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Histomorphometric Study of Animal Skin Exposed to Sulphur Mustard

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Bis(2-Chloroethyl) sulfide, commonly known as Mustard gas or Sulphur Mustard (SM) is a highly reactive alkylating agent and forms blisters in human skin. A number of in vivo and in vitro models have been utilized to study skin injury caused by SM exposure (Young et al 1923; Somani et al 1989). Relatively few studies have been attempted to document histological changes in animal skin (Herniques et al 1943; McAdams 1956; Vogt et al 1984). Athymic nude mice with human skin graft has proved to be an excellent model to investigate histopathological effect of SM (Papirmeister et al 1984a). Skin injury, regardless of the ultimate severity develops after a period of several hours. The initial injury consists of nuclear damage in the basal layer followed by intradermal infiltration of polymorphonuclear cells. Dose as well as time dependent histological studies indicated extensive cell damage in the form of pyknosis and edema followed by formation of perinuclear and cytoplasmic vacuoles in different animals.(McAdams 1956; Papirmeister et al 1984a&b).

However no attempt appears to have been made to quantify cellular changes that occur in response to SM. In this study we report histomorphometric measurement as well as histological changes produced by SM on mouse skin. An attempt has been made to quantify the changes in lipocytes as well as other cellular parameters to follow injury as well as the post-exposure recovery process.

MATERIALS AND METHODS

Swiss Albino male mice weighting 25 ± 3 g were kept in polypropylene cages. Animals had free access to food and water. SM (98% purity) synthesized in the DRDE laboratory was applied on the clipped area at the dorso

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caudal surface of the animals. The choice of doses used in the present study was selected from an earlier work (Vijayraghavan et al 1991). A single dose of 154.7mg/kg body weight was percutaneously applied to an area of one square cm without any vehicle. The animals were sacrificed on day 1,3 and 6 after the exposure. Each group consisted of 6 animals. In order that the morphometric data are comparable among animals of different groups a control skin sample was always taken from each animal from the unexposed dorsal portion. The only animals considered for the study were those in which the control skin had similar thickness as well as gross histological features so that the observed changes could be attributed only to SM and not to the variability in animals. Skin tissues (both control and treated) were fixed in 3% glutaraldehyde, dehydrated and embedded in resin for electron microscopy. Simultaneously the control skin was also fixed separately in 1% OsO_4 solution, so as to preserve the lipocytes effectively (Baker et al 1980). One micron thick sections were cut from resin blocks on an ultramicrotome. One set of sections was observed after staining with toluidine blue. The other set of sections was washed with a saturated solution of KOH in alcohol so as to remove the embedding resin. The sections were then stained with hematoxylin and eosin. Photomicrographs were taken at low magnification to demonstrate the changes in epidermis, and dermis simultaneously.

Mouse skin, comprised of epidermis and dermis, was divided into four zones for measurements. The lower part of the dermis, where the muscle fibres appeared, was taken as one of the reference points, the other being the upper part of the epidermis. The distance between the epidermis and the muscle fibres has been termed as the total thickness. Likewise the epidermis thickness, the thickness (or the space) between epidermis and lipocytes, as well as the thickness of lipocyte layer in the dermis, were measured with the help of a micrometer. These lipocytes were localised in the lower dermis more or less in a parallel array. The randomly placed lipocytes outside the array which were relatively few in number were not considered for measuring their thickness (spread). For tracing the lipocyte boundary the skin cross section were projected on the ground glass screen of a 'projectina' microscope at a magnification of 460X. Tracings of 100 lipocytes representing six animals in each group were obtained on tracing papers which could be fixed on the ground glass screen of the projectina. These tracings formed a permanent record of the samples. The area and perimeter of these cells were measured with the help of a planimeter. The data were analysed using the Student's

t-test. The significant threshold was fixed at $p < 0.05$.

