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A Less Toxic Heparin Antagonist—Low Molecular Weight Protamine

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Abstract—A new thirteen amino acid peptide, named low molecular weight protamine (LMWP), was obtained through the enzymatic digestion of native protamine. Both *in vitro* and *in vivo* results showed that LMWP fully maintained the heparin neutralization function of protamine but had much lower immunogenicity and antigenicity. Unlike protamine, neither LMWP nor LMWP/heparin complexes caused significant blood platelet aggregation in rats. These results suggest that LMWP can be used as a substitute for protamine for developing a new generation of nontoxic heparin antagonists.

Key words: low molecular weight protamine, heparin neutralization, immunogenicity, platelet aggregation

Despite its nearly universal use in clinical practice, protamine induces adverse reactions ranging from mild hypotension to idiosyncratic fatal cardiac arrest [1-4]. The toxicity of protamine is mediated through two pathways: nonimmunologic pathway, and immunoglobulin-mediated pathway. The mechanisms of protamine-induced adverse responses via the nonimmunologic pathway are attributed to the "cross-linking" ability of protamine due to its polycationic and polymeric nature. For instance, complement activation, which is one of the major events via this nonimmunologic pathway, is primarily due to the cross-linking of heparin by protamine to form antigen–antibody like large network structures [5]. Anaphylactoid type of reactions produced via this mechanism, which are manifested by complement activation, thromboxane generation, and histamine release, are more common. However, they can normally be aborted with slow administration of protamine and thus are less dangerous. On the contrary, anaphylactic types of responses produced via immunoglobulin-mediated pathway are unpredictable, not preventable, and always life threatening [3, 4].

It is well known that heparin neutralization by protamine results from the competitive binding of protamine with antithrombin III (ATIII) to heparin [6]. Because the binding between protamine and heparin is electrostatic and heparin binds ATIII via a small pentasaccharide sequence [7], it is very likely that only a certain domain on protamine which encompasses an essential sequence for favorable electrostatic interaction may fully maintain heparin neutralization function. Since small peptides with low molecular weight are usually associated with diminished or devoid immunogenicity [8], our hypothesis is that some low molecular weight protamine fragments derived from protamine may retain anti-heparin activity but be devoid of immunogenicity and "cross-linking" ability of the native protamine.

In this study, a protamine fragment, which fully maintains the heparin neutralization function of the parent protamine but with much less toxicity, was obtained by enzymatic digestion of protamine. Heparin neutralization activity and toxicity of this protamine fraction were examined both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials. Protamine sulfate (clupeine from herring), thermolysin (EC 3.4.24.4), Freund's adjuvant, and goat-anti-mouse IgG-alkaline phosphatase were purchased from Sigma (USA). Porcine intestine heparin

Abbreviations: LMWP) low molecular weight protamine; LMWH) low molecular weight heparin; ATIII) antithrombin III; APTT) activated partial thromboplastin time; NPH) neutral protamine Hagedorn; PZI) protamine zinc insulin.

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(169 IU/mg; average molecular weight of 13 kD), antithrombin III (ATIII), factor Xa, and chromogenic substrate S-2238 were obtained from Pharmacia Hepar Inc. (USA). Actin cephaloplastin was obtained from Dade (USA). Fresh frozen human plasma in citrate was obtained from the American Red Cross in Detroit (USA). Rats (Sprague–Dawley, 270 ± 23 g) and mice (ICR strain, 6-7 week-old) were supplied by Harlan Dawley Co (USA).

Preparation of LMWP. Thermolysin and protamine were mixed in a 1 : 100 ratio in PBS solution containing 20 mM $CaCl_2$. The reaction mixture was incubated for 30 min at room temperature, followed by the addition of EDTA (50 mM) to quench the protease activity. Low molecular weight protamine mixture was fractionated on a heparin affinity column (HiTrap) attached to HPLC by using a linear NaCl gradient prepared by mixing solutions of PBS and 2 M NaCl. A total of five peptide fractions were observed. A pure peptide fragment (termed as LMWP), which accounts for most of the heparin neutralization ability of low molecular weight protamine mixture, was obtained and its sequence (VSRRRRRRGGRRRR) was confirmed by mass spectrum and amino acid composition analysis.

