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Studies on the microcirculatory changes in non-freezing cold injury were extended to analogous types of vascular dysfunction in order to determine the possible role of local circulatory insufficiency as a common final pathway through which a variety of environmental factors may contribute to the severity of tissue damage. The technique of three-dimensional life-like visualization of the terminal vasculature by sudden in situ freezing was adapted for use on the ears of weanling rats to investigate the circulatory effects of dietary magnesium deficiency. This condition was of particular interest, since the characteristic erythema of the ears resembles that in cold injury, the need for magnesium has been found to be increased in the cold, and both conditions have been related to mast cell depletion. Although venular dilation was shown to be the principal cause of the erythema in both conditions, the vascular derangement in magnesium deficiency differed from the cold-induced dysfunction in the concomitant development of arteriolar dilation.

Preliminary observations were carried out on immersion freezing of the mouse ear to compare the resulting lesions with those of non-freezing cold injury and to develop an experimental model for rapid evaluation of therapy in frostbite. Exposure at -10°C to -70°C for 1 sec. to 2 min. were investigated. Irreversible circulatory impairment usually resulted only after more than 10 sec. at -20°C. Such lesions resembled non-freezing cold injury in being characterized by erythrostasis with some shunting through larger vessels near the margins of the frozen tissue. However, most arteries and veins tapered down sharply and became obstructed within a short distance after entering the frostbitten region, suggesting that segmental spasm of these vessels may have contributed to the pathogenetic ischemia.

LIST OF KEY WORDS FOR INDEXING: Cold injury, pathology, blood circulation, magnesium deficiency, rats, mice, nutrition.

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Physiopathology of Non-freezing Cold Injury

Previous studies on non-freezing cold injury of the mouse ear have shown that the irreversible tissue damage is a consequence of local microcirculatory stasis caused by the combined effects of arteriolar spasm, excessive capillary-venular dilation, and transendothelial leakage of plasma, which leads to hemocoagulation with increased viscous resistance to blood flow. Since a variety of exogenous and endogenous factors are known to produce similar microcirculatory changes, it was of interest to investigate other environmental conditions which might potentiate the cold-induced pathogenetic reaction. It has already been reported that dextran, commonly used as a plasma expander, will accentuate cold injury in mice, presumably because of the associated erythrocyte aggregation. In a similar manner, an increased viscous resistance to blood flow resulting from erythrocyte aggregation might account for the potentiation of cold injury by co-existing tissue damage or a dysproteinemia which would lower the suspension stability of the blood.

The present investigation was concerned particularly with the effects of dietary magnesium deficiency since this condition produces erythematous lesions similar to cold injury in the ears of weanling rats, and the need for magnesium during cold exposure is known to be greatly increased. Moreover, in both magnesium deficiency and cold injury, the release of vasoactive agents from mast cells may be concerned in the mechanism of tissue damage.

In order to correlate the microcirculatory changes during the course of the deficiency with histologic findings, the recently devised freeze-fixation technique for life-like visualization of the terminal vasculature in mouse ears was adapted for use on rats.
In addition, preliminary experiments were carried out on the local circulatory impairment caused by freezing in the mouse ear to determine the feasibility of producing a standard lesion which would be comparable to the non-freezing cold injury and might provide a rapid means for evaluating therapy in frostbite. Also the possible pathogenetic role of plasma proteases in non-freezing cold injury of the mouse ear was investigated by observations on the loss of metachromasia in ear cartilage.

Materials and Methods - The rats used for the magnesium deficiency experiment were male weanlings of the Sprague-Dawley strain with a weight range of 29 to 48 grams. They were kept in individual plastic cages with wire-mesh flooring which kept the animals from contact with the absorbent paper lining the cage bottom. A Hotpack walk-in environmental chamber served to maintain the temperature at 22°C ± 0.5°C. The low magnesium diet was obtained from Nutritional Biochemical Corp., Cleveland, Ohio. Control animals received the same diet with a supplement of 45.1 mg. magnesium (460 mg. MgSO4.7H2O) per 100 gram diet. Food and distilled water were supplied ad libitum. All mice were 6 to 8-week-old albino males of the Swiss-Webster strain housed and fed as in previous studies (1)(2).

The technique for life-like in situ fixation of the vasculature of rat ears by a sudden shower of -70°C acetone was similar to that described in prior publications (1)(7) except that subsequent submersion of the animal in a coolant bath was used to insure complete freezing. Dehydration in tetrahydrofuran at -15°C was prolonged from 18 hours to 40 hours. Stereomicroscopic examination was carried out on whole mounts of the ears cleared in Canada balsam. To visualize the plasma, 0.5 ml. of 1.4% Evan's Blue in saline
was injected one minute prior to termination.

Histologic sections were prepared from the whole mounts for correlative study by removing the balsam with xylol and embedding in paraffin. Other preparations were fixed in formal corrosive or a 10% solution of formaldehyde in 75% ethanol for optimum preservation of mast cells. In addition to hematoxylin and eosin, Wright's stain modified for tissue sections (8) was used to aid in identification of mast cells and to demonstrate the metachromasia of cartilage.

