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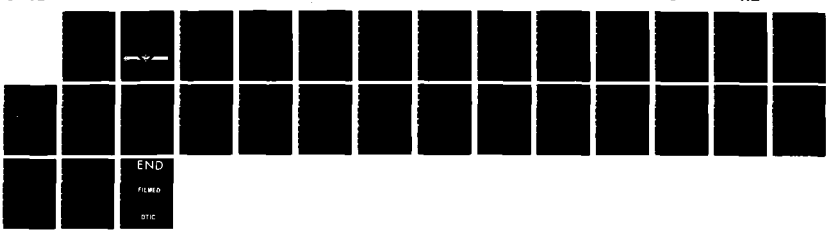

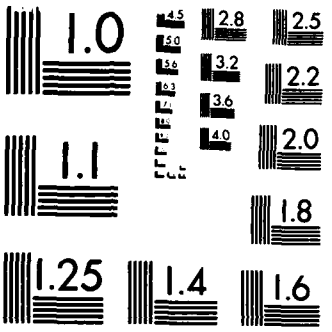

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REPORT NO T6/84

PLATELET-ENDOTHELIAL FUNCTION IN RELATION TO ENVIRONMENTAL TEMPERATURE

U S ARMY RESEARCH INSTITUTE OF ENVIRONMENTAL MEDICINE

Natick, Massachusetts

March 1984

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Thromboembolic phenomena are commonly observed in frostbite injury to the microvasculature. Large blood vessel endothelium was used to assess any alterations in platelet-endothelial interaction resulting from low environmental temperatures. Media from bovine endothelial cells (37°, 24°, 4°C) did not cause or enhance platelet aggregation. Yet ADP (120 μM) stimulated aggregation of all platelet samples. Bovine and porcine endothelial cells were incubated at 37°, 24°, 4°, and 0.5°C with homologous platelets suspended in Tyrodes

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buffer plus albumin, and the rate of ADP-stimulated platelet aggregation was compared with control platelets. Bovine venous endothelial cells (at 37°C) inhibited platelet aggregation more strongly than aortic endothelial cells. This inhibition was blocked by aspirin (7mM) and was independent of environmental temperature. Porcine endothelial cells also inhibited platelet aggregation. This inhibition (maximal at 37°C) was significantly attenuated by temperatures ≤ 24°C. A significant loss of inhibition of platelet aggregation could lower the threshold at which parenchymally-derived nucleotides or other substances from damaged cells can cause aggregation. Thus, in porcine endothelial cells > bovine endothelial cells, a possible mechanism for the thromboembolic action of cold is suggested. Keywords: Blood vessels; frostbite.

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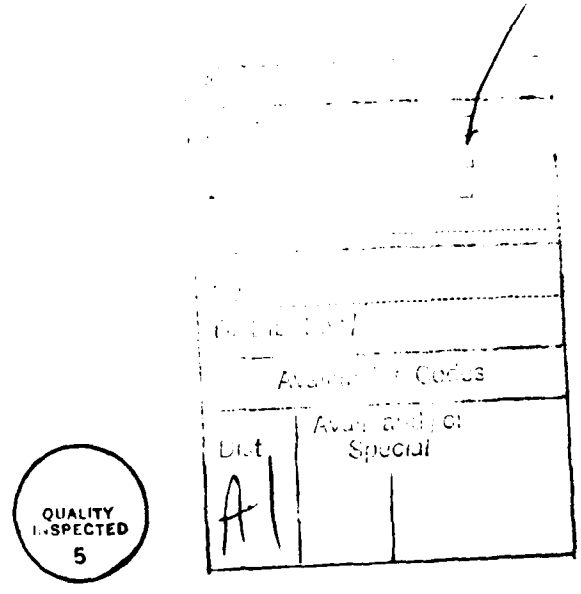

