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ULTRASTRUCTURAL CORRELATES OF THE PROTECTION AFFORDED BY
NIACINAMIDE AGAINST SULFUR MUSTARD-INDUCED
CYTOTOXICITY OF HUMAN LYMPHOCYTES IN VITRO

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19 ABSTRACT (Continue on reverse if necessary and identify by block number) We have previously shown that HD causes a concentration-dependent decrease in the viability of human lymphocytes as measured by dye exclusion. We have also shown that this decrease in viability was preventable by inhibitors of poly(ADP-ribose) polymerase, such as niacinamide. We are now gaining morphological correlates of the protection afforded by niacinamide through scanning and transmission electron microscopy study of human lymphocytes exposed to 1×10^{-6} M sulfur mustard (HD) incubated in the presence or absence of 1×10^{-6} M niacinamide for 24 hours at 37°C. Lymphocytes exposed to HD alone demonstrated 30%-40% viability and presented loss of microvilli, large cytoplasmic vacuoles, extensive blebbing of the perinuclear envelope, loss of cytoplasmic organelles, condensation of nuclear chromatin and multiple perforations of the plasmalemma. HD-treated lymphocytes in the presence of niacinamide had a viability of 87% and, except for blunting of microvilli, presented essentially normal ultrastructure. Although the sequence of the observed ultrastructural changes was not established, results of this morphologic study suggest that,					
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19. Abstract (cont'd)

in addition to the prevention of plasmalemmal deficits and dye infusion, the mechanism of niacinamide protection appears to include the preservation of morphologic and functional integrity of cellular organelles. (170)

PREFACE

This collaborative morphological study describing the pathology of sulfur mustard (HD)-induced lesion and its protection was performed under TASK AREA 875, PROTOCOL # 1-01-83-000-B-220 and satisfied JSA requirements STO-01, 02, 03. All technical protocols and morphologic data were recorded in laboratory notebook # 16-86 assigned to Dr. Petrali.

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INTRODUCTION AND REVIEW

In spite of the many reports since World War 1 on the pathology of mustard injury, no ultrastructural studies were performed until our laboratory published a report on the ultrastructure of the pathogenesis of blister formation following exposure to sulfur mustard of human-skin grafted to congenitally athymic nude mice (1). This study afforded us the opportunity for the first time to focus on several objectives: 1) to further delineate the histopathology which had been noted at light microscopy levels; 2) to identify possible early mustard-induced morphological changes which may occur during the latent asymptomatic phase; and 3) to promote our understanding of the temporal features of mustard pathology with the expectation that prophylactic and therapeutic strategies might be morphologically predictable. We now have the opportunity to describe HD-induced pathology in cells-in-culture, compare them with that of the skin lesion, and for the first time, describe the ultrastructural parameters of the protection afforded by candidate prophylactic compounds such as niacinamide.

By way of review, HD-induced pathology of human skin grafted onto congenitally athymic nude mice was initiated by damage to the basal keratinocyte of the stratum germinativum (Fig 1). These changes, obvious by light microscopy, beginning at 6 hours after HD exposure, included nuclear condensation and paranuclear vacuolation and led eventually to the separation of the dermal-epidermal junction at 24 to 48 hours post-exposure. By electron microscopy, basal cell changes at the same time frame included, sequentially, the condensation of nuclear chromatin with loss of euchromatin, blebbing and relaxation of the perinuclear envelope, appearance of paranuclear vacuoles, swelling of the endoplasmic reticulum, loss of mitochondria, progressive vacuolation, and perforation of the plasma membrane (Fig 2). Ultrastructural study of the separation of the dermal-epidermal junction at 24 to 48 hours following exposure provided evidence that separation occurred with the disabling of the anchoring filaments of hemidesmosomes between the altered plasma membrane of the basal cell and the basal lamina of the dermis (Fig 3). Microblisters thus formed were bounded by a roof composed of the basal cell membrane and a floor composed of the basal lamina. Although microblisters did not coalesce to form frank blisters, the temporal features of the developing pathology of microblister formation with this model system provided clear evidence that HD injury of human skin begins at the basal cell nucleus. This morphologic expression of HD injury supported our hypotheses linking morphological changes to underlying biochemical processes involving DNA damage and poly-adenosine diphosphate ribose polymerase-mediated depression of NAD, leading to the release of proteases which result in cell death and the induction of the associated microblister.