RESULTS AND DISCUSSION

Skin from the control animal showed a distinct epidermis with hair follicles and associated structures Fig 1a. In preparing the skin for embedding in resin, lipids were lost from lipocytes and were seen as a delicate network of empty polygonal shaped structures. The presence of lipocytes as well as their morphological features were reconfirmed by fixing the tissues separately with osmium tetraoxide. Osmium tetraoxide crosslinks the lipid components effectively. When viewed in the optical microscope the lipocytes appeared as dark polygonal shaped structures. The 24-h treated skin showed necrosis of cells which obliterated their normal cellular features. The number as well as the size of lipocytes was reduced considerably, and their rounding was evident Fig 1b. The collagen appeared more diffused than in the control. A few inflammatory cells were also observed in the dermal region. Pyknosis of cells was noticed. Cells around underdeveloped hair follicles also showed necrotic changes. These histological changes are in agreement with the reported work (Papirmeister et al 1984).

Figure 1c shows the effect of SM on third day. Detachment of the part of epidermis from dermis was noticed at certain points. Necrosis of cells in the entire area of dermis and epidermis was noticed. Many basal cells appeared rounded and the blood vessels were dilated. The number of inflammatory cells in the three day-old specimens showed an increase over 24-h treated tissue. The number as well as the size of lipocytes also registered an increase over the 24-h specimen.

The degenerative changes after the sixth day were less severe as compared to the third day lesions Fig 1d. Some of the cells around hair follicles in the dermal region had partially regained their shape. At places, signs of reepithelisation was noticed. The size of lipocytes began to change towards the normal tissue. Table 1 presents the data on epidermis thickness, distribution of lipocytes and the gross intercellular measurements done on mouse skin. The decrease in the thickness of the epidermis after the treatment was not significantly different than the control at the 95% confidence level.

On the first day after the treatment, the total thickness of the skin increased from an average value of 393 μm to 442 μm, and reached a value of 808 μm on the third day. Edema persisted until the last day of

