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Complement Depletion and Persistent Hemodynamic-Hematologic Responses in Protamine-Heparin Reactions

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Hypotension, bradycardia, pulmonary artery hypertension, neutropenia, and thrombocytopenia have been suspected to be due to complement activation following protamine reversal of heparin. This investigation examined these phenomena in complement-depleted animals. Eight dogs received intraperitoneal *naja n. naja* cobra venom factor (CVF), 20 U/kg, 48 and 24 hr prior to anticoagulation with sodium heparin, 150 IU/kg, and reversal 30 min later with protamine sulfate, 1.5 mg/kg. Decomplementation was confirmed in all dogs. Systemic blood pressure (BP), pulse (HR), pulmonary artery systolic and diastolic pressures, (PAS, PAD), cardiac output (CO), platelet count (PTC), and white blood count (WBC) with differential were monitored. The maximal mean changes for the entire group were BP, −43 mm Hg; HR, −16; PAS, +6 mm Hg; PAD, +3 mm Hg; CO, −27%; PTC, −49%; and WBC, −48%. These hemodynamic and hematologic responses, occurring in the face of CVF-induced decomplementation, support the conclusion that complement components C3 and C5–C9 are not influential factors contributing to these protamine-heparin-induced events.

Protamine sulfate is a useful agent for the reversal of the anticoagulant effect of sodium heparin. However, in both animals and patients, this drug may cause a number of undesirable hemodynamic and hematologic adverse responses, including hypotension, bradycardia, pulmonary artery hypertension, thrombocytopenia, and neutropenia [1–4]. Complement activation has been suspected to be the cause of these responses, although a direct relationship between hematologic changes and complement activation after protamine administration has not been observed in prior experiments undertaken by the authors [2, 3]. The purpose of this investigation was to produce a state of complement deficiency in animals utilizing cobra venom factor (CVF) and to quantitate subsequent responses to protamine reversal of heparin anticoagulation.

MATERIALS AND METHODS

Eight adult mongrel dogs (mean weight, 13 kg) were treated with cobra venom factor from the *naja n. naja* cobra 48 and 24 hr prior to the experimental study. The venom was given intraperitoneally for a total dose of 40 U/kg (20 U/kg with each administration). CVF was isolated from crude lyophilized cobra venom by ion-exchange chromatography and gel filtration [5].

The dogs were anesthetized with sodium pentobarbital (30 mg/kg intravenous) and mechanically ventilated. Anesthesia was maintained with supplemental sodium pentobarbital. Hydration was assured by administration of lactated Ringers solution, 20 cc/kg/hr. All animals had an arterial catheter placed in the right femoral artery, a Swan-Ganz catheter placed in the pulmonary artery by way of the right femoral vein, and a venous catheter inserted in the left femoral vein. Hemodynamic measurements included mean systemic blood pressure (BP), pulse (HR), pulmonary artery systolic pressure (PAS), pulmonary artery diastolic pressure (PAD), and cardiac output (CO) using thermodilution technique. Hematologic

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measurements included platelet count (PTC), complete blood count (CBC) with differential count, total hemolytic complement (CH_{50}) values, and crossed immunoelectrophoresis for monitoring of complement activation.

All animals were heparinized with sodium heparin (150 IU/kg, intravenous) and 30 min later the heparin was reversed with protamine sulfate (1.5 mg/kg, infused intravenously over a 10-sec period). These are standard doses administered clinically at our medical center. The rapid protamine infusion was chosen to magnify hemodynamic responses. Hemodynamic measurements including BP, HR, PAS, and PAD were monitored continuously. CO, PTC, and CBC were monitored at baseline, 3 min after heparin administration, 3 min prior to protamine reversal, and at 30 sec, 1, 3, 5, 10, and 30 min following completion of the protamine reversal.

Thrombin clotting times revealed full anticoagulation in all animals after heparin administration and complete reversal with administration of protamine sulfate. Hematologic assessments were also made before and after each administration of CVF.

Complement depletion was documented by assaying plasma samples for total hemolytic complement activity (CH_{50} units), employing a standard hemolysis test [6]. Plasma C3 levels were determined by crossed immunoelectrophoresis procedures using a specific antibody against canine C3. Since the height of the C3 peak in the gel slab relates directly to the amounts of C3 present in plasma, percentage changes in C3 concentrations were easily calculated. Data analyses included calculation of sample means with standard deviations, and comparisons using a paired *t* test. Animal care complied with the *Principles of Laboratory Animal Care* and the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 80-23, revised 1976).

