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Electron Microscopic Study of Guinea Pig Skin Exposed to Sulphur Mustard

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Sulphur Mustard (SM) 1-1′ thiobis (2-chloroethane) is an alkylating agent that readily reacts with protein and other biomolecules (Somani et al 1989).Several investigations have been attempted to experimentally define the pathogenesis of the cutaneous sulphur mustard lesion using animal models (Herniques et al 1943; Ginzler et al 1943; Papirmeister et al 1984).The pathogenesis has been defined in terms of edema, infiltration of polymorphonuclear cells, vascular alterations as well as ultrastructural changes in cells and their organelles. (Mc Adam 1956; Riviere et al 1991; Papirmeister et al 1991). Although the degenerative changes are easily noticed in cells, it is expected that the extracellular matrix(ECM) constituting the major component of the skin will also be affected. However no attempt appears to have been made to study the effect of SM on the extracellular matrix of G.pig skin. Recent studies on biologically active analogues of ECM have revealed that the ECM plays an important role in remodeling of damaged skin tissues (Yannas 1990). The ECM not only plays a mechanical role in supporting and maintaining tissue structures but also modulates many of cell functions such as development, migration and proliferation. It is recognized that the reepithelization of wounded skin takes place only when there is an underlying bed of ECM in the form of the dermal support. During wound healing or development, cells and matrix interact closely and probably iteratively (Yannas 1990; Shimizu et al 1991). The present study was undertaken to examine the effect of SM on the collagenous ECM in the Guinea pig skin in order to follow the course of injury as well as the onset of recovery. The morphological changes induced by SM were monitored with light, scanning and transmission electron microscopy.

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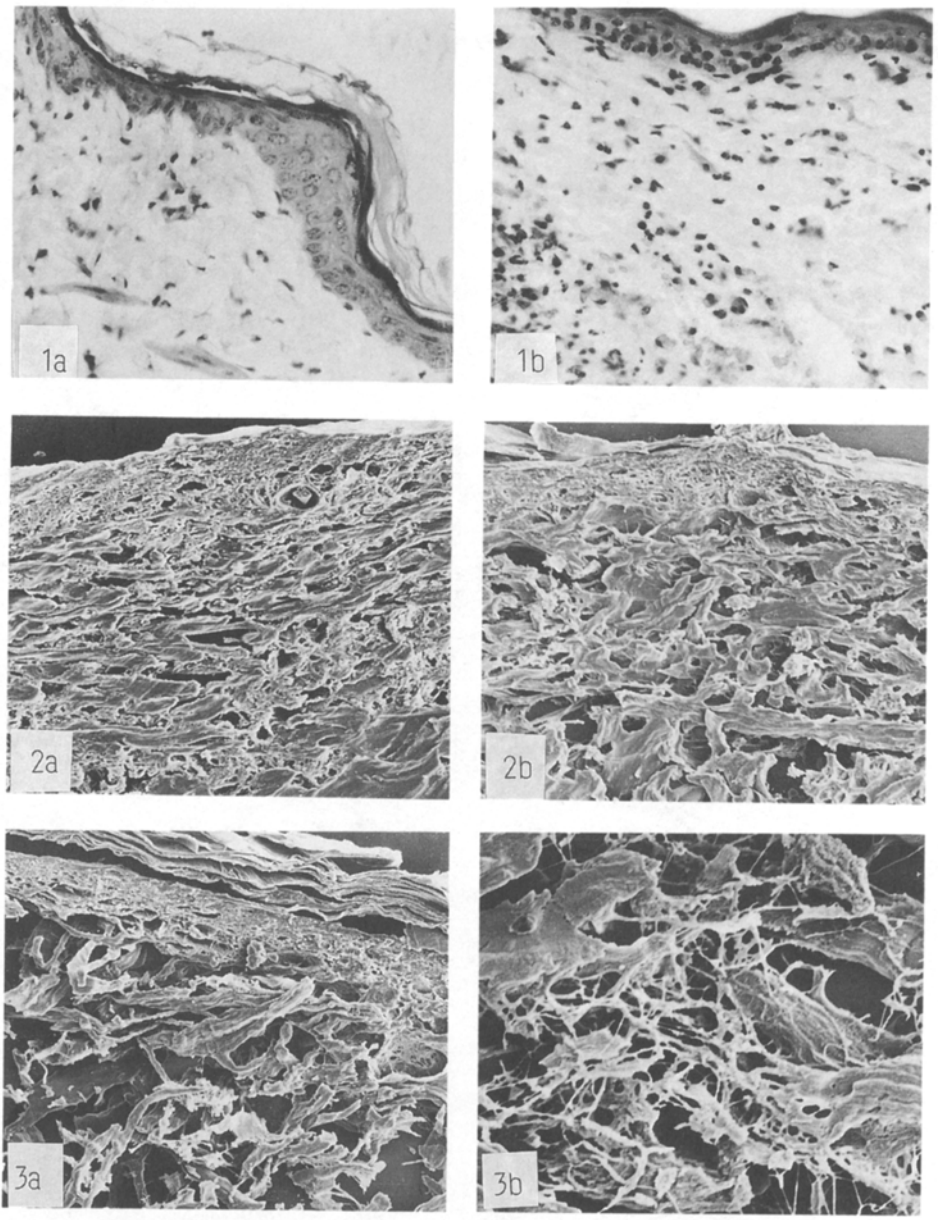
MATERIALS AND METHODS