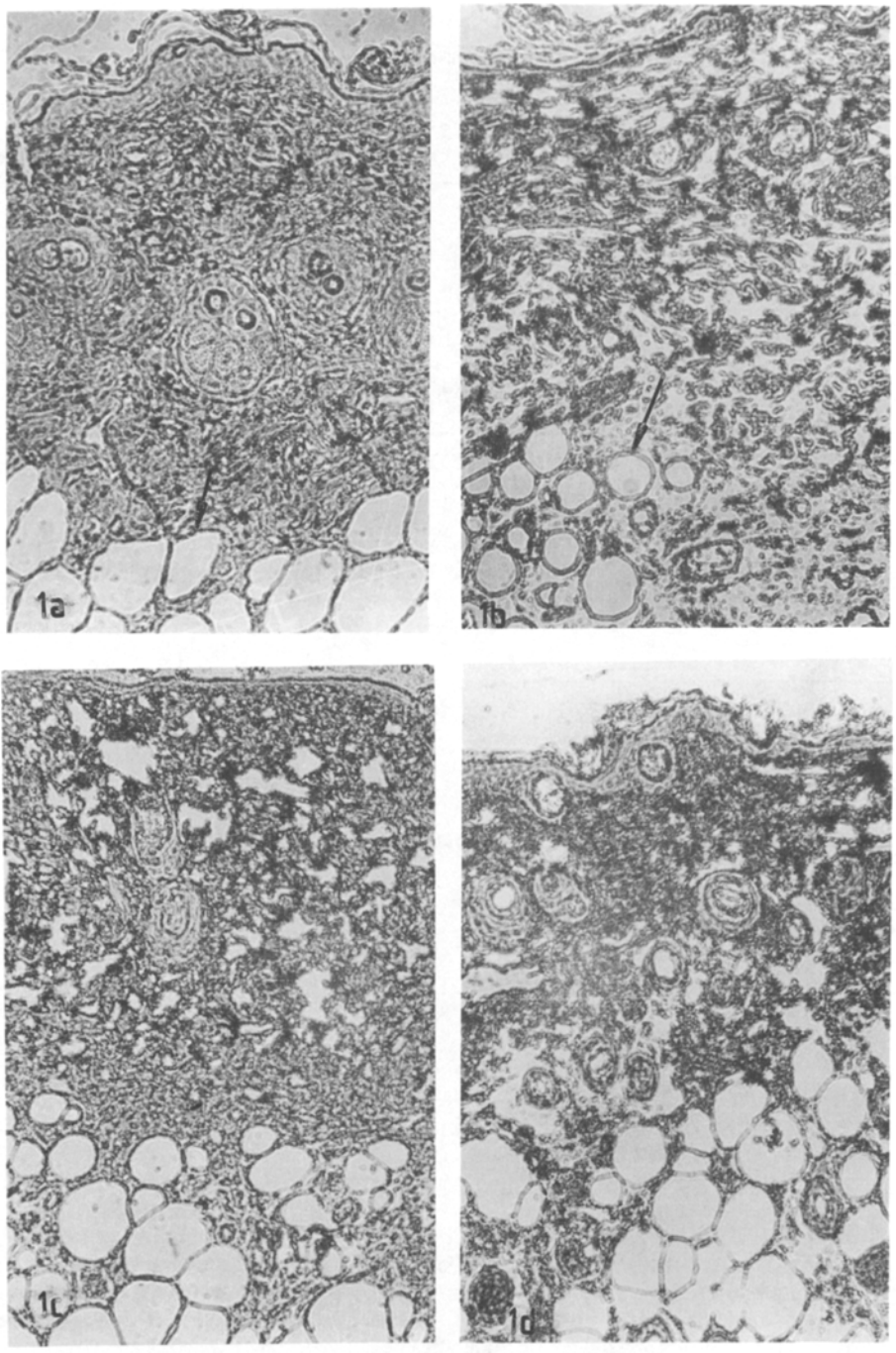

Figure 1. Effect of SM on skin - 1a. Distinct features of control skin including lipocytes (arrow). 1b. Depletion of lipocytes and their rounding. 1c. Reappearance of lipocytes on third day. 1d. Formation of outer boundary of hair follicle roots. (H & E - Original Mag. 80 x)

Table 1 Effect of sulphur mustard on skin thickness(μm)

Sample	Epidermis thickness	Distance between epidermis & lipocytes	Lipocyte layer thickness	Total thickness
Control	14.9±0.9	203± 5.9	168± 7.3	393± 5.6
SM exposed				
1 day	14.5±1.3	238± 5.5[a]	187± 6.4	442± 5.9[a]
3 day	14.4±1.2	387±13.0[b]	407±14.8[b]	808±17.0[b]
6 day	13.8±1.2	310± 9.2[b]	471±12.7[b]	792±11.6[a]

N = 20, Values are given as MEAN ± SE
a = significant from control at $p < 0.05$
b = significant from control & other groups at $p< 0.05$

Table 2. Perimeter and area of lipocytes

Sample	Perimeter (μm)	Area (μm^2)
Control	123.1 ±6.1	1008.7 ± 62.6
SM exposed		
1 Day	66.3 ±1.9[b]	338.4 ± 17.0[b]
3 Day	442.0 ±15.8[b]	1809.0 ±117.0[b]
6 Day	115.0 ±4.0	976.0 ± 67.0

N = 100, Values are given as MEAN ± SE.
b = significant from control & other groups at $p < 0.05$

the observation. The increase in the total thickness was significant for all the three sets of observations. However the change in the total thickness was not significant between the third and sixth day, though a decreasing trend was observed. The injury induced by SM was very pronounced on lipocytes.

As a result the distribution of lipocytes, their number and size were altered, which consequently changed the morphometric data. The distance between the epidermis and the fat cells increased from 203 μm to 238 μm after 24-h. This increase indicated edema between these two reference points. Table 1 shows that the distance further increased in the three day old lesions. The lipocytes after regeneration were dispersed over a larger area and remained so until the sixth day. Table 2 shows the morphometric data on perimeter and area of

