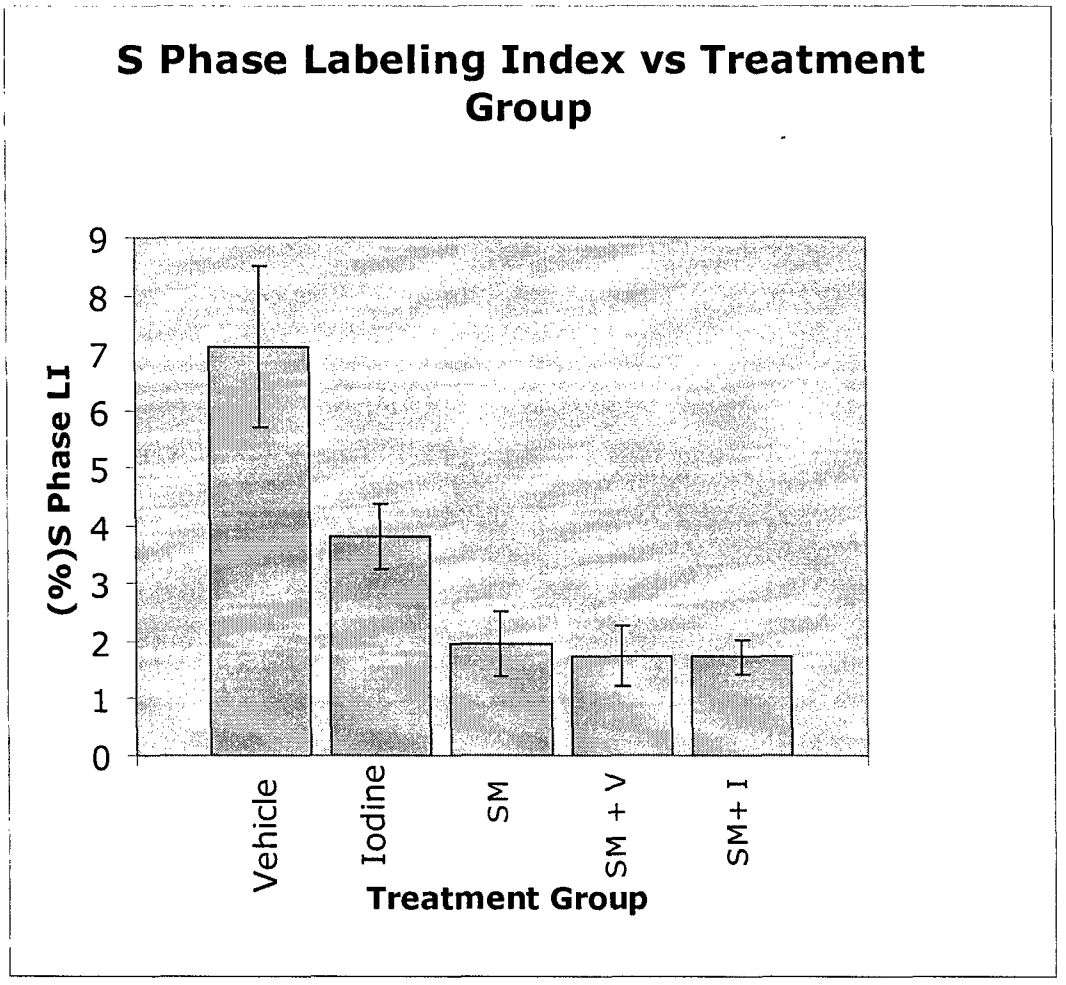


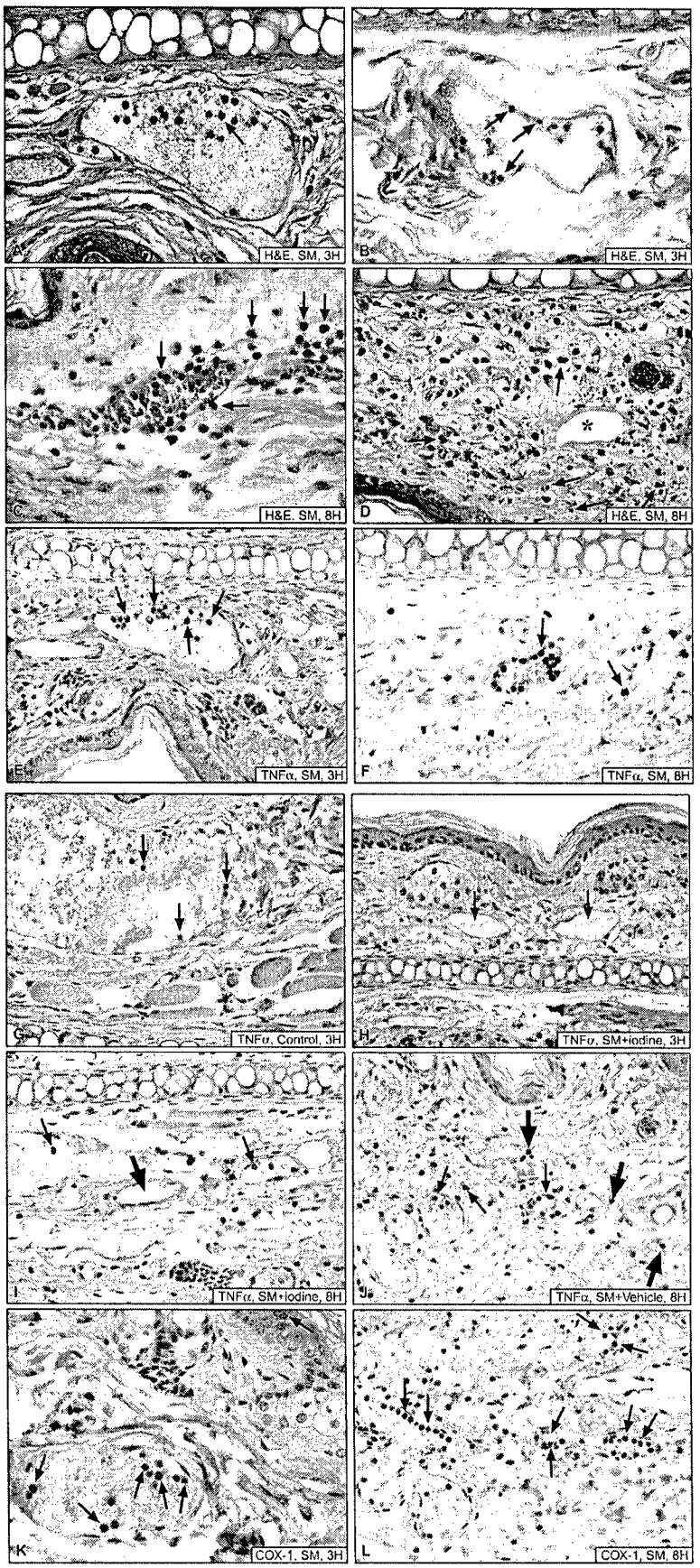
Fig. 3: Effect of SM followed by iodine treatment on levels of TNF-alpha and other inflammatory mediators in the mouse ear skin at short time intervals after exposure