Determination of heparin neutralization ability of LMWP *in vitro*. Heparin neutralization ability of LMWP was measured in human plasma using the HEPTest® clotting assay. In brief, 15 µl of protamine or LMWP solution (20-200 µg/ml) was mixed with 100 µl of heparinized human plasma (1 U heparin/ml). A mixture of 15 µl of saline with 100 µl of heparinized human plasma (1 U heparin/ml) was taken as control. To the mixture, 100 µl of ATIII was added. After 2 min of incubation, 100 µl of RECALMIX® (preheated to 37°C) was added, and the clotting time was measured immediately using a fibrometer (Fibrosystem; Becton Dickinson Company, USA).

Determination of heparin neutralization ability of LMWP *in vivo*. Female Sprague–Dawley rats (mean weight 270 ± 23 g) were anaesthetized (50 mg/kg sodium pentobarbital) and a single jugular vein cannula was inserted into the right jugular vein. Blood (0.5 ml) was drawn as a control at 5 min after the injection of 0.4 ml saline. Heparin (25 U in 0.2 ml saline) was dosed intravenously and blood samples (0.4 ml) were drawn at 5 min after heparin injection. Right after that, 0.2 ml protamine (100 µg/100 g body weight) or LMWP (100-250 µg/100 g body weight) was injected intravenously at a period of 2 min. Blood (0.5 ml) was drawn at 5 min after protamine injection. Heparin activities in these rat plasmas were determined by activated partial thromboplastin time (APTT) test using a fibrometer (Fibrosystem; Becton Dickinson Company).

Immunogenicity assay of LMWP in mice. The immunogenicity of protamine and LMWP was examined in mice. The production of polyclonal antibodies was performed according to the method of Cooper and Paterson [9]. Twenty-six ICR mice (6-7 week-old), 12 for protamine and 14 for LMWP, were included in this study. Each mouse was immunized with 50 µg of protamine or LMWP in complete Freund's adjuvant (CFA). The first booster was given at the fourth week after primary immunization with 5 µg of protamine (or LMWP) in incomplete Freund's adjuvant (IFA). Animals were bled at 2-week intervals. Blood was collected, allowed to clot, and centrifuged to collect serum.

Determination of LMWP-mediated platelet count drop. Female Sprague–Dawley rats (mean weight 270 ± 23 g) were anaesthetized (50 mg/kg sodium pentobarbital) and a single jugular vein cannula was inserted into the right jugular vein. Blood specimens (0.09 ml) were obtained prior to heparin (10 IU/100 g body weight) administration, and 5 and 10 min after administration of protamine (100 µg/100 g body weight) or LMWP (250 µg/100 g body weight). Platelet count was determined using phase contrast microscopy.

Detection of anti-protamine and anti-LMWP antibodies by ELISA. High binding 96-well ELISA plates were first coated with 100 µl of equivalent concentration of protamine or LMWP (100 µg/ml) in PBS/Tween 20 (pH 7.5) and allowed to incubate overnight at 4°C. The unbound protamine or LMWP was removed by draining the plate and washing 4 times with PBS/Tween 20. The remaining binding sites in the wells were blocked by incubating the plates with 120 µl/well PBS/Tween 20 containing 1.0% human serum albumin for 1 h at 37°C. Anti-protamine and anti-LMWP antibodies in diluted serum of immunized mice were detected by the routine ELISA method using goat anti-mouse IgG-alkaline phosphatase as the detection antibody. In competitive ELISA, diluted serum was replaced with a fixed dilution of serum (100 times) containing increasing concentrations (1 to 1000 µg/ml) of free protamine or LMWP.

RESULTS

A total of eight peptide fractions were obtained after passing of enzyme digested protamine mixture through a heparin column (Fig. 1a). Since protamine neutralization of heparin results from its stronger heparin affinity than that of ATIII [7], small protamine fractions with weaker heparin binding strength is predicted to be unable to neutralize anticoagulant activity of heparin. In agreement with this assumption, small protamine fragments with low heparin affinity (peaks *1-7*, eluted before 0.80 M NaCl concentration) showed hardly any heparin neutralization function (data not shown). Only peak number eight showed high heparin binding strength (eluted at about 0.95 M NaCl concentration) and accounted for most of the heparin neutralization ability of the enzyme-digested protamine mixture (table). The mass spectrum and amino acid composition analysis (Fig. 1b) showed that this peak