The focus of the present study is to describe, also for the first time, HD-induced pathology of human lymphocytes in vitro and the ultrastructural parameters of the protection afforded by an inhibitor of

polymerase, niacinamide, already proven in a companion study (2) to be effective in maintaining the viability of HD-treated lymphocytes.

MATERIAL AND METHODS

Human lymphocytes incubated for 24 hours at 37°C with niacinamide (1×10^{-3} M), HD (1×10^{-3} M), or HD in the presence of niacinamide were washed in suspension medium and centrifuged for 10 minutes at 250xG. The resultant cell pellets were fixed for one hour in 1.6% formaldehyde and 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.34 and 192 mOsm with respect to the buffer. Following primary fixation, the pellets were post-fixed in buffered 1% osmium tetroxide, dehydrated in graded ethanol, recentrifuged to minimize losses and embedded in epoxy resin. Replicate semithin sections, 1 micron thick, were differentiated with Humphrey's stain (3) and analyzed by light microscopy. Ultrathin sections, counterstained nonspecifically with uranyl acetate and lead citrate, were examined by transmission electron microscopy. Those portions of fixed pellets not embedded in epoxy resin were critically point dried, sputter-coated with gold-palladium to 150 angstrom thickness and processed for scanning electron microscopy.

RESULTS

Semithin sections of lymphocytes exposed to HD alone showed clearly the loss of viability and cytotoxicity induced by this compound (Fig 4). Viability of this group as determined by dye exclusion was 30% of control values. Morphologically, cells presented condensed nuclear chromatin, pyknosis, large paranuclear vacuoles which haloed the nuclei of affected cells. Fragments and ghosts of necrotic cells appeared in the surround. HD-treated cells in the presence of niacinamide, on the other hand, were intact with a viability of 87% of control values and, otherwise, showed the protection afforded by niacinamide. Lymphocytes of the niacinamide group were essentially unchanged from that of controls.

By scanning electron microscopy lymphocytes of the niacinamide group presented essentially normal cellular surface features of medium-sized lymphocytes, to include abundant microvilli, cell processes and intact plasmalemma (Fig 5). Lymphocytes of the HD-exposed group showed the adverse affects of their surface features, i.e., cells became rounded, lost their microvilli and presented many perforations of the plasma membrane. These changes were especially obvious at higher magnifications.

Transmission electron microscopy was most revealing of the HD-induced pathology and its similarities to the pathology seen on basal cells of the human skin graft study (Fig 6.) Niacinamide-treated lymphocytes showed essentially normal ultrastructure with well-developed chromatin networks, intact cytoplasmic organelles, and a cytoplasm rich in monoribosomes. Lymphocytes exposed to HD alone demonstrated fine-structural changes indicative of cytotoxicity and cell death. These

changes, involving 7 out of 10 cells included condensation of nuclear chromatin with loss of euchromatin, extensive blebbing of the nuclear envelope, paranuclear vacuolation, loss and electron opacity of cytoplasmic organelles, and fragmentation of the plasma membrane. Fragments of cytoplasm, ghost cell membranes and other cellular debris of spend cells made up most of the surround. Lymphocytes exposed to HD in the presence of niacinamide presented essentially normal ultrastructure, with the exception of stunted microvilli, and appeared protected from the cytotoxic effects of HD. A persistent feature of the protected cell, in addition to the paucity of microvilli, was the appearance of unusually clustered vacuoles in various regions of the cytoplasm. The significance of these vacuoles is not known at this time, although they appear not to affect the viability of the cells.