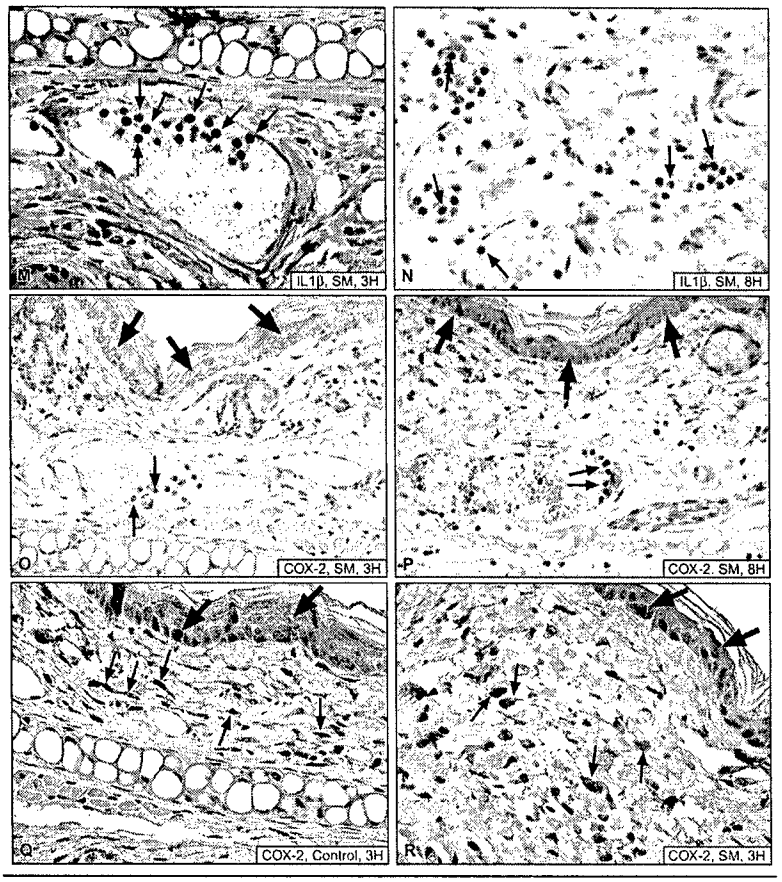
Assessment of mouse ear 3 h after SM exposure showed numerous strongly TNF-alpha-positive neutrophils within the lumina of the blood vessels (Fig. E). The inflammatory response became intensified 8 h after exposure; the strongly TNF-alpha-
Histopathology and TNF-alpha, COX-1, COX-2 and IL-1beta immunostaining of sulfur mustard (SM)-exposed and iodine-treated skin. The ears were exposed to diluted SM, treated with iodine 20 min later, sacrificed 3 and 8 h after SM exposure, processed, and stained as described in Methods. (A) – (C): Succeeding stages of histomorphological changes following exposure to the irritant.

(A) Animal treated with sulfur mustard and scarified 3 hours following exposure. Note – increased presence of neutrophils within blood vessels (neutrophilia, arrows). This reflects the earliest stage of histomorphlogical changes following exposure to the irritant, in which acute inflammatory cells aggregate in the vascular bed close to the irritant-exposed area. Hematoxylin and Eosin (H&E), X600.

(B) Animal treated with sulfur mustard and scarified 3 hours following exposure. Note – neutrophils adhere to the endothelium (arrows) and/or cross through the vessel wall. This reflects the next stage (to that shown in Fig. A) of histomorphlogical changes following exposure to the irritant, in which acute inflammatory cells adhere and cross the vascular bed close to the irritant-exposed area. H&E, X600.

(C) Animal treated with sulfur mustard and scarified 8 hours following exposure. Note – neutrophils adhere to the endothelium and/or cross through the vessel wall, and infiltrating the adjacent dermis (arrows). This reflects the next stage (to that shown in Fig. B) of histomorphological changes following exposure to the irritant, in which acute inflammatory cells adhere, cross the vascular bed, and infiltrate the dermis close to the irritant-exposed area. H&E, X600.


A
H&E, SM, 3H
B
H&E, SM, 3H
C
H&E, SM, 8H
D
H&E, SM, 8H
E
TNFα, SM, 3H
F
TNFα, SM, 8H
G
TNFα, Control, 3H
H
TNFα, SM+Iodine, 3H
I
TNFα, SM+Iodine, 8H
J
TNFα, SM+Vehicle, 8H
K
COX-1, SM, 3H
L
COX-1, SM, 8H



(D) Animal treated with sulfur mustard and scarified 8 hours following exposure. Note – neutrophilic infiltration in the dermis (arrows) and empty lumen of blood vessel (asterisk). There is apoptosis of some of the inflammatory cells. H&E, X600.

(E) Animal treated with SM, sacrificed 3 h following exposure. Arrows indicate numerous strongly (grade 3) TNF-alpha-positive neutrophils within lumen of blood vessel. TNF-alpha immunostaining, X400.

(F) Animal treated with SM and sacrificed 8 h later. Arrows indicate numerous strongly (grade 3) TNFalpha-positive neutrophils within lumen of blood vessel, adhering to the endothelium and within the dermis. TNF-alpha immunostaining, X600.

(G) Control anima sacrificed 3 following exposure to vehicle. Arrows indicate few neutrophils within blood vessel, most negative for TNF-alpha. TNF-alpha immunostaining, X600.

(H) Animal treated with SM and iodine, sacrificed 3 h following exposure. Arrows indicate lumen of blood vessel with no inflammatory cells. TNF-alpha immunostaining, X400.