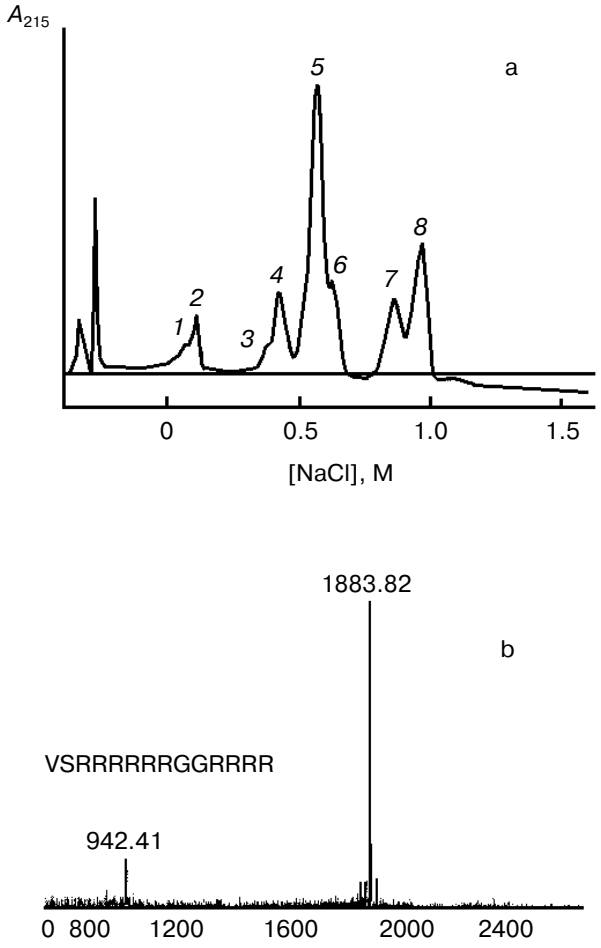

Fig. 1. Characterization of fractions from enzymatic digestion of protamine: a) elution profile of low molecular weight protamine fraction; b) mass-spectrometry and amino acid composition assay results of fraction No. 8 (LMWP).

containing a single peptide with the sequence of VSRRRRRRGGRRRR was from the C-terminal of protamine and was termed low molecular weight protamine (LMWP). Anti-heparin assay results from both *in vitro* and *in vivo* experiments showed that although the dose required for complete heparin neutralization was about two times higher than that of protamine, this peptide fully maintained the heparin neutralization function of protamine (table).

It is known that protamine-mediated toxicities are mainly through an immunoglobulin-mediated pathway because of the production of anti-protamine antibodies [3, 4]. Since a small peptide with 20 amino acids or less is usually associated with low immunogenicity, we further compared the immunogenicity of protamine and LMWP by monitoring anti-protamine or anti-LMWP antibody production in protamine or LMWP-immunized mice. As shown in Fig. 2, antibody titers in pooled sera from protamine-immunized mice were much higher than those from LMWP-immunized mice over the entire experimental duration (8 weeks). The greatest difference in antibody production between these two groups was observed six weeks after the primary immunization, and

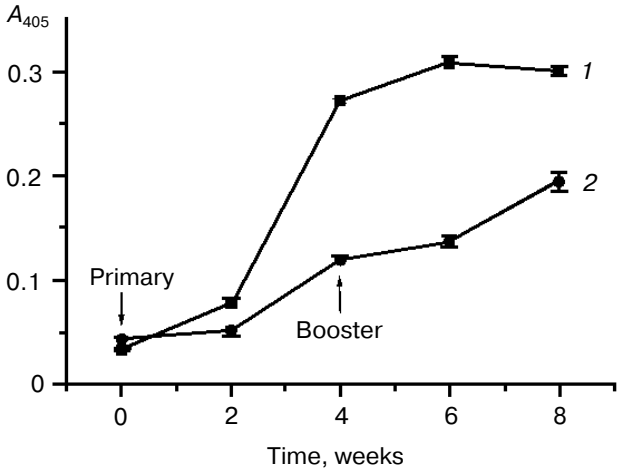

Fig. 2. Time course of antibody induction by protamine (*1*, n = 12) and LMWP (*2*, n = 14) in mice. Mice received protamine (50 μg) or LMWP (50 μg) in primary inoculation, and then were boosted with protamine (10 μg) or LMWP (10 μg) at 4 weeks after primary immunization. Blood samples were collected every 2 weeks after immunization and antibodies were detected on protamine- or LMWP-coated plates, respectively, using ELISA.

