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Bethesda, MD 20814-5044

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Dear Captain Melaragno:

Enclosed is the Third Triannual Report for Contract No.: N00014-91-C-0044, which is entitled "Cellular and Tissue Injury During Nonfreezing Cold Injury and Frostbite". This Report covers the period from September - December, 1991. If you have any questions about the Report or the research, please contact me at 404-952-1660.

As you will see from the report, we have started to study the role of platelets in the no reflow phenomenon, which is one of the most detrimental events during nonfreezing cold injury and frostbite. Discussion of these concepts with physicians who are experienced in treating cold injury would aid us in designing mechanistic studies that have clinical relevance. We would greatly appreciate it if you or your staff could provide us with the names and phone numbers of military physicians who have experience in this area.

Sincerely yours,

John F. Carpenter, Ph.D.
Senior Scientist

cc: Mrs. Mellars, DCMDS-GAACA
DCMAO Atlanta

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Cellular and Tissue Injury During Nonfreezing Cold Injury and Frostbite

Third Triannual Report: September - December, 1991

Introduction. Our recent research on the mechanisms of cell damage during nonfreezing cold injury and frostbite has provided some interesting insights into potential methods for ameliorating damage to the affected areas. At this point, the clinical relevance of these ideas is only hypothetical. However, as the following paragraphs demonstrate, our work on the contribution of membrane and metabolic perturbations to cold injury should soon provide a solid foundation for future preclinical experiments in animal models and for subsequent clinical studies.

Role of platelets in the no reflow phenomenon. One of the primary causes of tissue damage during cold injury appears to be the failure of vascular flow to be re-established to the affected area after warming. Consequently, the region is subjected to continued ischemia at elevated temperatures, which is ultimately lethal to those cells that may have survived the prior cold ischemia. Histopathological studies have indicated that thrombosis, especially in the microvasculature, plays a dominant role in the no reflow phenomenon. These observations are supported further by a recent clinical study on a small number of frostbite victims. It was found that treating the patients with the "clot buster" tissue plasminogen activator led to increased circulation to and recovery of the thawed tissue.

Thus, the activation and aggregation of platelets to form thrombi during cold injury appear to be major causes of the extensive tissue injury that is noted. If the mechanism of this phenomenon were understood, then a rational approach could be taken for design of potential clinical intervention and/or prophylactic treatment. Based on our recent laboratory experiments, in combination with a review of the relevant literature, we have formulated testable hypotheses for the mechanism of platelet hyperaggregability during cold injury.

Several observations made by previous researchers are relevant to this discussion: 1) Platelets that are exposed to low temperatures can spontaneously aggregate upon rewarming and stirring in vitro. 2) Similarly, platelets are more sticky after exposure to cold, as documented by adhesion to glass beads. 3) While at low temperature, and after rewarming, platelets are hypersensitive to aggregating stimuli (e.g., collagen, thrombin, ADP). 4) It is thought that vascular endothelium is damaged during cold exposure (see below). This could result in a reduced production of prostacyclin, which attenuates platelet aggregation. In addition, loss of endothelium would expose the underlying collagen and provide a focal point for initiation of thrombus formation.

Using Fourier transform infrared microspectroscopy, we have found that platelet membrane phospholipids undergo a phase transition from liquid crystalline to the gel state during cooling. We propose that this increase in the viscosity of the membrane may lead to the increased sensitivity of chilled platelets to agonists and perhaps serve as a primary lesion leading to spontaneous aggregation. There are numerous examples in the literature of modification of platelet sensitivity to agonists by changes in membrane viscosity. All of these studies were

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conducted isothermally at $> 22^{\circ}\text{C}$ using chemicals to modify membrane fluidity. Compounds such as cholesterol that increase membrane viscosity at physiological temperature make platelets hypersensitive. The opposite is seen for a wide variety of membrane fluidizers, including Vitamin E and anesthetics. The mechanism for transduction of this physical change into an alteration in platelet function is not known. However, it seems reasonable that increasing membrane viscosity by lowering temperature should have the same acute effects as those noted when chemicals are used to modify fluidity. We will test this hypothesis by determining the sensitivity of platelets to agonists, their stickiness, and their capacity to spontaneously aggregate, when they are incubated above and below the transition temperature. In addition, we have recently found that membrane fluidizers (e.g., chloroform) serve to broaden and lower the temperature of thermotropic phase transition. Thus the hyperaggregability of platelets incubated at low temperatures should be attenuated in the presence of these compounds.