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by

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and Murray P. Hamlet

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TABLE OF CONTENT

LIST OF FIGURES

ABSTRACT

Thromboembolic phenomena are commonly observed in frostbite injury to the microvasculature. Large blood vessel endothelium was used to assess any alterations in platelet-endothelial interaction resulting from low environmental temperature. Media from bovine endothelial cells (37^{o}, 24^{o}, 4^{o}C) did not cause or enhance platelet aggregation. Yet ADP (120 μM) stimulated aggregation of all platelet samples. Bovine and porcine endothelial cells were incubated at 37^{o}, 24^{o}, 4^{o}, and 0.5^{o}C with homologous platelets suspended in Tyrodes buffer plus albumin, and the rate of ADP-stimulated platelet aggregation was compared with control platelets. Bovine venous endothelial cells (at 37^{o}C) inhibited platelet aggregation more strongly than aortic endothelial cells. This inhibition was blocked by aspirin (7 mM) and was independent of environmental temperature. Porcine endothelial cells also inhibited platelet aggregation. This inhibition (maximal at 37^{o}C) was significantly attenuated by temperatures $< 24^{o}$C. A significant loss of inhibition of platelet aggregation could lower the threshold at which parenchymally-derived nucleotides or other substances from damaged cells can cause aggregation. Thus, in porcine endothelial cells > bovine endothelial cells, a possible mechanism for the thromboembolic action of cold is suggested.

INTRODUCTION

For a variety of reasons, a significant proportion of the population suffers from cold and frostbite injury every year. The debilitation caused by these injuries is due in large measure not only to tissue injury caused by freezing, but also by the stasis of tissue blood flow (13,18). Frostbite, in particular, is characterized by a substantial loss in nutrient blood flow. When the tissue is rewarmed, although blood flow to the affected area may resume, this flow is due primarily to the shunting of blood through the larger, non-nutrient microvessels (9). Thus, it is probable that much of the observed tissue injury could be ameliorated, upon thawing, if adequate nutrient blood flow could be re-established.

Histologic examination of frostbitten tissue often reveals aggregates of either red blood cells or platelets lodged in the capillary network of the affected tissue (9). It is unclear whether these aggregates form as the result of temperature per se; whether they result from massive tissue damage; or from some alteration of platelet-endothelial interaction.

Normally, the endothelial cell production of prostacyclin (PGI_2) is sufficient to inhibit platelet aggregation (19). The fact that one observes platelet aggregation in microvessels which are primarily endothelium, raises questions about the efficiency or integrity of the platelet-endothelial interaction (which includes prostacyclin production) at low temperatures. Therefore, our research proposed that cold exposure may cause some alteration in the normal interaction between platelets and endothelial cells. As a result, we elected to use clinical tests for platelet aggregation as a means of evaluating the normalcy of platelet solutions, and the incubation of platelet solutions with cultured endothelial cell monolayers as a means of investigating platelet-endothelial interaction at low temperatures.

MATERIALS AND METHODS

Cultured Cells. Endothelial cells were removed from freshly dissected aortas of both calves and pigs by a collagenase (1 mg/ml) digestion, as previously described by others including Gimbrone et al. (5), Jaffe et al. (6) and Joyner and Strand (7). In addition, endothelial cells were also cultured from bovine portal vein. Primary cultures were maintained in Medium 199 plus 10% fetal calf serum (both obtained from Grand Island Biological, Grand Island, NY) at 37°C and in an atmosphere of 5% CO_2, 95% air. These cells, which were used at days 7-10 in culture, exhibited normal endothelial morphology by light, scanning and transmission electron microscopy. In addition, they stained postively for Factor VIII antigen.

Platelet Solutions. Blood was obtained either from calves or from pigs by bleeding directly into acid citrate dextrose. This mixture was centrifuged at 1,250 rpm for 15 minutes to remove red blood cells and leukocytes, and the resulting platelet-rich plasma was re-centrifuged at 6,500 rpm to obtain a platelet pellet. Platelets were re-suspended in Tyrodes' buffer which contained 0.3% bovine serum albumin (T-BSA), and kept at room temperature (24°C) until used (2-5 hours). In some instances, platelets were isolated and concentrated not by centrifugation, but by gel filtration. In these studies, platelet-rich plasma was applied to a column (53 mm diameter x 170 mm in height) of Sepharose 2B (Pharmacia Fine Chemicals, Piscataway, NJ) previously equilibrated with NaCl and then with T-BSA. Elution of the platelet fraction was performed with Tyrodes' buffer (pH 7.4).