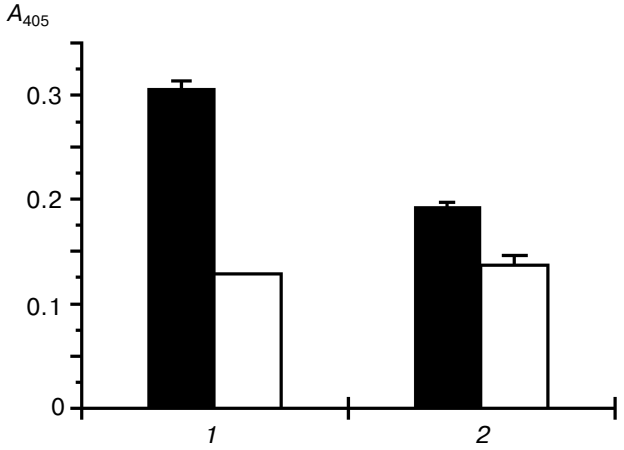

Fig. 3. Cross-reactivity assay of anti-protamine and anti-LMWP antibodies (dark and light columns, respectively) by ELISA. The cross-reactivity of anti-protamine (*1*) and anti-LMWP antibodies (*2*) to LMWP and protamine was detected on protamine- or LMWP-coated plates, respectively.

Neutralization of the anticoagulant activity of heparin by protamine and LMWP

Neutralization			
control	heparin	protamine	LMWP
In vitro			
13.5 ± 2.8	128 ± 3.4	15.2 ± 2.3 (13)	13.8 ± 1.7 (28)
In vivo			
12.5 ± 2.1	> 300	11.8 ± 1.5 (300)	13.1 ± 2.4 (700)

Note: Data listed in the table are clotting time (seconds). Data in brackets are the amount (μg) of protamine or LMWP required for the complete neutralization of heparin (1 IU/ml *in vitro* and 25 IU/rat *in vivo*). Other experimental conditions are as described in "Materials and Methods".

the antibody level in LMWP-immunized mice was three times lower than that in protamine-immunized mice (Fig. 2). The cross-reactivity of anti-protamine and anti-LMWP to LMWP and protamine was also examined. Although anti-LMWP showed a slightly higher reactivity to protamine than LMWP, anti-protamine exhibited much lower cross-reactivity to LMWP (Fig. 3).

Protamine-mediated adverse responses via the non-immunologic pathway are attributed to the "cross-linking" ability of protamine due to its polycationic and polymeric nature. Either protamine or its complex with heparin can bind to molecules on the cell membrane and thus affect cell function [5]. For example, the binding of protamine or protamine/heparin complexes to platelets has proved to cause platelet aggregation and induce thrombocytopenia [10]. To examine if LMWP would cause less cross-linking reactions as compared to its parent protamine, the effect of LMWP on platelet aggregation and thrombocytopenia induction in mice was further tested by intravenous injection of LMWP in the presence or absence of heparin. As shown in Fig. 4a, protamine itself could cause a significant platelet count drop (~25%) during the period of 5-15 min after injection, but LMWP had hardly showed any such aggregation induction function during the same period. In agreement with this result, LMWP only induced about 10% of platelet count drop when it was used for heparin neutralization, but there was no statistical difference between LMWP and the control group (Fig. 4b). On the contrary, protamine neutralization of heparin caused more than 30% of platelet count drop under the same experimental conditions.

DISCUSSION

In spite of its Food and Drug Administration approval, adverse reactions of protamine sulfate range from mild hypotension to idiosyncratic fatal cardiac arrest because of its immunogenicity and "cross-linking" ability. In fact, aside from its well-known use in heparin neutralization, protamine is used widely in insulin formulations to produce long-acting insulin (neutral protamine Hagedorn (NPH) and protamine zinc insulin (PZI)) allowing insulin-dependent diabetic patients to achieve euglycemia with less frequent insulin injections. This previous exposure to protamine renders diabetic patients highly susceptible to severe protamine hypersensitivity. It was reported