Young male Guinea pigs of English strain (weighing 280 ± 20g) were kept in polypropylene cages with free access to food and water. Sulphur Mustard (98% pure; by GLC analysis) synthesized in the chemistry laboratory of our establishment was applied on the hair clipped area on the dorso caudal surface of the animals. A single dose of SM, equivalent to one LD50 (51.3 mg/kg percutaneous] was applied on an area of one square cm without any vehicle. The animals were sacrificed on day 1, 3, 6 and 10 after the exposure. In each experimental group five animals were taken. The control skin sample was taken from the unexposed dorso caudal portion of the corresponding animals. However only one representative control micrograph has been given in this study. Skin tissues were processed for electron as well as light microscopy at $4^{o}C$ for 12 h using formaldehyde glutaraldehyde fixation (Karnovsky. 1965). Tissues were post-fixed at $20^{o}C$ for 2 h with 1% osmium tetroxide aqueous solution. Both resin as well as paraffin wax blocks were prepared for the study. One micron thick sections were also cut from the resin tissue blocks and examined in a light microscope following staining with toludine blue. Ultrathin sections were cut on Reichert-Jung Ultracut E from the epoxy resin blocks, stained with uranyl acetate and lead citrate and examined at 80 kV in a JEOL JEM-1200EX Transmission Electron Microscope. From the paraffin tissue blocks 10-12 micron thick sections were also prepared. Sections were dewaxed, coated with a thin layer of gold in a JEOL JFC-1100 sputter coating unit and examined at 5 kV in a JEOL JSM-840 Scanning Electron Microscope. Guinea pig skin comprised of epidermis and dermis was divided into three zones for thickness measurement. The upper part of the epidermis was taken as one of the reference point, the other being the muscle fibres. The distance between the epidermis and the muscle fibre has been termed as total thickness. Likewise the epidermal thickness was also measured. These measurements were done in order to quantify the extent of edema induced by SM exposure. All measurements were done on semi thin plastic sections with the help of a micrometer. The data were analyzed using the students t test. The significant threshold was fixed at $P<0.05$.

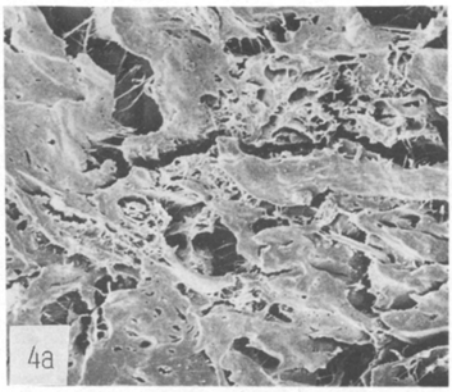
RESULTS AND DISCUSSION

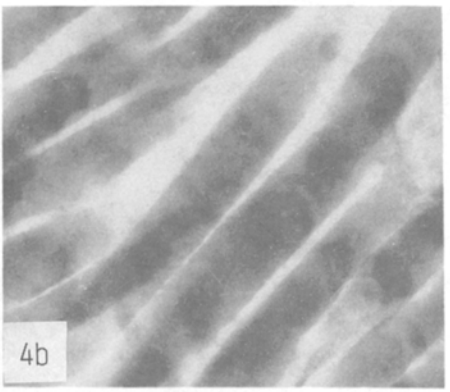
Skins from the unexposed portion of the animal showed a distinct epidermis as well as the associated collagen matrix. The dermis was free from any preferred accumulation of polymorphonuclear leukocytes (Fig 1a). Appearance of mild erythema was observed