Platelet Aggregation. Aggregation was performed in a Payton Aggregometer (Payton Assoc., Inc., Buffalo, N.Y.), 37°C and 900 rpm. The one minute slope (decrease in absorbancy of the aggregating platelets; mm on the chart moving at 1 inch/min.) for aggregation was expressed as a percentage of

the appropriate control solution of platelets. Except for construction of dose-response curves, all aggregation studies were performed with ADP (Sigma Chemical Co., St. Louis, MO) solutions which were 120 μM (final concentration).

RESULTS

Characterization of the Platelet Populations. The aggregation of both porcine and bovine platelets in response to stimulation by a 120 μM dose of ADP, occurred in a manner similar to the aggregation of other platelets in other systems. The platelets were stable (in an unaggregated state) until the application of ADP. Then, aggregation (measured as the increase in light transmittance through a stirred cuvette) proceeded rapidly (Fig. 1). The comparison of aggregation curves was made on the basis of the rate of aggregation in the first minute following introduction of the agonist (= minute slope).

By these techniques, a difference in aggregation was observed between unfiltered and gel-filtered platelets (Fig. 2). Both the slopes and the intercepts differed significantly from each other ($P < 0.05$) with the unfiltered platelets being more sensitive to the agonist (ADP). In addition, it was noted that porcine platelets at 24°C were more sensitive to ADP than were bovine platelets (Fig. 3), but this difference in sensitivity was less apparent at either 37°C or at 4°C. Finally, incubation temperature (> 0°C) per se had no significant effect on the rate of aggregation of either porcine or bovine platelets (Fig. 3).

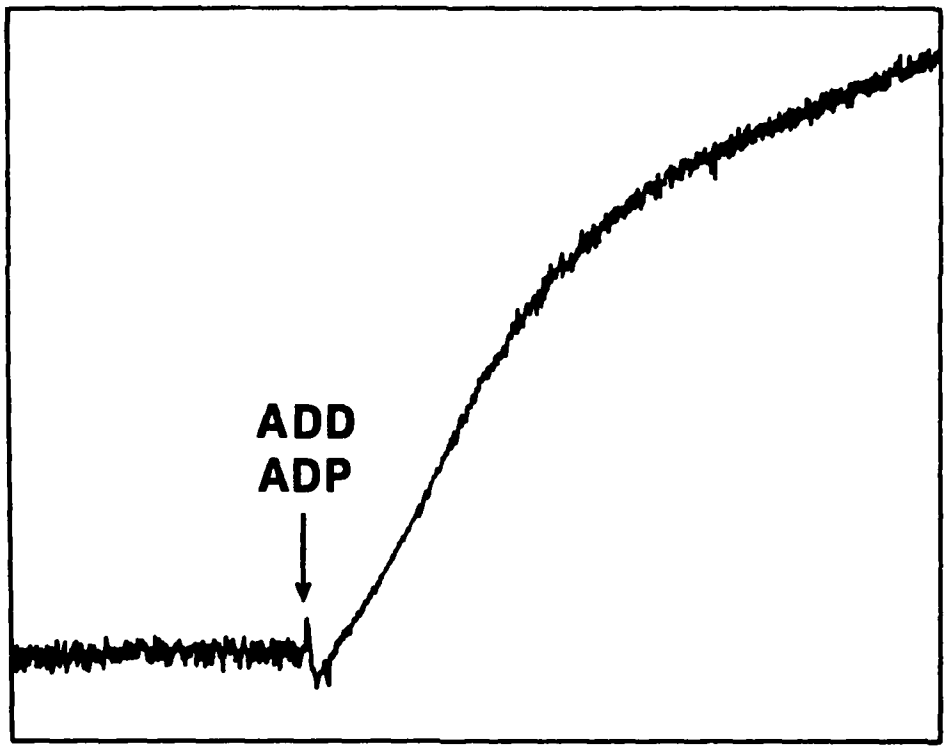

Figure 1. A platelet aggregation curve typical of either bovine or porcine platelets. A solution of platelets added to the aggregometer shows a steady baseline indicative of a non-spontaneously aggregating platelet population. Addition of ADP (arrow) initiates the aggregation response. The rate of aggregation was measured as the height in mm of the aggregation curve, one minute after the addition of ADP.