Vascular and histologic changes in the ears of magnesium deficient rats (9):

The vascular and histologic effects of magnesium deficiency were studied in the ears of 55 weanling rats after 2-14 days on the test diet. Thirty matched control animals received the same diet supplemented with magnesium sulfate. The onset of the reaction occurred 3 to 4 days after the beginning of deprivation and was characterized by rapidly progressive vasodilation involving arterioles as well as venules and, to a lesser extent, capillaries. There was no evidence of preceding vasoconstriction. Associated findings were a protein-rich edema and a predominantly eosinophilic cellular infiltrate. After 5 to 7 days of deprivation vasodilation was maximal and the dominant tissue changes were mononuclear cell infiltration and proliferation together with connective tissue and epidermal hyperplasia. After 7 to 8 days, the inflammatory process subsided spontaneously. However, interstitial fibrosis with diffuse thickening of the ears resulted in pallor by 14 days. Degranulation of mast cells was not prominent, but there appeared to be a slight progressive decrease in their number. Intravenous and dietary administration of magnesium sulfate, after four days of deficiency, reduced the hyperemia gradually over a period of more than 2 days. The sudden onset of the inflammatory process, the slow regression after magnesium supplementation and the spontaneous reversal despite continuation of the deficiency, support the concept that the de-
Efficiency triggers the release of an endogenous vasoactive factor (e.g., serotonin) which is ultimately exhausted.

Freezing Injury of the Mouse Ear. The ears of mice anesthetized with ether were frozen for periods of 1 second to 10 minutes by immersion in liquid coolant ranging from -13°C to -70°C. Six hours to 4 days after freezing the ears were examined to determine the course of the lesions. Exposures of more than 5 seconds at -25°C or below resulted in total microcirculatory arrest within 6 to 25 hours. After less than 5 seconds at -25°C, or more than 10 seconds at -20°C, there usually was rapid development of confluent capillary-venular erythrostasis with occasional persistence of circulation through interarteriolar and intervenous communications at the proximal margins of the frozen tissue. The principal arteries and veins typically became obstructed within a short distance after entering the frostbitten region. The lumens tapered down sharply proximal to the point of occlusion and dilated again in the occluded portions, suggesting that segmental spasm of these vessels at the border of freezing may have contributed to the pathogenetic circulatory impairment. Exposures of less than 10 seconds at -20°C, or as long as 2 minutes at -18°C, resulted in little or no permanent tissue damage. Thus the most favorable conditions for producing a standard lesion of moderate severity appeared to be an exposure of 10 to 15 seconds at -20°C, but even under such well-defined circumstances the degree of injury was remarkably variable.

Extracellular Proteolysis in the Mediation of Non-freezing Cold Injury. Since the early vascular as well as connective tissue damage in cold injured extremities is typically focal despite uniform exposure to low temperature, it was of interest to determine if interstitial activation of focally extravasated plasma protease was concerned in the pathogenetic mechanism. The action of such an extracellular proteolytic enzyme is known to be manifested by reduction in cartilage metachromasia. In a preliminary study on 10 mice, the effect of exposure at 3°C, for 7 days on metachromasia of ear cartilage and vascular
lesions was observed. At the end of cold exposure the animals were etherized, and one ear was amputated and frozen at -70°C, after intravenous injection of Evan's blue dye, while the other ear was fixed in Zenker-formol. The frozen-fixed ears were prepared as cleared whole mounts for stereomicroscopic examination of the microvascular changes and the Zenker-fixed ears were sectioned and stained with hematoxylin and eosin, Lendrum's micro-Mallory method for fibrin, and Bensley's method for metachromasia. Results were compared with those in mice kept at room temperature.

The ears of all cold-exposed mice showed decreased metachromasia of cartilage as well as extensive capillary-venular leakage, stasis, erythrocyte extravasation, and segmental fibrinoid necrosis of patent venules. These results suggest that extracellular proteolysis is a factor in cold-induced impairment of cartilage. Further work is needed to determine if a similar proteolytic mechanism is involved in fibrinoid necrosis and to establish if the proteases concerned are derived from the plasma or from lysosomes.

COMMENT

The findings presented suggest that a capillary-venular insufficiency analogous to that in non-freezing cold injury constitutes a common final path in the pathogenesis of such diverse inflammatory conditions as those caused by magnesium deprivation or tissue freezing. The microcirculatory derangement in magnesium deficiency differs from cold-induced vascular dysfunction in the absence of vasoconstriction. However, the exaggerated venular dilation produces a similar increase in the ratio between the respective cross-sectional areas of the venular bed and the corresponding arteriolar supply with consequent slowing of venular flow and increased transendothelial plasma leakage. The microvascular derangement in freezing injury resembles that
of non-freezing cold injury in the predominance of erythrostasis as the ob-
structive process, but thrombosis is more common and segmental spasm of
veins as well as arterioles appears to contribute to the production of patho-
genetic ischemia.

Since the mechanism of tissue damage in magnesium deficiency as well as
in freezing and non-freezing cold injury appears to depend on a similar pro-
gressive microvascular impairment, these pathogenetic processes are likely
to potentiate each other and their combined effects might result in irreversible
ischemia, even when their independent action would be subliminal. A similar
potentiation of circulatory insufficiency could result from other injurious
factors such as mechanical irritation and secondary bacterial infection.
Thus a better understanding of this possible common final path in inflammatory
reactions is basic to improving the prophylactic and therapeutic management
of cold injury.
REFERENCES

N.B. References which are capitalized received support under this contract; those marked with an * were accepted for publication during the period covered by the report.