(I) Animal treated with SM and iodine sacrificed 8 h following exposure. Arrows indicate that lumen of blood vessel has only very few inflammatory cells that are TNF-alpha positive (thin arrow), in contrast to the SM- treated in which blood vessels have numerous inflammatory cells that are TNF-alpha-positive. Blood vessels devoid of inflammatory cells can be seen (thick arrow). TN-Falpha immunostaining, X400.

(J) Animal treated with SM and vehicle sacrificed 8 h following exposure. Arrows indicate that lumen of blood vessel (thin arrows) -and subcutis (thick arrows) have numerous TNF-alpha-positive neutrophils. TNF-alpha immunostaining, X400.

(K) Animal treated with SM sacrificed 3 h following exposure. Arrows indicate numerous COX-1- negative or slightly (grade 1) -positive neutrophils adhering to the blood vessel endothelium. COX-1 immunostaining, X600.

(L) Animal treated with SM sacrificed 8 h following exposure. Arrows indicate numerous COX-1-negative or slightly (grade 1) -positive neutrophils within lumen of blood vessel. COX-1 immunostaining, X400.

(M) Animal treated with SM sacrificed 3 h following exposure. Arrows indicate numerous IL-1beta-negative or slightly (grade 1) -positive neutrophils adhering to blood vessel endothelium. IL-1beta immunostaining, X600.

(N) Animal treated with SM sacrificed 8 h following exposure. Arrows indicate numerous IL-1beta-negative neutrophils adhering to blood vessel endothelium. IL-1beta immunostaining, X600.

(O) Animal) treated with SM sacrificed 3 h following exposure. Arrows indicate numerous COX-2-negative neutrophils within lumen of blood vessel (thin arrow). Epidermal cells are moderately positive (thick arrows). COX-2 immunostaining, X400.

(P) Animal treated with SM sacrificed 8 h following exposure. Arrows indicate numerous COX-2-negative neutrophils adhering to endothelium of blood vessel (thin arrows). Epidermal cells are moderately positive (thick arrows). COX-2 immunostaining, X400.

(Q) Control animal sacrificed 3 h following exposure. Thin arrows indicate fibroblasts within dermis having dense and homogenous brownish (COX-2-positive) cytoplasm. Epidermal cells are moderately positive (thick arrow). COX-2 immunostaining, X600.

(R) SM-treated animal sacrificed 3 h following exposure. Arrows indicate swollen fibroblasts within dermis having moderate amount of cytoplasm that contains dispersed brownish (COX-2-positive) granules. Epidermal cells are moderately positive (thick arrow). COX-2 immunostaining, X600.

Fig. 4: Effect of anti TNF-alpha antibodies on SM-induced skin lesions in the mouse ear model.

Ear swelling is the difference in ear thickness between values obtained pre-and 2 days postexposure. AntiTNF-alpha antibodies were injected 30 min after SM exposure (diluted in methylene chloride 1:80); 5 microliters were applied on inside area of each ear. AntiTNF-alpha antibodies were injected intracardial at the indicated doses after dilution with saline. Volume of injection: 0.25ml. Controls received 0.25ml 0.9% NaCl. $*p <$ 0.03, $**p < 0.005$.