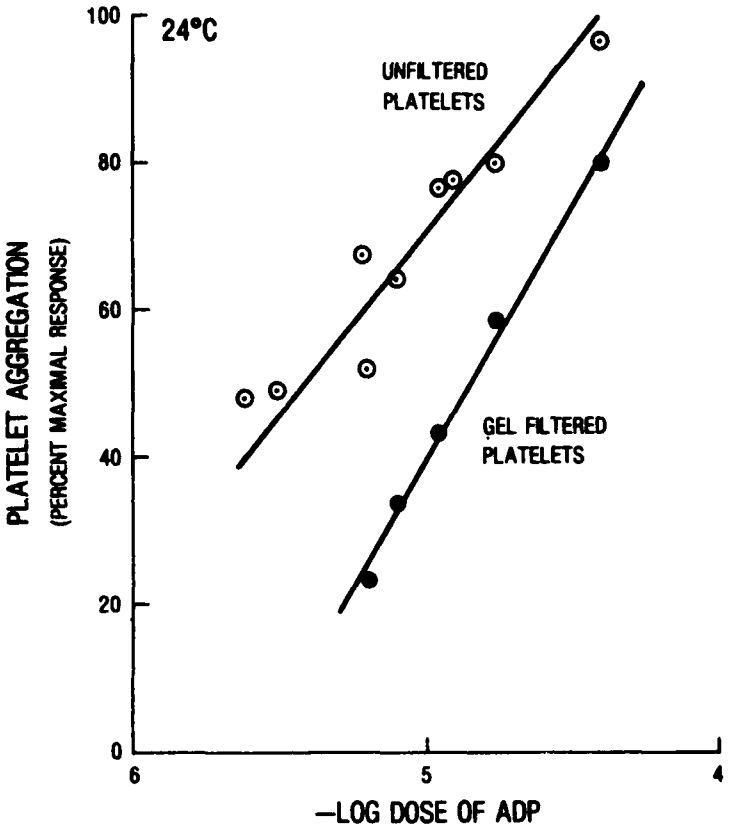

Figure 2. The dose-response relationship between gel-filtered (GFP) and unfiltered (UNF) porcine platelets is represented. Y(GFP) = 70.090 (X) + 389.730, r = 0.996; Y(UNF) = 49.970 (X) + 320.730, r = 0.972. ED_{50} values predicted from regression analyses are: GFP, 1.65×10^{-5} M; UNF, 5.85×10^{-6} M.