RESULTS

Protamine reversal of heparin produced a maximal fall in BP of 43 ± 24 mm Hg at 150 sec with a persistent decline of 9 ± 16 mm Hg at 30 min (Fig. 1). Statistically significant differences from baseline BP were noted from 75 to 180 sec after reversal. Interestingly, two dogs showed only minimal hypotension with BP decreases of 10 and 12 mm Hg, respectively. The HR dropped 15 ± 18 at 135 sec and 16 ± 14 at 5 min (Fig. 2). Significant differences from baseline HR were found at 135 sec, as well as at 5 and 10 min.

Elevations of both systolic and diastolic pulmonary artery pressure occurred. The maximal PAS rise was 6 ± 9 mm Hg at 60 sec after protamine administration, and the maximal PAD rise was 3 ± 6 mm Hg at this same time interval. These values were not statistically different from baseline pressures (Fig. 3). Finally, CO declined 27% from initial baseline values at 180 sec after protamine administration, although there was an initial rise at 30 sec (Fig. 4).

Hematologic changes were more marked. PTC fell 49% from initial counts at 60 sec after protamine administration and remained 30% below baseline at 30 min (Fig. 5). Significant PTC differences occurred at all time periods. Similarly, white blood cell counts fell 48% from baseline at 60 sec following protamine administration (Fig. 6). Full recovery of the latter was noted by 5

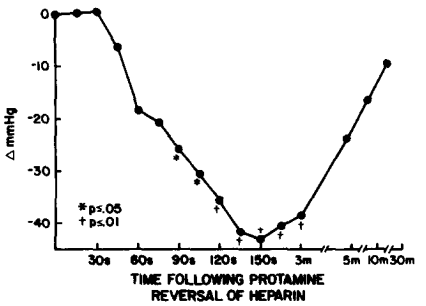

FIG. 1. Mean systemic arterial blood pressure change after protamine reversal of heparin.

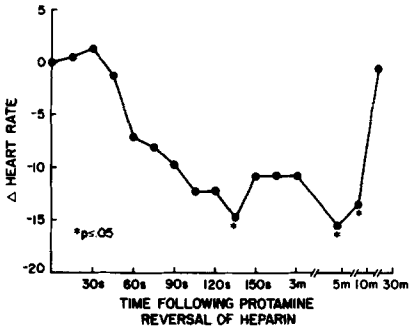

FIG. 2. Pulse change after protamine reversal of heparin.

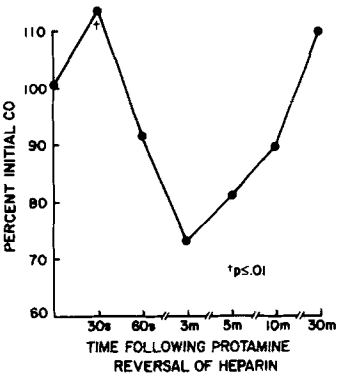

FIG. 4. Cardiac output change after protamine reversal of heparin.

min. There was a slight decline in the percentage of granulocytes, from 47 to 38% at 30 sec after protamine reversal. The percentage of lymphocytes rose initially from 36 to 47% at 30 sec, and declined to 30% at 5, 10, and 30 min after the protamine reversal. Eosinophils accounted for 5% of the white cells initially, a percentage that increased to 12% at 60 sec following protamine reversal. Monocyte, basophil, and stab analysis did not reveal any significant changes, probably reflecting their initial low levels of 4, 2, and 1%, respectively.

Baseline PTC before the first CVF administration was 284,000 ± 98,000. PTC just prior to protamine reversal 48 hr later was 251,000 ± 81,000, representing a slight decrease of 12%. The baseline white blood cell count before the first CVF administration was 24,000 ± 19,000 and 48 hr later it had fallen to 5000 ± 3900, a decrease of 79%. Interestingly, the white blood cell count 24 hr after the first CVF administration had increased 31% to 31,400 ± 8900.

Total hemolytic complement activity in plasma was 95.3 units before CVF treatment, but only 10 units after the second CVF administration. At the same time, there was an 83 ± 3% reduction in plasma C3 levels compared to pretreatment values.

DISCUSSION

Complement activation has been suggested as a cause of the adverse hemodynamic and hematologic effects of protamine sulfate [7–13]. It has been known for more than a decade that protamine, when added to

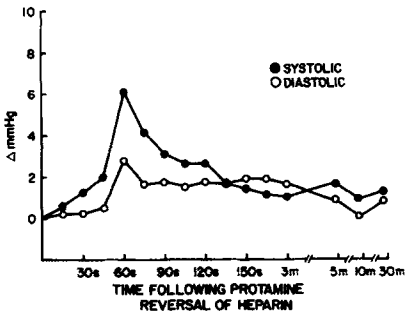

FIG. 3. Pulmonary artery blood pressure change (systolic, diastolic) after protamine reversal of heparin.