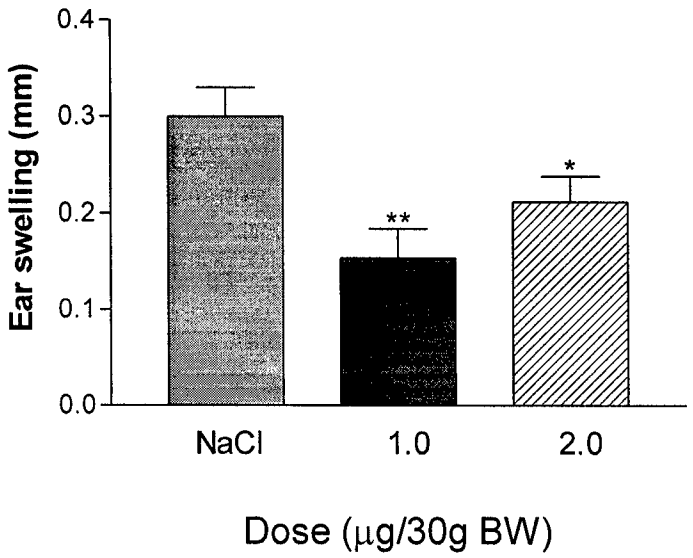


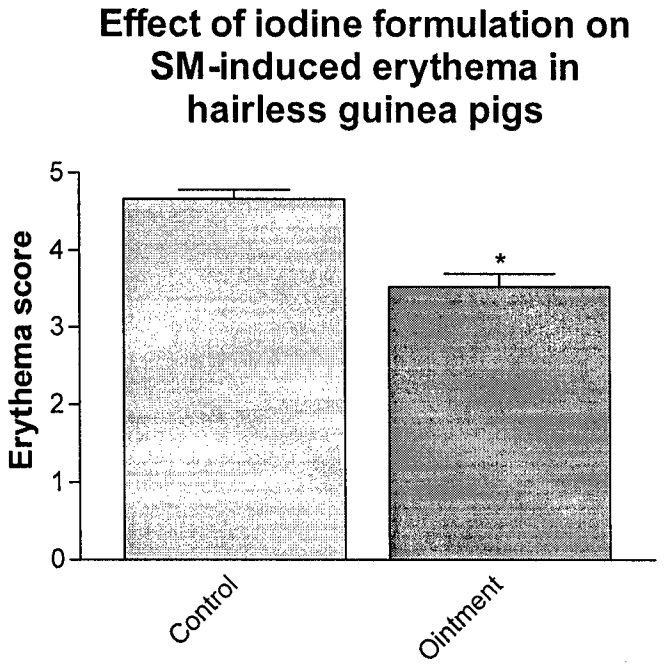
Table 1: Effect of Anti TNF-alpha Antibodies Against Sulfur Mustard-Induced Skin Lesions

Type of lesion	**SM**	**SM+Ab**
Subepidermal microblister formation	0.83±0.40	0.25±0.16
Epidermal ulceration	0.66±0.42	0.50±0.27
Epidermal Necrosis	2.16±0.47	1.87±0.35
Acute inflammation	2.33±0.21	1.25±0.31*
Neutrophilia	0.83±0.16	0.50±0.189
Subacute inflammation	0.33±0.33	0.0±0.0
Hemorrhage	1.83±0.30	1.37±0.26
Dermal necrosis	2.50±0.22	1.75±0.31

AntiTNF-alpha antibody solution was iv-injected (1μg in 0.25ml/30g BW) into mice 30 min after SM exposure. Animals were sacrificed 48 h after exposure; ears were fixed, embedded in paraffin, sectioned, and stained with H&E for histopathological evaluation as described in Methods. The reactive and inflammatory changes in the epidermis and dermis were assigned a severity grade of 0-4 representing unremarkable, minimal, mild, moderate, and marked changes, respectively. Results are expressed as mean ±SE using the Mann Whitney test for statistical evaluation of the differences between the control and antibody-injected groups. SM (n=6); SM + Ab (n=8).

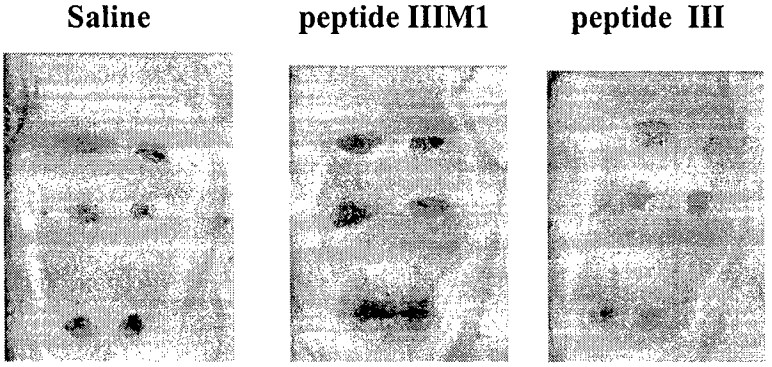
*$p < 0.03$.