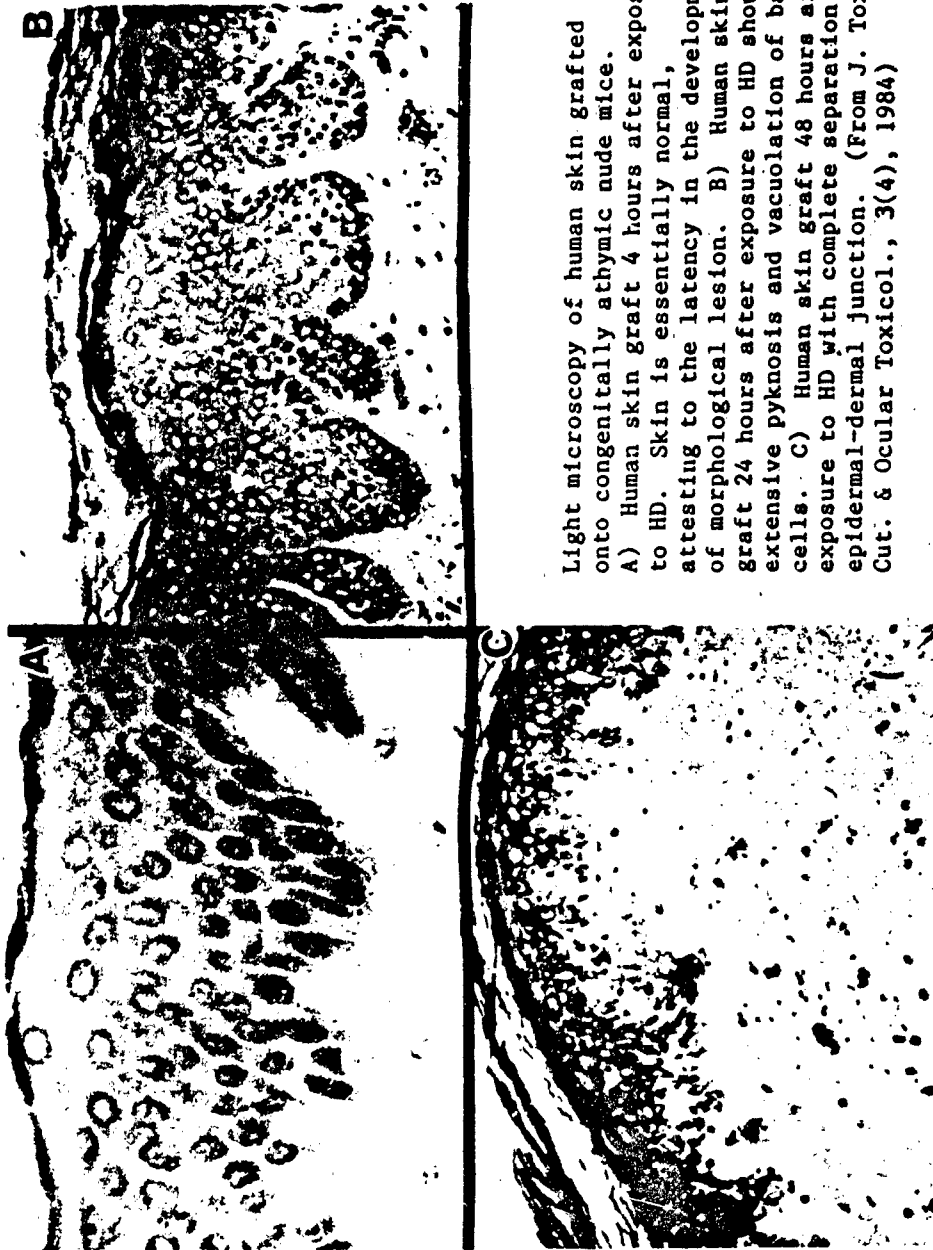
DISCUSSION

The results of this in vitro ultrastructural study indicate that the fine structural events correlate strongly with the basal cell pathology induced by HD in human skin grafted to congenitally athymic nude mice (1). In both types of tissue there was the development of initial nuclear pathology, followed by cytoplasmic changes leading to the death of the cell. Although sequential events could not be established in this single time study of lymphocytes, the developing ultrastructural pathology appears identical to that of human skin and points out that the protection afforded these cells by niacinamide could be extended to include a similar mechanism of protection in skin.