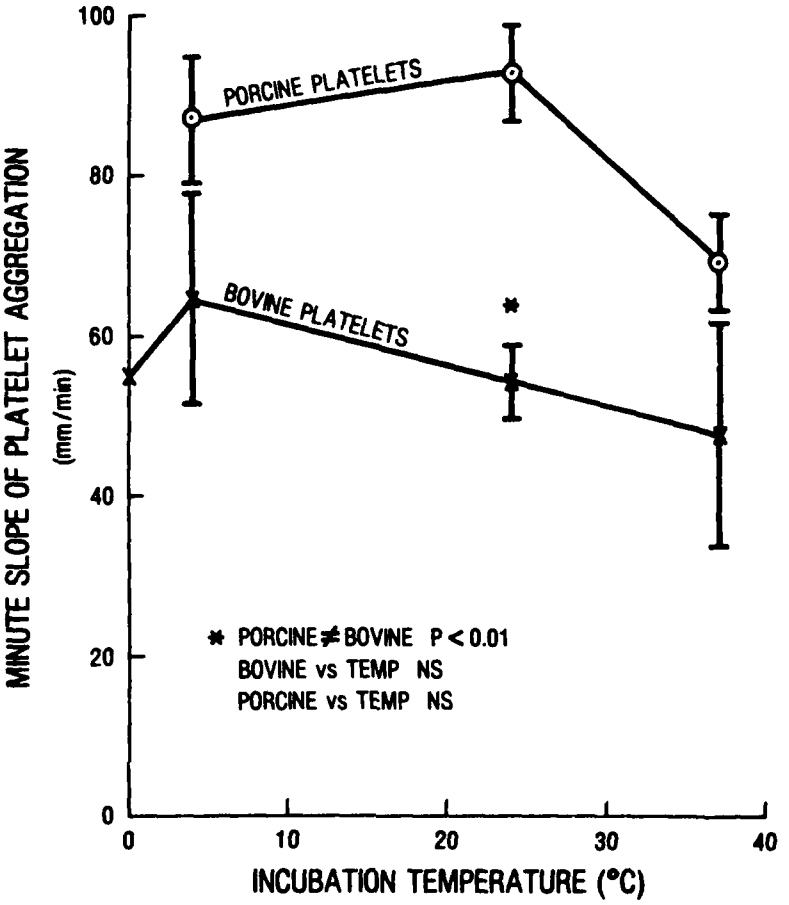

Figure 3. The relationship between incubation temperature and platelet aggregation in response to a standard dose (120 μM) of ADP. The height in mm corresponds to the light transmitted through a platelet sample. Increases in height correspond to increases in aggregation. * indicates statistical significance, $P < 0.05$.

Platelet-Endothelial Interaction. The effect of environmental temperature on any interaction between platelets and endothelial cells was assessed in two ways. First, incubation media from cultured endothelial cells (at 37°, 24°, or 4°C for 15 minutes) was applied as a potential agonist to platelet solutions in the aggregometer. This mixture was monitored for at least two minutes to determine if any aggregatory response would result. At the end of this observation period, all platelet-media mixtures were stimulated with ADP to determine the capability of the platelets to aggregate. The results of a typical experiment (Fig. 4) illustrate the overall observation that: a) the endothelial cell incubation media contained no agonist sufficient to cause aggregation; b) the aggregability of the platelets was unaffected by media from the endothelial cell. Thus, there was no difference in the minute slope of the ADP-induced aggregation following incubation with media derived from endothelial cells at any temperature (Temp $< 0.5^{\circ}C$).

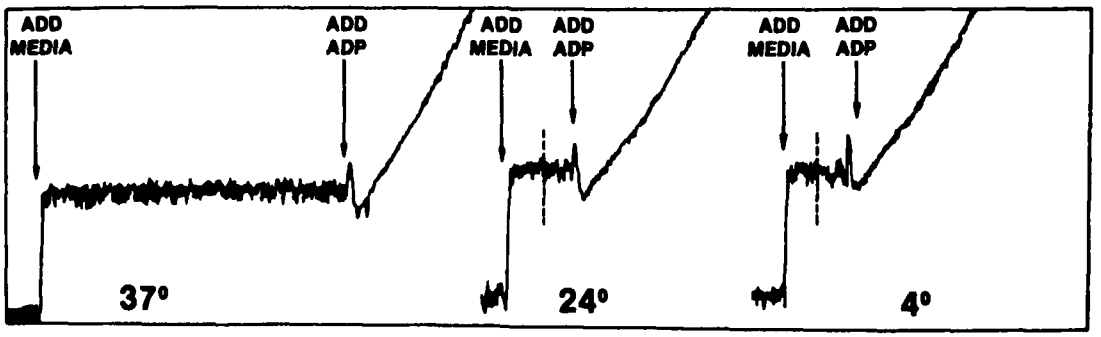

Figure 4. A composite of typical aggregation patterns before media is added as an agonist (first arrow) and after ADP is added as an agonist (second arrow). These profiles are samples from each environmental temperature (within a single experiment). Only 1 to 1.5 minutes of aggregation following ADP are presented, as this is the portion of the aggregation curve considered throughout the study.

A second set of experiments to determine any alterations in platelet-endothelial interaction were performed by incubating platelet-containing solutions on endothelial cell monolayers. A comparison of the inhibitory effects of endothelial cells on platelet aggregation can be seen in Figure 5. Recalling from Figure 3 that porcine platelets are more sensitive to ADP than bovine platelets, these results are significant in that bovine endothelial cells inhibit platelet aggregation to a much greater extent than porcine endothelial cells. Figure 5 also compares the effect of non-endothelial vascular cells (smooth muscle from the azygous vein), and a tumor-producing cell line whose use is planned in future experiments (to stimulate growth of capillary endothelium). Remarkably, neither of these cell types had any effect on platelet aggregation. Therefore, the inhibitory effect on platelet aggregation may reside primarily with endothelial cells.