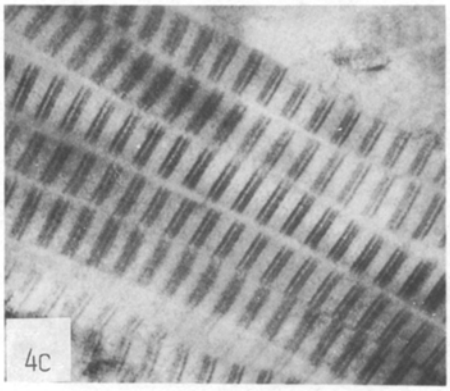
within an hour at the site of SM application. However no blisters were noticed on the skin until the end of the study. An increase in the number of polymorpho-nuclear (PMN) cells was visualized on the first day following the SM exposure (Fig 1b). The lower part of dermis(not shown) also exhibited several foci of invading PMN cells.Cells around underdeveloped hair follicles showed necrotic changes. Many pyknotic fibroblasts were noticed. Structural details of the control skin obtained by SEM showed an ordered and compact arrangement of the epidermis. Adjoining to the epidermal layers the ordered collagenous structures of dermis were evident (Fig 2a). Structural changes revealed by the SEM in the skin on the first day showed signs of loosening of structure resulting in the formation of gaps in the dermis (Fig 2b). However the extent of separation varied in all five animals studied. Such changes were more pronounced on the third day of post-exposure in the form of widespread increased gaps and extensive fibrillation (Figs 3a 3b). It was noted that the fibrillations were more intense in the deep dermal region as compared with upper dermis. The observed changes on the sixth day of post exposure showed a reverse tendency in that the enlarged intercellular gaps in the dermal region underwent a noticeable reduction. At many places the collagen fibres became less fragmented. Overall improvement was observed in the alignment of the collagenous bundles of the ECM leading to a compact structure as compared to the three day old changes (Fig 4a). The cellular components around numerous roots of hair follicles exhibited a concentric symmetry. Although the process of recovery could be noticed on the sixth day, it was far from complete. The collagenous bundles were still loosened and fibrillated at many sites. When examined at high magnification in the TEM these fibres failed to show characteristic periodicity of collagens (Fig 4b). Unexposed fibres revealed characteristic periodicity of collagens (Fig 4c). Cellular damage induced by SM in the form of margination of nuclear chromatin and rarefaction of the nucleus were observed (Fig 4d). Figure 4c shows normal skin cells. On the tenth day following exposure the morphology of the guinea pig skin displayed over all improvements in the compactness of the dermis (Fig 5). The cellular components around the roots of hair follicle were found to attain concentric symmetry. Cells from the basal layer showed normal structural details. The surface texture of sixth and tenth day old skin exhibited a hard crust and inelastic tissues at the sites of SM application. Table 1 presents the data on epidermis thickness and gross intercellular measurement done on G. pig skin. It indicates that the application of SM induces edema which continued to

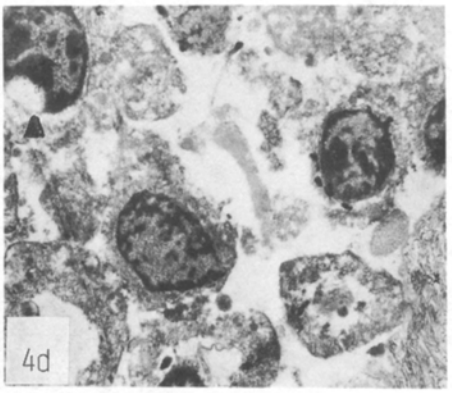


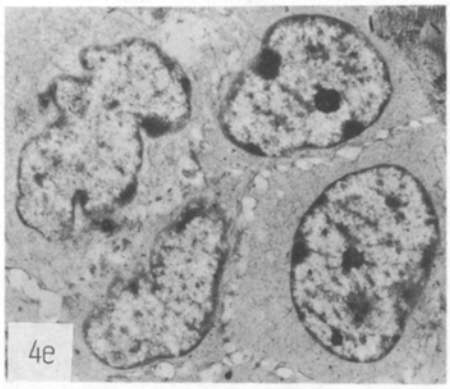
Figures 1a-3b ; 1a: 250X,Control G.P skin with normal histological details ; 1b: 250X Infiltration of PMN cells on first day post exposure ; 2a: 1000X Scanning micrograph of control skin showing compact structure; 2b: 1000X formation of gaps in the ECM, collagen bundles disrupted ; 3a: 1000X breaking up of collagen support of skin on third day ; 3b: 2000X extensive fibrillation of collagenous fibre bundles.