It is not possible to conclude whether the ultrastructural changes observed in this study and in the previous human skin graft study (1) are specific for HD-cytotoxicity. Responses of the basal cell to exogenous toxins and proteases such as papain (4), collagenase (5) pronase (6), and the reported ultrastructural pathology of other skin lesions (7) are all strikingly similar to the HD-induced pathology of the skin basal cell and, now, to that of the human lymphocyte in vitro. In addition, the temporal features of HD-induced lesion, to include the effect on the epidermal-dermal junction, are also similar. If the response is nonspecific, it points to the vulnerability of the basal cell, as well as to the epidermal-dermal junction in the skin, as a site of primary lesion in most skin pathologies.

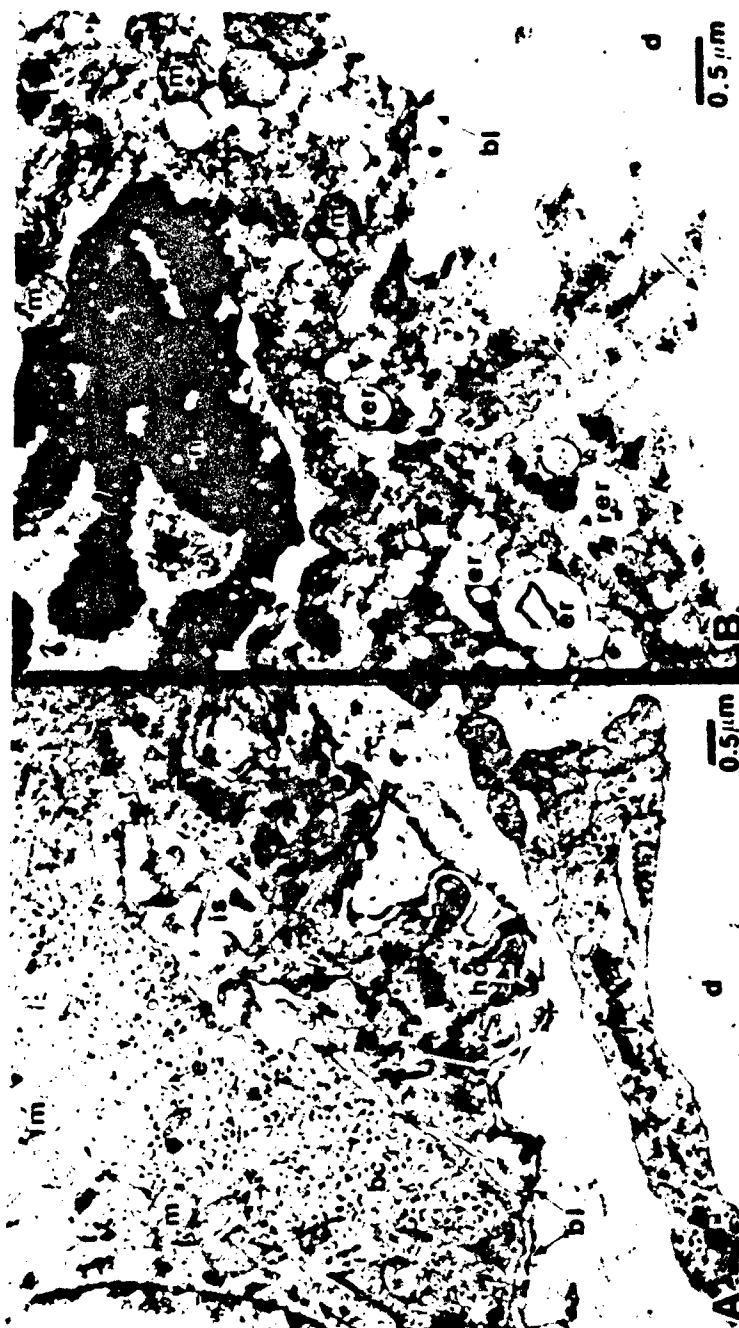
Irrespective of the specificity or nonspecificity of the cellular response to HD toxicity, this study validates the usefulness of the human lymphocyte in vitro model as an alternate tissue of study for HD toxicity, and presents morphological support of the protection afforded by niacinamide.