Membrane phase transitions may also play a role in the retention of the capacity of chilled platelets to spontaneously aggregate upon rewarming and stirring in vitro. Interestingly, if they are held at 37°C without stirring for 4-5 minutes, spontaneous aggregation is much reduced upon stirring. This period could be sufficient for the hysteretic transition of the membrane phospholipids back into the liquid crystalline phase. Alternatively, spontaneous aggregation may be a manifestation of increased levels of intracellular calcium that could result due to cold damage to the platelet. Upon rewarming and stirring the calcium could induce aggregation. The loss of the capacity to spontaneously aggregate with incubation at 37°C may reflect the return of calcium homeostasis in the platelet.

We are going to measure the levels of intracellular calcium in platelets after chilling and recovery. If the levels do increase as proposed, we will then investigate the role of thermotropic membrane phase transitions in this process. That is, there could be leakage of calcium from internal membrane-bound stores due to the transition itself and/or to the dysfunction of the Ca^{2+} -ATPase induced by the gel phase lipid.

Finally, it has been noted that chilled platelets lose their disc shape due to depolymerization of microtubules. This process can be inhibited with taxol and other microtubule stabilizers. We will also test the contribution of this process to cold-induced hyperaggregability of platelets.

If these hypotheses prove correct, then we can start to test them more rigorously in vivo using an animal model, such as the chilled rabbit hindlimb. In this model if the leg is maintained at 0°C for more than 20 minutes, there is no reflow upon rewarming. The contribution of platelet aggregation to this process can be tested by injecting platelet inhibitors prior to cold treatment. These compounds range from aspirin to prostacyclin analogues, as well as membrane fluidizers. If the animal studies were encouraging, we could then collaborate with physicians to design a prophylactic treatment to be given to military personnel at risk to cold injuries. The challenge is to design a regime that inhibits cold-induced platelet aggregation while not compromising normal hemostasis.

Metabolic perturbation studies. Our continued studies on metabolic regulation in red blood cells have demonstrated that as temperature is lowered the activity of phosphofructokinase (PFK) is inhibited at progressively higher pH's. That is, our data support the hypothesis that it is the degree of protonation of key

histidine residues and not the actual pH that determines the degree of inhibition of PFK. Thus a level of acidosis that would not be inhibiting at 37°C can greatly arrest PFK activity at 4°C. A manuscript describing these data is in preparation.

Also, we have found that at any temperature there is a reserve of glycolytic flux that can be activated at alkaline pH. For example, the metabolic heat production of red cells at an intracellular pH (pHi) of 7.0 at 37°C (i.e., "normal" physiological conditions) is only about 50% of the maximum total metabolic heat production, which is noted at pHi 7.8. Thus, under anaerobic conditions, where glycolysis provides the sole source of metabolic energy, much greater energy production can be realized by alkalinizing the system. This may have relevance to humans exposed to environmental cold, the extremities from whom can be subjected to ischemia and acidosis. Exercise physiologists have found that pHi-induced inhibition of PFK arising during exhaustive exercise can be attenuated by pre-alkalinizing the blood of subjects. This is accomplished by ingesting alkaline solutions. Theoretically the same approach could aid in the maintenance of glycolytic flux in regions affected by cold-induced vascular stasis.

We will now investigate in vitro the molecular mechanisms responsible for the pH- and cold-induced inhibition of PFK. The experiments will be designed to document the contributions of: the acute effects of protonation of key histidine residues on catalytic activity; dissociation of active tetramers into inactive dimers; and the binding of PFK to structural proteins.

For our next cell model we will work with vascular endothelium, which is known to be destroyed by cold ischemia. For chilling sensitive cells, depletion of metabolic energy and membrane damage are thought to work in concert to foster increased intracellular calcium levels and a concomitant activation of proteases and phospholipases. Our initial studies will characterize the contribution of metabolic inhibition to this process. In addition, in parallel with our platelet studies we will assess how prostacyclin production is affected during exposure to and recovery from cold and acidosis.

Effect of acidosis and inorganic phosphate on skeletal muscle fibers. We have found that increased concentrations of protons and inorganic phosphate synergistically decrease the strength of contraction in skeletal muscle fibers. Our most recent experiments indicate that this effect is amplified at lowered temperature. This observation may account for the progressive loss of skeletal muscle function in areas subjected to long-term cold ischemia.

Stress proteins and chilling damage. We have begun a new collaborative effort with Prof. Abraham Novogrodsky and Dr. Harry Landers (The Rogosin Institute, Cornell University Medical Center). We would like to study the role of stress proteins during hypothermia. That is, we plan to determine if proteins such as Hsp 70 are induced in human cells during cold shock and if synthesis of these proteins increases cellular resistance to this stress. For the latter work, we will attempt to induce synthesis prior to hypothermic exposure (e.g., either with drugs or with sublethal heat shock). There is one example in the insect literature documenting that sublethal heat shock increased the resistance of the organisms to subsequent cold shock. These sorts of experiments have not been performed with human cells.



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