Fig. 5:


Effect of iodine formulation on SM-induced erythema in hairless guinea pigs
Erythema score
0
1
2
3
4
5
*
Control
Ointment

*p<0.05

Fig. 6: Protective effect of peptides against SM-induced skin lesions in haired guinea pigs



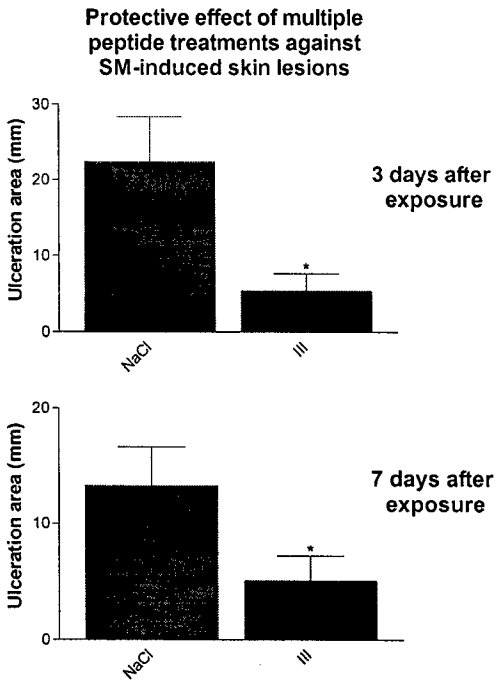
Quantification of the ulceration area in the different treatment revealed that peptide III significantly reduced the size of ulceration area as compared to the vehicle (saline)-treated group.

Peptide III: H-Lys-Gly-Asn-Tyr-Ala-Glu-Arg-Ileu-Ala-OH (KGNYAERIA)

Peptide IIIM1: H-Lys-Gly-His-Tyr-Ala-Glu-Arg-Val-Gly-OH (KGHYAERVG)

Fig. 7: Quantification of the protective effect of multiple treatments of peptides against SM

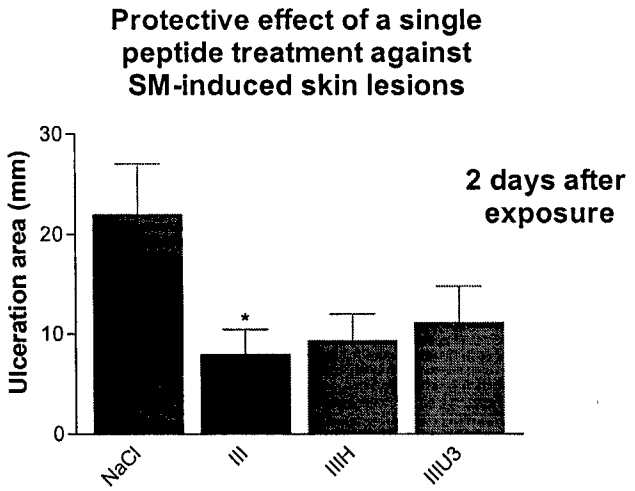
Peptides (III, IIIM1 or vehicle—saline) were administered (iv) into guinea pigs at a dose of 1mg/kg. The animals were injected 8, 6, 4, 2 days and 20 min before exposure to SM. Six sites on each shaved back were exposed to 1µl (1.27 mg) neat SM. The protective effect of peptide III in the guinea pig skin model is demonstrated whereas the mouse sequence IIIM1, did not protect the guinea pig skin against SM.



Peptide III: H-Lys-Gly-Asn-Tyr-Ala-Glu-Arg-Ileu-Ala-OH (KGNYAERIA)

Peptide IIIM1: H-Lys-Gly-His-Tyr-Ala-Glu-Arg-Val-Gly-OH (KGHYAERVG)

Fig. 8: Quantification of the protective effect of a single treatment of peptides against SM Male guinea pigs were intracadially injected with the peptide (1mg/kg) or the vehicle (0.9%Nacl) 7 days (single treatment) prior to SM exposure. The backs of the animals were shaved 24 hours prior to exposure. The back was divided into six sites, each was exposed to 1µl (1.27mg) neat SM. The size of ulceration area of each exposure site was measured and expressed as squared mm. Results are the mean ±SE of 18 sites in the control group and 18 sites of the peptide-treated groups.



Peptide III: H-Lys-Gly-Asn-Tyr-Ala-Glu-Arg-Ileu-Ala-OH (KGNYAERIA)

Peptide IIIH: H-Lys-Gly-Asn-Tyr-Ala-Glu-Arg-Val-Gly-OH (KGNYAERVG)

Peptide IIIU3: H-Lys-Ala-Asn-Tyr-Ala-Glu-Arg-Val-Gly-OH (KANYAERVG

Fig. 9: Lysophosphatidic acid (LPA)-induced luminescence in peptide-transfected and control cells.