FIGURE 1



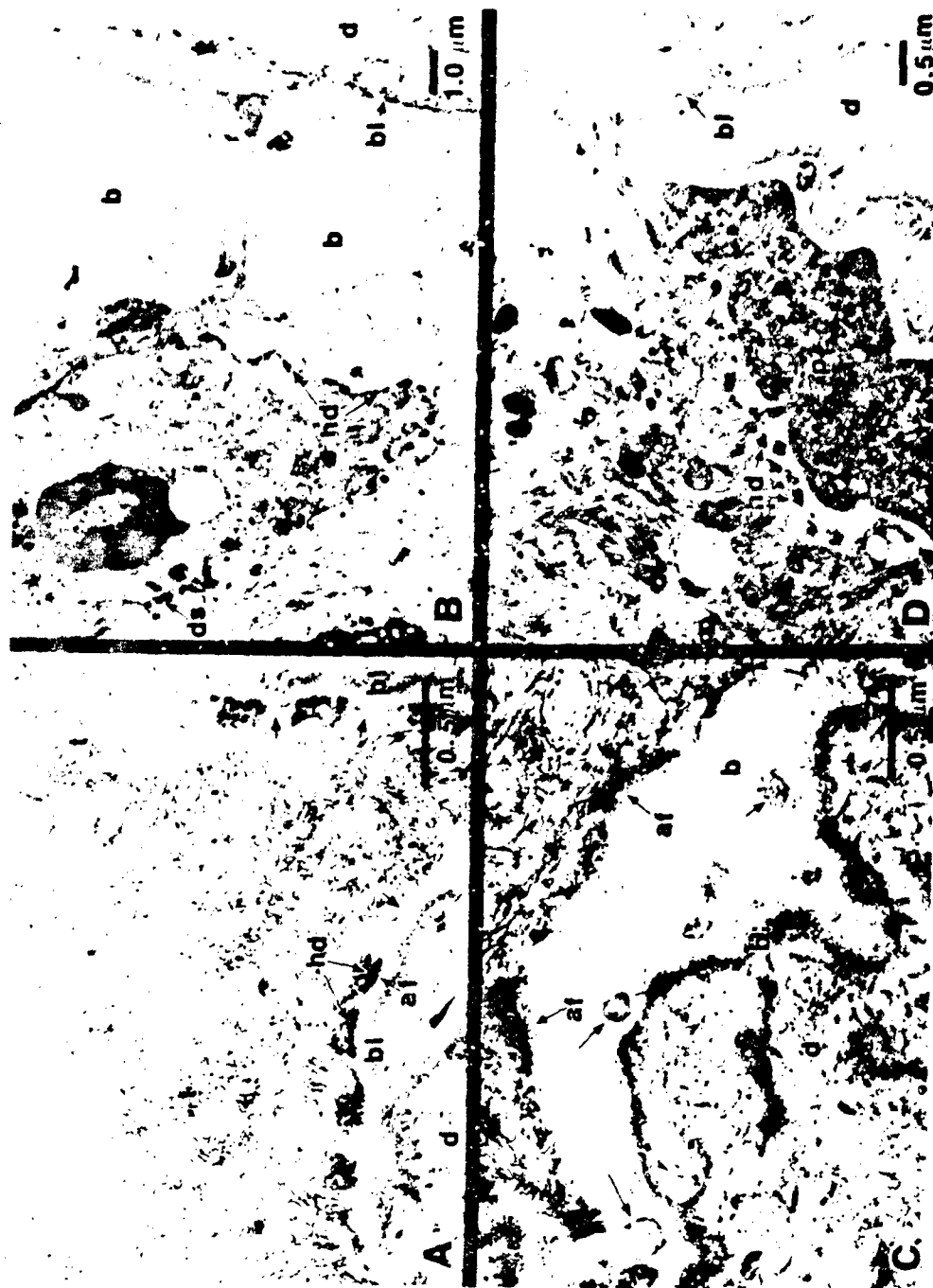
Light microscopy of human skin grafted onto congenitally athymic nude mice. A) Human skin graft 4 hours after exposure to HD. Skin is essentially normal, attesting to the latency in the development of morphological lesion. B) Human skin graft 24 hours after exposure to HD shows extensive pyknosis and vacuolation of basal cells. C) Human skin graft 48 hours after exposure to HD with complete separation of epidermal-dermal junction. (From J. Toxicol. Cut. & Ocular Toxicol., 3(4), 1984)