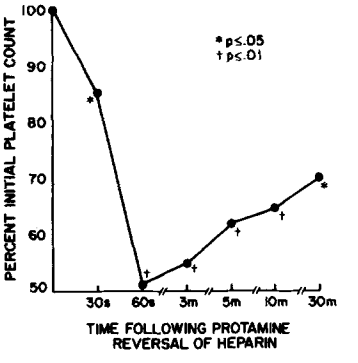

FIG. 5. Platelet count change after protamine reversal of heparin.

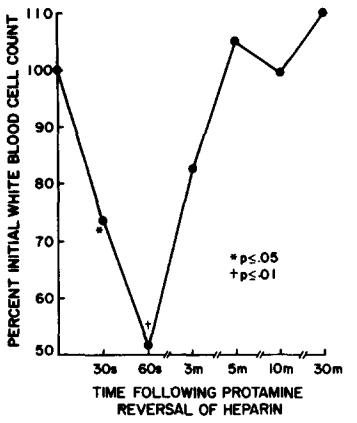

FIG. 6. White blood cell count change after protamine reversal of heparin.

acute phase serum, consumed the complement cascade components C1, C4, and C2, with little consumption of C3–C9, in a reaction that was dependent on time, temperature, pH, and salt concentration, including the presence of calcium ions [9]. This reaction occurred even in the absence of heparin, as well as in normal sera with high doses of protamine. C-reactive protein appears to be the essential component in acute phase serum, which acts with protamine to facilitate this response that has been postulated to begin at the level of C1q.

Combinations of heparin and protamine, at levels below those causing complement activation if either substance is given separately, deplete total hemolytic complement *in vitro* [10]. Heparin was found to be approximately 20 times more anticomplementary in the presence of protamine sulfate, and protamine was 5–10 times more anticomplementary in the presence of sodium heparin [10]. Heparin, alone, minimally lowered C1 and C3–C9 levels, but augmented C4 and C2 activity, an effect thought to be due to heparin's action on antithrombin III. Protamine, alone had little effect on complement activation in the aforementioned study. However, heparin plus protamine depleted total hemolytic complement in the fashion of an antibody–antigen complex with classical pathway activation beginning at the level of C1.

Earlier *in vitro* studies were followed by *in vivo* experiments using rabbits in which generation of C5a was implied by existance of a transient granulocytopenia produced by heparin–protamine interaction [11]. This response was also noted to occur following protamine administration alone in animals having elevated C-reactive protein levels, albeit not to a statistically significant degree. Depletion of complement factors with CVF completely abolished the granulocytopenic response. This indirectly supported the contention that complement, up to and including C5, could be activated by protamine.

Humans undergoing cardiopulmonary bypass exhibited elevated C3a and C4a levels after protamine reversal of heparin, implicating classical pathway activation [12]. These elevations were distinct from those occurring in the cardiopulmonary bypass circuit itself. It was postulated that complement activation, pulmonary sequestration of leukocytes, lysosomal enzyme release, superoxide generation, histamine release from mast cells, and finally increased capillary permeability occurred in response to protamine reversal of heparin. However, it should be noted that the 100 patients in this study did not exhibit reversal hypotension and thus correlations of hemodynamic data to complement values were not possible. In addition, the measurements in the aforementioned study were obtained 10 min after protamine reversal, a time beyond that when the adverse responses have been documented to occur in peripheral vascular surgery patients with protamine reversal of heparin [2]. Similar findings have been noted by Kirklin and his colleagues [13].

In contrast to these two former studies, that both suggest complement activation associated with heparin–protamine interactions, is a third report on humans undergoing cardiopulmonary bypass [14]. In this study no consumption of complement, particularly C3 and C4, could be documented

following protamine reversal of heparin anticoagulation. In this regard, we were unable to document activation of total hemolytic complement with protamine reversal of heparin activity in an earlier canine study [3], and in a patient study no correlation existed between hemodynamic parameters and C3a generation [2].