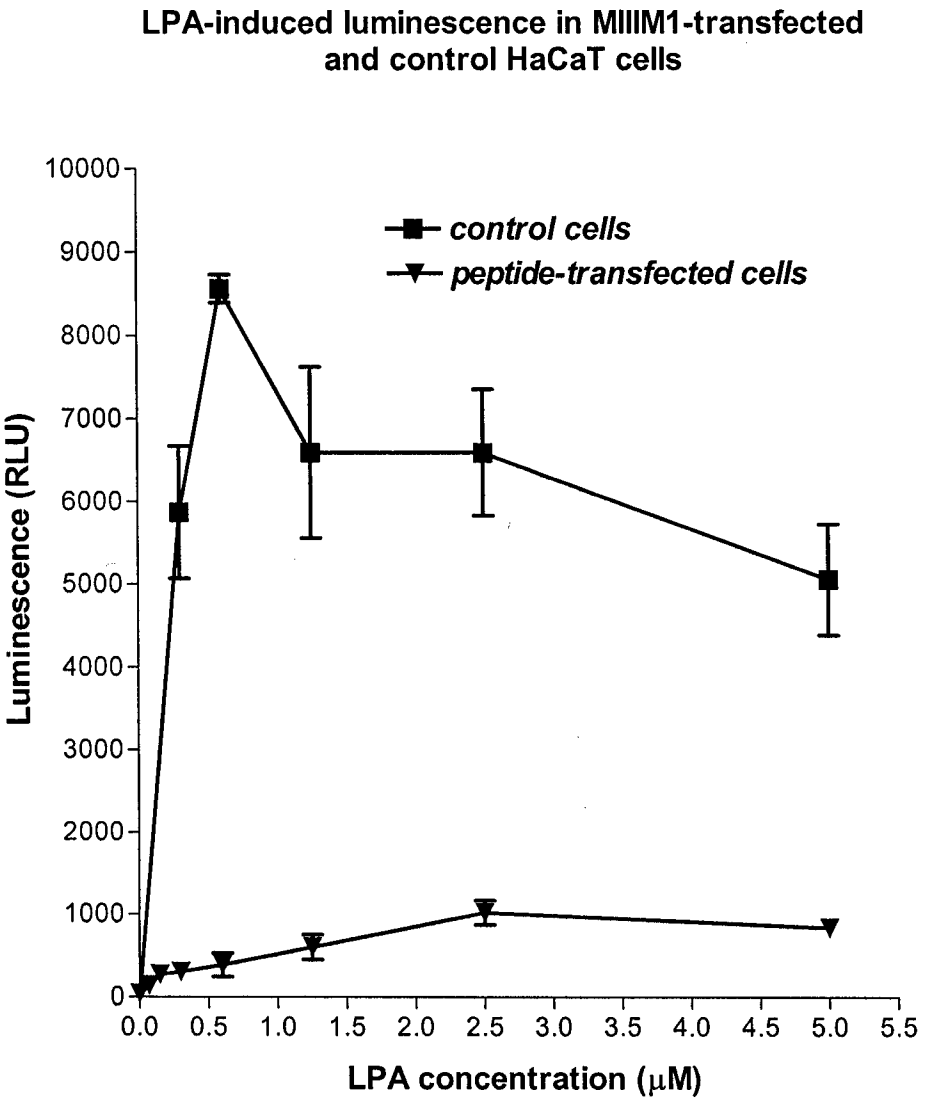


Fig. 10: Effect of hydrogen peroxide on peptide-transfected cells and control HaCaT cells.

The cells were prepared and maintained as describes in Fig. 7. Cells ($0.5x10^6$) were exposed to 500μM hydrogen peroxide. MTT viability test was carried out after 24 hours. It is shown that the control cells are more vulnerable to hydrogen peroxide, namely, reduction of 71% in viability of the control cells whereas the peptide-transfected cells were more resistant and showed reduction of only 38% upon exposure to hydrogen peroxide.