FIGURE 2



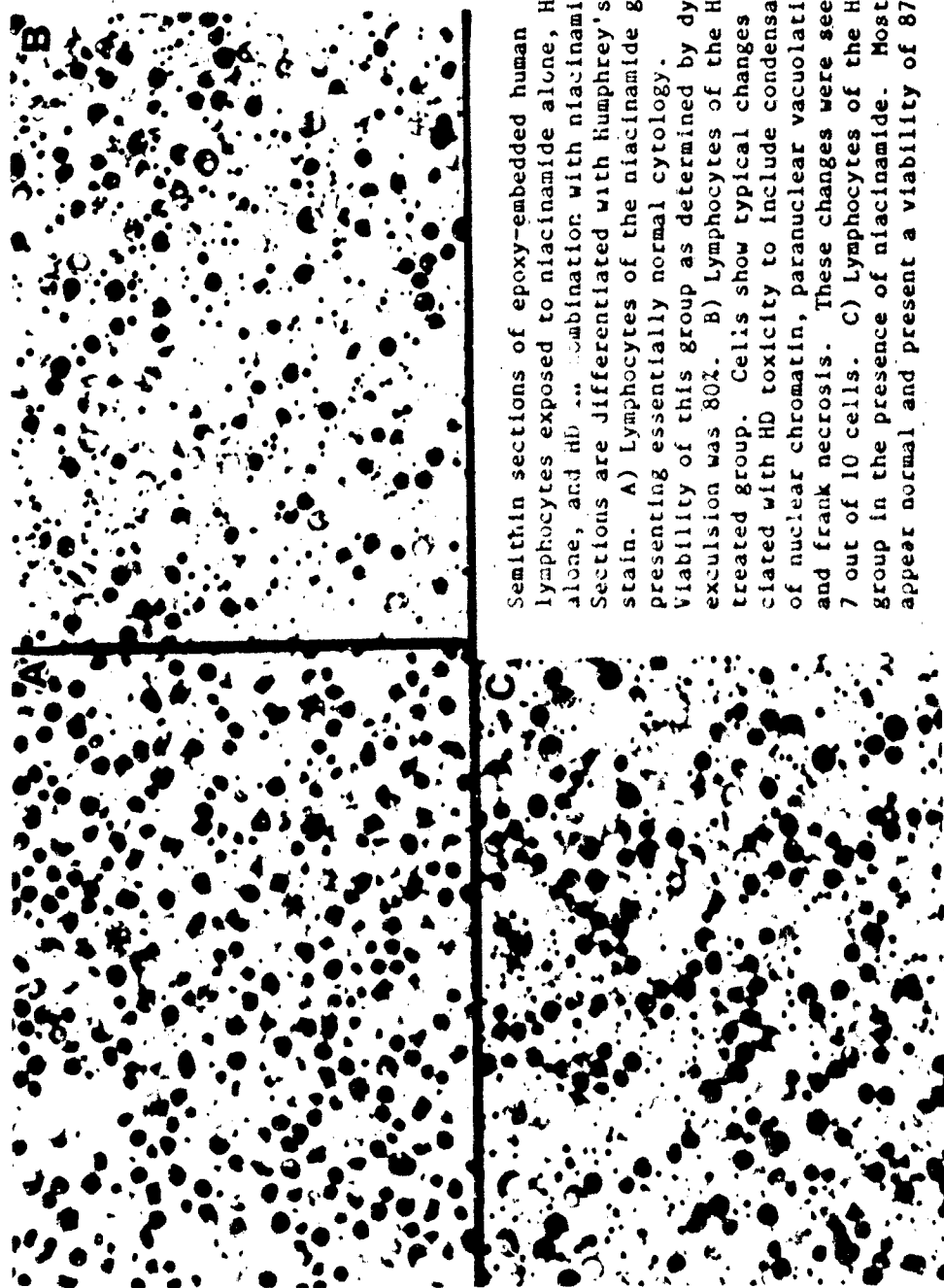
Ultrastructural changes produced by HD in the basal cell. A) Basal cell not involved in the toxic process; (m) mitochondria, (e) endoplasmic reticulum, (bc) basal cell cytoplasm, (bl) basal lamina, (is) intercellular space, (t) tonofibrils, (er) rough endoplasmic reticulum, (sm) smooth endoplasmic reticulum, (n) nuclear chromatin condensation, (f) fibroblast, (d) dermis. B) Early changes at 12 hours following exposure include nuclear chromatin condensation (n), breaks in the plasma membrane (arrows), widening of perinuclear spaces, and swelling of rough endoplasmic reticulum (rer) and dilation of smooth endoplasmic reticulum (er). (From J. Toxicol. Cut. & Ocular Toxicol., 3(4), 1984).