In the present investigation, we chose to study dogs depleted of complement factors by treatment with CVF in an attempt to resolve the controversy surrounding this topic. CVF is a 144,000 Da protein that works with a cofactor (factor B of the alternative complement pathway) to cause complement activation [15, 16]. CVF interacts with factor B in a reversible, Mg^{2+}-dependent complex, in order to be cleaved by another cofactor of the alternative pathway (factor D). From this interaction, factor B is cleaved to a smaller fragment (Ba) which is released and a larger fragment (Bb) which remains bound to the CVF. This binding is important for C3 cleavage and perpetuation of the alternative pathway. Thus, by this means CVF leads to massive activation of the alternative complement cascade and decomplementation for both the classical and alternative pathways from C3 to C9. The low complement levels achieved by CVF may remain reduced for as long as 5 days [15, 17]. Decomplementation was complete in all animals of the current investigation.

We have previously studied the hemodynamic and hematologic responses in dogs to protamine reversal of heparin using standard doses as used in the current study [1, 3, 18]. In complement-intact animals, the blood pressure typically dropped 40 mm Hg at 75 and 120 sec after reversal along with profound bradycardia of −19 at 90 to 105 sec postreversal. Pulmonary artery hypertension occurred some 90 sec after reversal up to +9 mm Hg PAS, and cardiac output diminished by 30% at 60 sec postreversal. Platelet count typically fell 75%, while white blood cell count dropped 60% at 60 sec after reversal. Finally, only a 7% activation of CH_{50} was noted in animals 30 sec and 5 min after reversal.

Certain differences were apparent when comparing the results of this study to our earlier work. First, the typical two-phase hypotensive response as noted previously did not occur [3]. Second, pulmonary artery pressure elevations occurred earlier than would have been expected and were slightly less than those reported previously [3]. Third, although neutropenia did develop, the expected granulocytopenia was not observed [3]. Finally, the magnitude of the thrombocytopenia was slightly less than predicted. [3]. A few comments regarding our understanding of the events surrounding the responses usually accompanying heparin–protamine reactions deserve comment.

As mentioned, protamine-induced hypotension is typically a two-phase response after a 10-sec rapid bolus of protamine, phase one being maximal at 75 sec and phase two at 120 sec. Bradycardia and pulmonary artery hypertension are most marked 90 and 105 sec after reversal. Each phase may be mediated by different mechanisms. The first phase seems most likely to be due to a direct toxic effect of protamine on vessels or their cell membranes. This effect leads to vasodilation with a corresponding decrease in total peripheral resistance. Although thrombocytopenia does develop during this first phase, thrombocytopenic dogs still demonstrate early hypotension [19]. On the other hand, platelet aggregation and release of vasoactive substances most probably are responsible for phase two reactions, causing pulmonary artery hypertension, bradycardia, and further hypotension [3, 20]. Thrombocytopenic dogs do not develop bradycardia and pulmonary artery hypertension [19]. The second phase may be thrombin dependent in that platelets subjected to protamine are inhibited in their aggregation to thrombin but not ADP [19, 21, 22]. In addition, we have demonstrated that pulmonary accumulation of platelets can be completely blocked by protamine pretreatment in the dog [18].

Complement activation in animals and humans following protamine administration appears to be a secondary event rather than a cause of major hemodynamic and hematologic changes. That adverse hemodynamic and hematologic responses to protamine occur in dogs with documented decomplementation supports this contention. Although C1, C4, or C2 components of the cascade could contribute to the adverse responses, inasmuch as these components are not inhibited by the CVF, it is unlikely that one of them is responsible for all the observed reactions without further generation of activated C3, C5, and the rest of the cascade. However, C2 activation is known to generate a kinin-like moiety, and C4a anaphylatoxin exists, both of which can cause vasodilation and increased capillary permeability [23]. The influence of these specific component factors in heparin–protamine reactions must still be investigated. Nevertheless, this study documents that inhibition of complement was not protective against the adverse reactions noted with protamine reversal of heparin activity although the magnitude of some of the responses was slightly less than previously reported from our laboratory in studies of this canine model [3].