Cultured Cells versus Tissues. To verify that the phenomena observed for cultured endothelial cells had basis, in fact, at the tissue level, we chose one cell system, bovine portal vein, to investigate more closely. Utilizing cultured endothelial monolayers or pouches constructed from portal vein (by ligating one end), we could demonstrate that endothelium could inhibit platelet aggregation (Fig. 6) and the vein exerted a stronger inhibition than did the cells in culture. In addition, this inhibition was almost completely reversed, if endothelial monolayers were pre-incubated for 30 minutes with acetylsalicylic acid (7 mM). These results, similar to those of others (19), indicate that the endothelial cell-mediated inhibition of platelet aggregation is a function of prostaglandin synthesis. In fact, Weksler et al. (19) and others (15) have shown that the specific prostaglandin appears to be prostacyclin (PGI_2) whose production appears to be restricted to the endothelial cell.

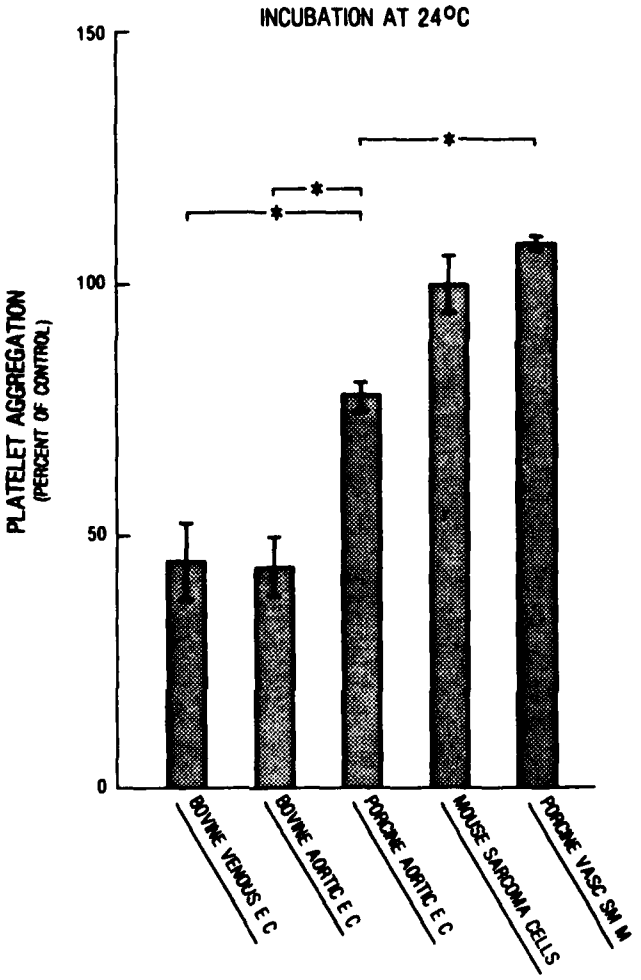

Figure 5. Comparisons of platelet aggregation, relative to aggregation of control populations of platelets, to demonstrate the inhibition of platelet aggregation by endothelial (but not by non-endothelial) cells. * indicates statistical significance, $P < 0.05$.