FIGURE 3



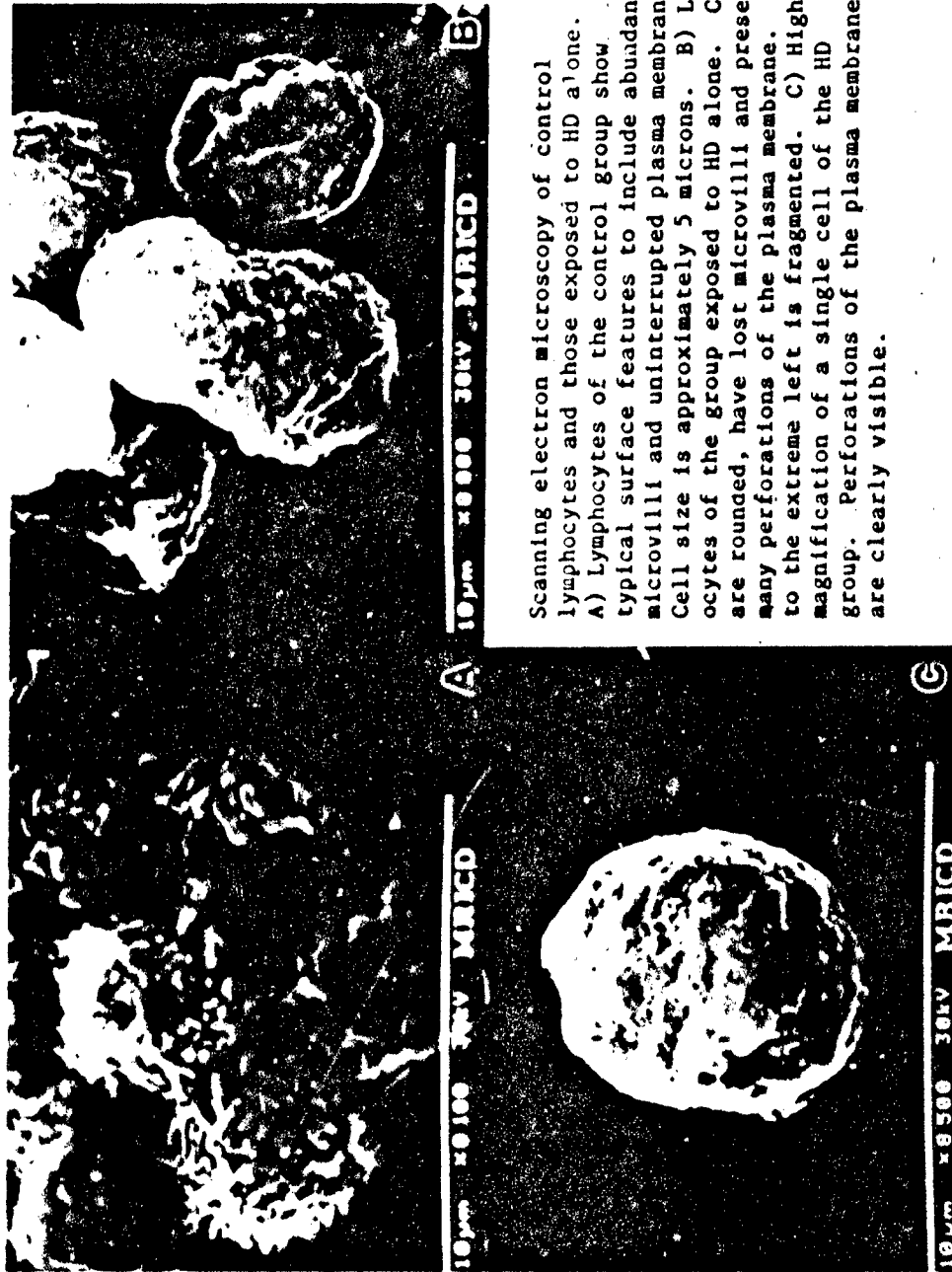
Ultrastructural changes produced by HD at the epidermal-dermal junction. A) The junction prior to vesication at 24 hours following HD. Except for perforations of the plasmalemma (arrows) other structures appear normal, such as tonofilaments (t), hemidesmosomes (hd), anchoring filaments (af), basal lamina (bl), and collagen fibers in the dermis (d). B) Basal cell changes to include coalescing vacuoles (b), separation of cell from dermis (d) by disruption of hemidesmosomes (hd). C) Microblister formation at 48 hours following HD. Blister is formed by disablign of anchoring filaments (af) of hemidesmosomes. D) Invasion of blister cavity by phagocyte (p) 48 hours after HD exposure. (from J. Toxicol. Cut. & Ocular Toxicol., 3(4), 1984)