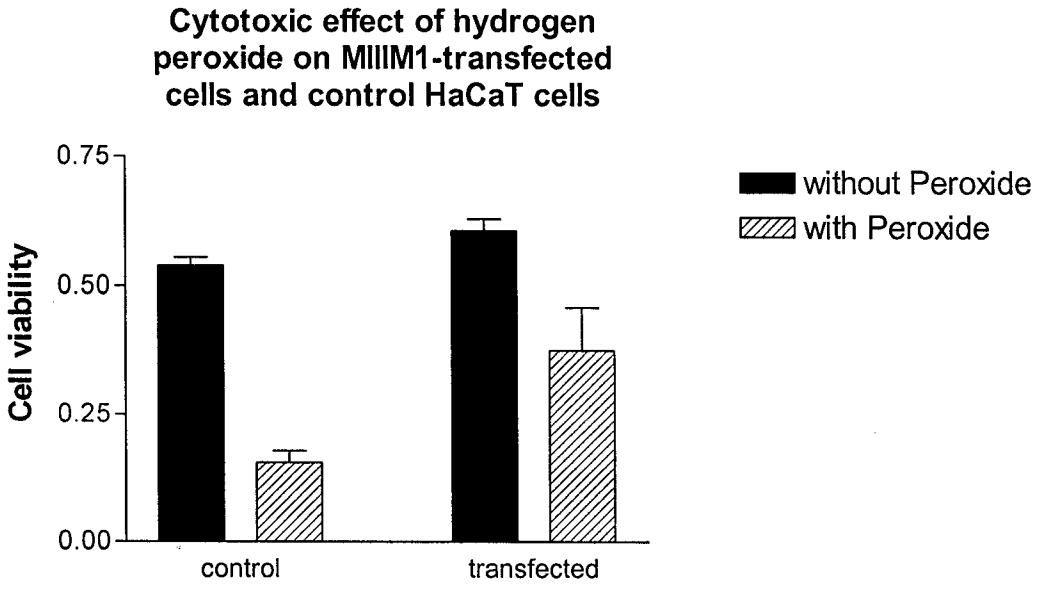


Fig. 11: Effect of sulfur mustard (SM) on peptide-transfected cells and control HaCaT cells.

The cells were prepared and maintained as describes in Fig. 10. Cells ($0.5x10^6$) were exposed to 1μM SM. The bars represent peptide-transfected cells without (peptide) or with exposure to SM (peptide+SM) and control cells without (control) or with exposure to SM (control+SM). MTT viability test was carried out after 24 hours.

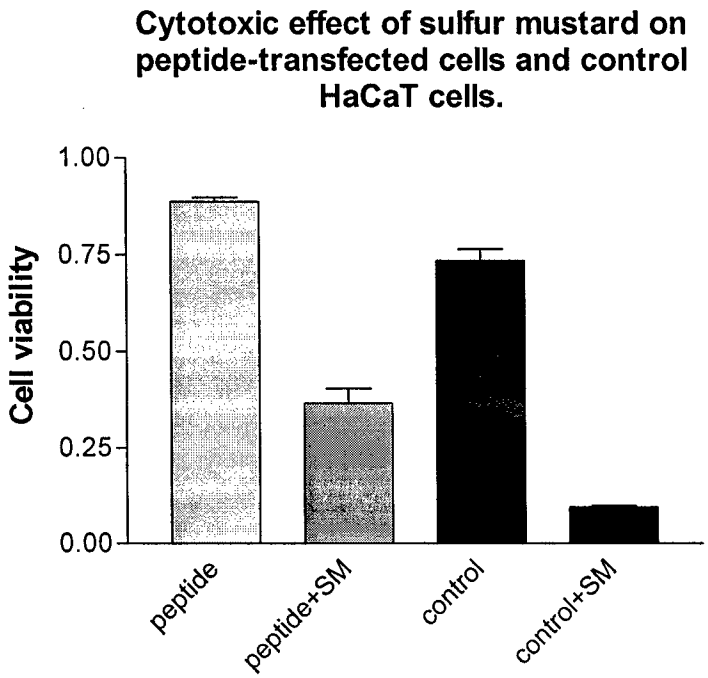


Fig. 12: Effect of IIIM1 on SM cytotoxicity in HaCaT cells.

The cells were preincubated with 30μM peptide for 4 hours then exposed to 1μM SM. MTT viability test was carried out after 24 hours.