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REFERENCES

1. Wakefield, T. W., Whitehouse, W. M., Jr., and Stanley, J. C. Depressed cardiovascular function and altered platelet kinetics following protamine sulfate reversal of heparin activity. *J. Vasc. Surg.* **1:** 346, 1984.
2. Wakefield, T. W., Hantler, C. B., Lindblad, B., Whitehouse, W. M., Jr., and Stanley, J. C. Protamine pretreatment attenuation of hemodynamic and hematologic effects of heparin–protamine interaction: A prospective randomized study in human beings undergoing aortic reconstructive surgery. *J. Vasc. Surg.* **3:** 885, 1986.
3. Wakefield, T. W., Lindblad, B., Whitehouse, W. M., Jr., Hantler, C. B., and Stanley, J. C. Attenuation of hemodynamic and hematologic effects of heparin–protamine sulfate interaction after aortic reconstruction in a canine model. *Surgery* **100:** 45, 1986.
4. Horrow, J. C. Protamine: A review of its toxicity. *Anesth. Analg.* **64:** 348, 1985.
5. Ballow, M., and Cochrane, C. G. Two anticomplementary factors in cobra venom: Hemolysis of guinea pig erythrocytes by one of them. *J. Immunol.* **103:** 994, 1969.
6. Mayer, M. M. Complement and complement fixation. In Kabat and Mayer, (Eds.), *Experimental Immunochemistry.* Springfield, IL: Thomas, 1961. Pp. 133–240.
7. Kirkland, J. K. in discussion of Shapira, N., Schaff, H. V., Piehler, J. M., White, R. D., Sill, J. C., and Pluth, J. R. Cardiovascular effects of protamine sulfate in man. *J. Thorac. Cardiovasc. Surg.* **84:** 505, 1982.
8. White, J. V. Complement activation during cardiopulmonary bypass. *N. Engl. J. Med.* **305:** 51, 1981.
9. Siegel, J., Rent, R., and Gewurz, H. Interactions of C-reactive protein with the complement system. 1. Protamine-induced consumption of complement in acute phase sera. *J. Exp. Med.* **140:** 631, 1974.
10. Rent, R., Ertel, N., Eisenstein, R., and Gewurz, H. Complement activation by interaction of polyanions and polycations. I. Heparin-protamine induced consumption of complement. *J. Immunol.* **114:** 120, 1975.
11. Fehr, J., and Rohr, H. *In vivo* complement activation of polyanion–polycation complexes: Evidence that C5a is generated intravascularly during heparin–protamine interaction. *Clin. Immunol. Immunopathol.* **29:** 7, 1983.
12. Cavarocchi, N. C., Schaff, H. V., Orszulak, T. A., Homburger, H. A., Schnell, W. A., and Pluth, J. R. Evidence for complement activation by protamine–heparin interaction after cardiopulmonary bypass. *Surgery* **98:** 525, 1985.
13. Kirklin, J. K., Chenoweth, D. E., Naftel, D. C., Blackstone, E. H., Kirklin, J. W., Bitran, D. D., Curd, J. G., Reves, J. G., and Samuelson, P. N. Effects of protamine administration after cardiopulmonary bypass on complement, blood elements and the hemodynamic state. *Ann. Thorac. Surg.* **41:** 193, 1986.
14. Chiu, R. C. J., and Samson, R. Complement (C3, C4) consumption in cardiopulmonary bypass, cardioplegia, and protamine administration. *Ann. Thorac. Surg.* **37:** 229, 1984.
15. Vogt, W. Factors in cobra venoms affecting the complement system. *Toxicon* **20:** 299, 1982.
16. Smith, C. A., Vogel, C. W., and Muller-Eberhard, H. J. Ultrastructure of cobra venom factor-depen-

dent C3/C5 convertase and its zymogen, factor B of human complement. *J. Biol. Chem.* **257:** 9879, 1982.

17. Zabern, I. V., Przyklenk, H., Damerau, B., and Zimmermann, B. Isolation and properties of complement inhibitor from *Naja haje* venom, distinct from known anticomplementary factors in cobra venom. *Scand. J. Immunol.* **14:** 109, 1981.
18. Wakefield, T. W., Bouffard, J. A., Spaulding, S. A., Petry, N. A., Gross, M. D., Lindblad, B., and Stanley, J. C. Sequestration of platelets in the pulmonary circulation as a consequence of protamine reversal of the anticoagulant effects of heparin. *J. Vasc. Surg.* **1:** 187, 1987.
19. Lindblad, B., Wakefield, T. W., Whitehouse, W. M., Jr., and Stanley, J. C. The effect of protamine sulfate on platelet function. *Scand. J. Thorac. Cardiovasc. Surg.*, in press.
20. Radegran, K., Taylor, G. A., and Olsson, P. Mode of action of protamine in regard to its circulatory and respiratory side effects. *Eur. Surg. Res.* **3:** 139, 1971.
21. Cobel-Geard, R. J., and Hassouna, H. I. Interaction of protamine sulfate with thrombin. *Amer. J. Hematol.* **14:** 227, 1983.
22. Okajima, Y., Kanayama, S., Maeda, Y., Urano, S., Kitani, T., Watada, M., Nakagawa, M., and Ijichi, H. Studies on the neutralizing mechanism on antithrombin activity of heparin by protamine. *Thromb. Res.* **24:** 21, 1981.
23. William, B. D. The complement system. *Brit. J. Anaesth.* **51:** 7, 1979.