FIGURE 4



Semithin sections of epoxy-embedded human lymphocytes exposed to niacinamide alone, HD alone, and HD in combination with niacinamide. Sections are differentiated with Humphrey's stain. A) Lymphocytes of the niacinamide group presenting essentially normal cytology. Viability of this group as determined by dye exclusion was 80%. B) Lymphocytes of the HD-treated group. Cells show typical changes associated with HD toxicity to include condensation of nuclear chromatin, paranuclear vacuolation and frank necrosis. These changes were seen in 7 out of 10 cells. C) Lymphocytes of the HD-treated group in the presence of niacinamide. Most cells appear normal and present a viability of 87%.

FIGURE 5



Scanning electron microscopy of control lymphocytes and those exposed to HD alone. A) Lymphocytes of the control group show typical surface features to include abundant microvilli and uninterrupted plasma membrane. Cell size is approximately 5 microns. B) Lymphocytes of the group exposed to HD alone. Cells are rounded, have lost microvilli and present many perforations of the plasma membrane. Cell to the extreme left is fragmented. C) Higher magnification of a single cell of the HD group. Perforations of the plasma membrane are clearly visible.

FIGURE 6



Transmission electron microscopy of human lymphocytes exposed to niacinamide alone, HD alone and HD in the presence of niacinamide. A) Lymphocyte of the niacinamide group. Ultrastructure is typical of a medium-sized lymphocyte with well developed chromatin network, intact mitochondria (m), microvilli (Mv), uninterrupted plasma membrane (PM) and cytoplasm rich in monoribosomes (Rb). B) Lymphocyte of the HD-treated group presenting nuclear pyknosis (N), cytoplasmic vacuoles (V), blebbing of the perinuclear envelope (NB), rarefaction and electron opacity of cytoplasm and perforations of the plasma membrane (arrows). Cellular viability of this group was 30%. C) Lymphocyte of the HD-niacinamide group presenting, except for stunted microvilli (Mv), essentially normal ultrastructure. Cellular viability of this group was 87%.

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