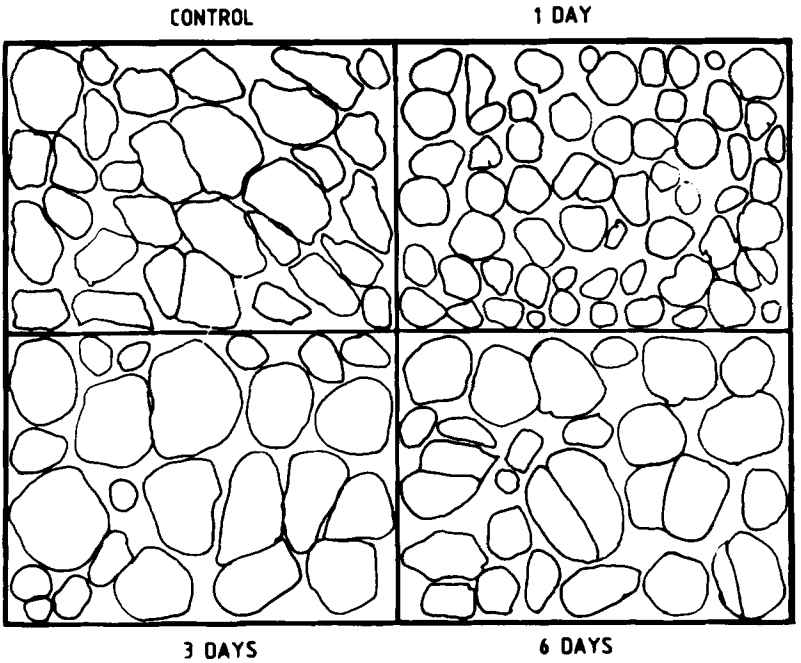

Figure 2. Representative tracing of lipocytes showing change in shape and size on different days. Original mag-460 x.

lipocytes of control as well as treated skins. Figure 2 presents the lipocyte boundaries illustrating change in shape and size as a result of SM treatment. The figure does not represent the actual numerical density of lipocytes present in a cross section of the tissues. However maximum number of lipocytes were drawn randomly in the figure from sections so as to show all shapes and sizes. On the first day after the application, the area as well as perimeter of lipocytes showed a drastic reduction. On the third day the lipocytes showed an increase in number as compared to the one day old lesion and their size increased significantly both with respect to control and the one day old lesions. Since skin is an active lipid synthesizing organ, the formation of lipocytes could have begun anywhere between the first and the third day. Newly formed cells were larger. The perimeter as well as the area of lipocytes on third day were significantly increased. However on the sixth day after the treatment both the perimeter and the area registered a significant decrease from the third day values. The changes in the skin on the sixth day were suggestive of regeneration as many cells appeared to have regained their shape. The hair follicle roots again reappeared. The values of the perimeter and the area of lipocytes also were

close to the control value, and they were not significantly different.

The present study shows that the application of SM induces edema which continued to develop until the third day. Due to the filling of edematous fluid, various histological changes occurred. The morphometric data recorded in this study enabled us to quantify these changes. Most of the measured parameters were affected by the distribution and shape of lipocytes. It is well known that SM is a lipophilic agent and due to the high electrophilic property of the sulphonium ion it may not only affect DNA but also membranes and proteins (Wormser 1991).

The combined effect of these two factors, namely the dissolution of lipid by SM and the disintegration of collagen bundles surrounding the lipocytes (due to the forces generated by the edematous fluid) could be responsible for the decrease of lipocytes in terms of dimensions and number on the first day after the treatment. The electron microscopical observations revealed that after the treatment the collagens surrounding the lipocytes were distorted on the first day *(unpublished observation)*. In spite of the edema between the first and the third day, the lipocytes had regained the outer cell boundary and recovered in number. The newly formed lipocytes were larger in size but of varied shapes. The presence of inflammatory cells as well as the reappearance of lipocytes were suggestive of a recovery process at the cellular level. The shape and size of the lipocytes on the sixth day appeared closer to normal. Histological details presented in this report are comparable to those previously reported, in spite of different animal models used (Papirmeister et al 1984a).

This study indicates that SM depletes and distorts the lipocytes due to its lipophilic nature. The reappearance of lipocytes was taken as one of the early indications of the post-recovery process along with the other histological changes. The recovery observed in the present study lends support to the earlier report that healing rates of cutaneous SM lesions in animals are somewhat faster than those reported in humans (Papirmeister et al 1984a).

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