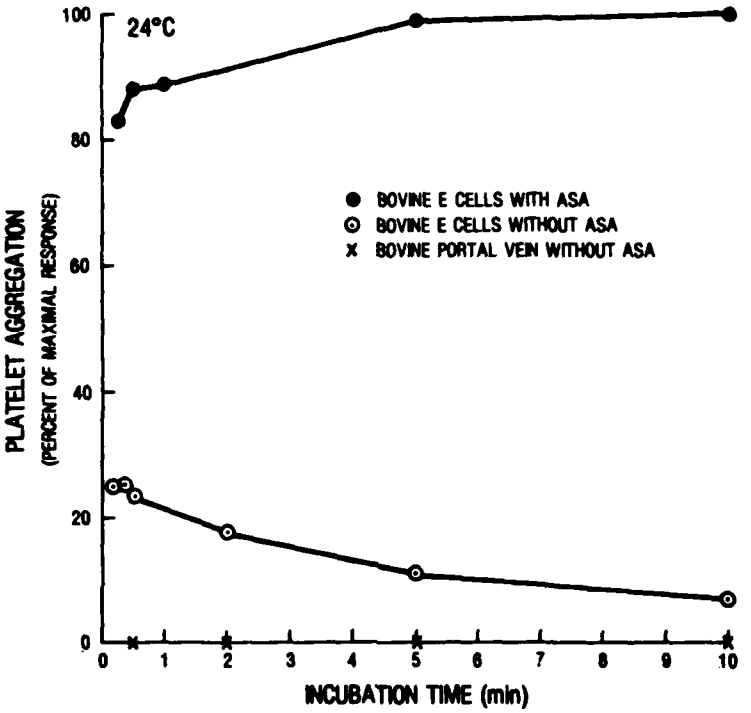

Figure 6. Comparisons of platelet aggregation, relative to control platelet populations, to demonstrate: 1) the effect of aspirin (= ASA, 7 mM) in blocking the endothelial cell-induced inhibition of aggregation; 2) the difference in inhibition of aggregation between cultured endothelial cells and pouches of portal vein, complete with an endothelial lining.

Effects of Environmental Temperature on Platelet-Endothelial Interaction.

We used the anti-aggregatory effect of cultured endothelial monolayers to investigate the effect of environmental temperature on platelet-endothelial interaction. Bovine platelets were incubated either with arterial or venous endothelial cells (Fig. 7), and results indicate that the inhibition of aggregation occurs remarkably quickly, especially at 37°C. In addition, these results (at 37°C) indicate that bovine venous endothelial cells inhibit platelet aggregation more strongly than do arterial endothelial cells. These results show a trend towards loss of anti-aggregatory effect with decreasing environmental temperature, but no predictable pattern can be extracted. In contrast, the porcine platelet-endothelial interaction appears significantly affected by environmental temperatures $\leq$ 24°C (Fig. 7). Moreover, while porcine endothelial cells at 37°C strongly inhibit platelets from aggregating, they require 20 minutes of incubation to accomplish this inhibition. However, with incubation times greater than 3 minutes, there is considerable disparity between cells incubated at 37°C and those incubated either at 24°C, or at 4°C.

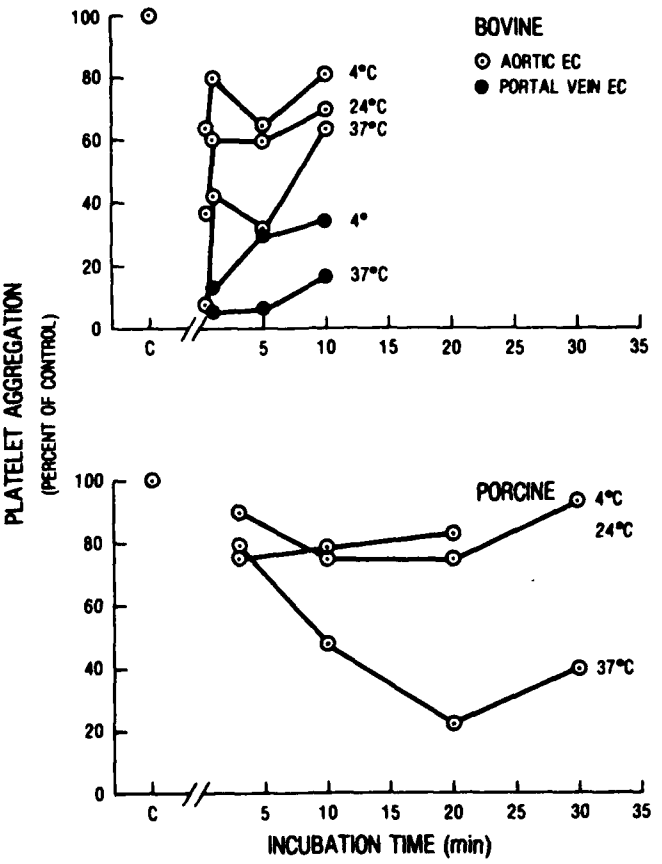

Figure 7. Comparisons of platelet aggregation, relative to control platelet populations, to demonstrate: upper panel - the differences in aggregation between bovine platelets, incubated on arterial or venous endothelial cells (EC), as a function of environmental temperature; lower panel - the differences between porcine platelets incubated on aortic endothelial cells at various temperatures. C = control value = 100%.