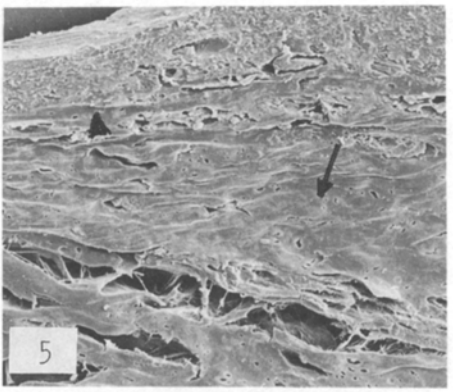












Figures 4a-5; 4a: 2000X GP skin on sixth day after the exposure shows less intense fibrillation and relative compact configuration; 4b:50,000X Transmission electron micrograph of SM exposed collage fibres showing loss of periodicity; 4c: 50,000X TEM of normal collagen fibre showing their characteristic periodicity; 4d:2500X skin on sixth day showing margination of nuclear chromatin and rarefaction of the nucleus (arrow head); 4e: 2500X control cells; 5:1000X compact and ordered dermal (arrow) and epidermal region (arrow head) on the tenth day.

Table-1 Effect of Sulphur Mustard on Skin Thickness(μM)

Sample	Epidermis thickness	Total thickness
Control	52.0±3.0	1000±8.2
SM exposed		
1 Day	90.2±4.2[a]	1602±21.4[a]
3 Day	120.0±8.1[b]	1734±32.9[a]
6 Day	66.6±6.6	1528±25.6[b]
10 Day	59.0±4.2	1350±15.3[b]

N = 20,values are given as Mean ± SE
a = Significant from control at $p<0.05$
b = significant from control and other groups at $p<0.05$

develop until the third day, and thereafter it slowly reduced. On the first day after the treatment the epidermis thickness increased from an average value of 52 μ to 90.2 μ and reached a value of 120 μ on the third day. Likewise the total thickness also increased from an average value of 1000 μ to 1602 μ and reached a value of 1734 μ on the third day. The increase in epidermis thickness as well as total thickness is significant at 95% confidence level from their respective controls. On the sixth day after the treatment the epidermal thickness and the total thickness showed a decreasing trend. Although the decrease in the epidermis thickness was significant compared to the third day values, the reduction in the total thickness of the skin was not significant from the third day values. The decrease in these two parameters continued until the tenth day and the decrease was significant from the third day values at 95% confidence level. However the edema (as measured by the total thickness) still persisted until the tenth day and was significantly different from the control values. Dermal exposure to SM is known to mediate two initial inflammatory responses; infiltration of PMN cells and edema (Papirmeister et al 1984; Dennenberg et al 1985; Chauhan et al 1993a). While the identities and sources of inflammatory mediators involved in SM injury are largely unknown, the histopathology of cutaneous SM lesion in different animal models indicates that vasoactive and chemotactic mediators are produced within the lesional area starting within a few hours of exposure (Papirmeister et al 1991). SM also induces the disruption of ECM and involved collagenous fibres in it. The disruption of ECM could be due not

only to the forces generated during the course of retention of edematous fluid but also the action of SM on the collagenous components of the ECM. It is known that collagenases are contained in healing wounds to remove collagenous debris and they inturn can degrade collagenous fibres (Shimizu et al 1991; Yannas et al 1990). In the present study it could not be substantiated whether the loss of periodicity was due to the action of SM per se, collagenases were responsible for the degradation or the combined effect of these two factors led to the loss of periodicity. It was assumed that proteinases are activated at some stage in SM induced skin injury and that they contribute to both cytopathology and tissue destruction. The cellular changes observed in the present study (Fig 4d) are in agreement with the reported work, though on different animals and are perhaps characteristic to SM toxicity (Vogt 1984; Papirmeister et al 1990, Riviere et al 1991). As with any biological activity eliciting various responses at different levels of organisations, the process of recovery in skin like wise showed varied responses in cells, individual collagen fibres and the extracellular matrix on a temporal scale. The changes in thickness of the epidermis (Table 1) suggest that at the dose used SM even affects the viable layers of epidermis. Structural changes induced by SM in the passive epidermal layers have also been reported in another animal model (Chauhan et al 1993b). The results of the present study indicate perhaps for the first time that SM affects the collagenous extracellular matrix in the skin of G-pig. SM induced intra-fibre damages degrading the periodic structure of collagen. The initiation of recovery could be noticed on the sixth day when the intracellular gaps in the dermal region underwent a noticeable reduction. However the recovery was far from complete as collagen fibres were still found damaged. The recovery on the tenth day was more easily noticeable both at the macroscopic level as well as ultrastructurally. Since all known alkylation products exhibit great stability under physiological conditions therefore it is likely that the cross-linked collagenous ECM will take a long time to return to normal structure. As a result it is expected that the process of healing may continue beyond the tenth day.

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