DISCUSSION

The specific interaction of platelets with damaged endothelial cells is not well known. However, mural thrombi, emboli and aggregates of platelets in the microvasculature are commonly described features in the pathology of frostbite (9,17). The platelet aggregation seen in these areas of blood flow stasis can be caused by a number of physiological factors (e.g., adenosine diphosphate = ADP, epinephrine, collagen, thrombin, etc.; 14), but whether these or other factors or their effects are perturbed in frostbite is unknown. There is evidence to show that some of the clotting factors are altered in the systemic circulation in response to frostbite (4), but since the damage due to frostbite is a local phenomenon, there is no information concerning these factors in the local vascular bed. In this investigation, primary attention was focused on the effects of non-freezing cold on the interaction between platelets (bovine or porcine) and large blood vessel endothelium (bovine aortic and portal venous; porcine aortic). Specific attention was focused on alterations in ADP-induced platelet aggregation, when platelets were exposed either to endothelial cell media from various temperatures, or to the cells themselves at various temperatures.

These studies demonstrate again (16) that a significant dose-response relationship exists between the adenine nucleotide ADP and the platelet, and that the platelet bathed in its own serum (unfiltered) is more sensitive to ADP than the platelet which may be stripped of its homologous serum microenvironment (gel-filtered platelet). In fact, the differences in both slope and intercept for the linearized dose-response relationship lead one to suspect that the relationship between serum and the platelet is complex.

The fact that prolonged (15-20 minute) exposures of isolated platelets to non-freezing cold (to $0^{o}C$) have no effect on platelet aggregation, leads one to suspect something other than cold per se to be involved in the formation of the microemboli, observed with frostbite damage. In addition, it appears clear that cooled endothelium does not release anything which either initiates platelet aggregation or enhances nucleotide stimulation of aggregation. Thus, non-freezing cold does not appear to precipitate events within the vasculature which lead to the thromboembolic phenomena considered to be responsible for the cessation of nutrient blood flow in frostbite.

Although the inhibition of platelet aggregation by endothelial cells is now widely known (19), the interaction between platelet and endothelial cell in cold/freezing environments has not been tested. This study describes an abberation in that interaction (in the porcine system) which develops with reduced environmental temperature. However, this study is unable to address the question of whether this abberation resides in PGI_2 production, platelet-PGI_2 receptor function, or both. Nonetheless, the data show that the normal anti-aggregatory ability of porcine endothelial cells is significantly attenuated with temperatures $\leq 24^{o}C$.

The loss of anti-aggregatory "protection" of the porcine platelets by the endothelial cells at temperatures lower than $24^{o}C$ may have significance if these cells resemble the human platelet-endothelial cell system. If this analogy exists, it would predict that as the vasculature is cooled (not frozen), platelets would become more and more susceptible to aggregation in response to physiologic (ADP) stimulation. Further, it is known that damaged cells in general release a variety of substances including nucleotides such as ADP. Thus, nucleotides or

other substances, derived from damaged parenchyma may be the stimuli which initiate the in vivo aggregatory phenomena leading to microvascular occlusion.

The pig has several physiologic characteristics which make it an ideal model for human physiologic studies (3). The pig has been used as an appropriate model for the human cardiovascular system, owing to similarities in vascular anatomy (1,2,12), stress reaction (8,10,11) and the like. Thus, it seems likely that other porcine vascular phenomena may resemble those observed in man, although this is yet to be shown. Therefore, if the porcine platelet-endothelial cell system represents the human predicament, the conditions exist to explain some of the embolic phenomena observed in the frostbitten microvasculature.

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