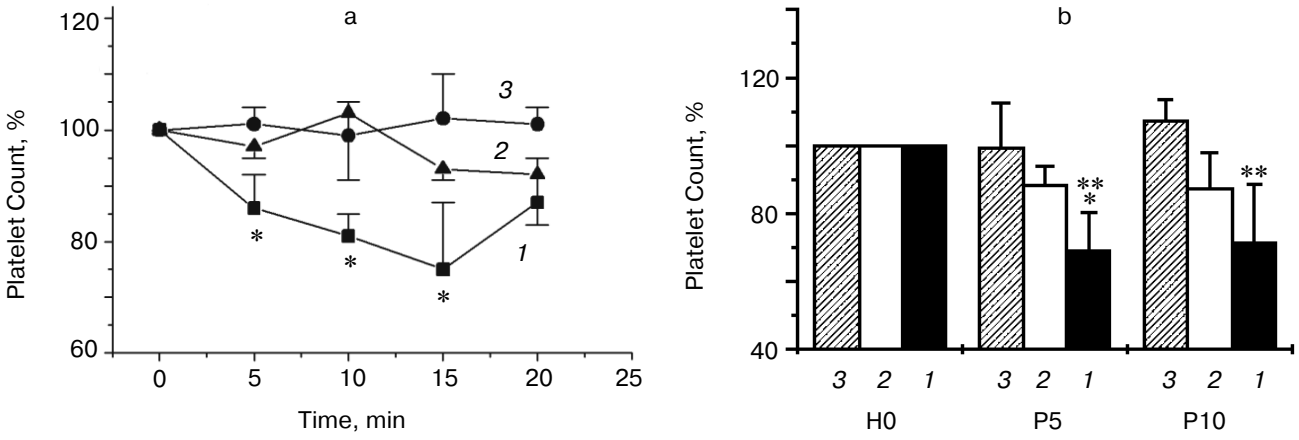

Fig. 4. a) Comparison of platelet count drops in the blood of rats after intravenous injection of protamine (100 μg/100 g body weight) (*1*) and LMWP (250 μg/100 g body weight) (*2*). Five, four, and two rats were used in protamine (*1*), LMWP (*2*), and control (saline) (*3*) groups, respectively. Data represent the average of these experiments; * $p < 0.05$. b) Comparison of protamine (100 μg/100 g body weight) (*1*) and LMWP (250 μg/100 g body weight) (*2*) induced platelet count drops during its neutralization of heparin (10 IU/100 g body weight) in rats. Blood platelet numbers were measured at 5 min (P5) and 10 min (P10) after protamine or LMWP injection following the heparin injection. Five rats were used for both protamine and LMWP group. Blood platelet levels before heparin injection (H0) were taken as controls (100%); * $p < 0.01$ (in comparison with LMWP group); ** $p < 0.01$ (in comparison with saline group (*3*)).

that 30% of NPH-treated diabetics had IgG anti-protamine antibodies, and the risk of a hemodynamically significant protamine reaction at the time of cardiac surgery in insulin-dependent diabetics was approximately ten times higher than that in non-diabetic controls [11]. For this reason, protamine toxicity has drawn considerable attention recently and various protamine-like peptides obtained by either chemical or recombinant synthesis methods have been tested as substitutes to protamine for their heparin anticoagulant neutralization activity [12-15]. However, except for the expensive platelet factor 4 (1 mg/US $2800), none of these peptides have proved to be safe for clinical use regardless of their heparin neutralization abilities.

Low molecular weight protamine (LMWP) that we obtained here fully maintains the heparin neutralization function of its parent protamine (table); it is less immunogenic than the parent protamine (Fig. 2); it lacks cross-reactivity to anti-protamine antibodies (Fig. 3) and had hardly any effect on platelet aggregation (Fig. 4). Therefore, compared to protamine, LMWP possesses much less toxicity and is highly likely to become a new nontoxic heparin antagonist. Since anti-protamine antibodies exhibits very low cross-reactivity to LMWP, the use of LMWP as a substitute for protamine in heparin reversal will enable a large population of diabetic patients which already have anti-protamine antibodies in their bodies to avoid the risks of immunoglobulin-mediated, fatal protamine responses. In fact, LMWP has also proved to be an ideal substitute for protamine in its formulation with insulin and in DNA condensation (data to be published). Therefore, in addition to heparin neutralization, less toxic LMWP also possesses great potential for its applications in other pharmaceutical products such as insulin formulation and gene therapy.

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REFERENCES

1. Metz, S., and Horrow, J. C. (1994) in *Pharmacology and Physiology in Anesthetic Practice* (Stoelting, R. K., ed.) Vol. 1, J. B. Lippincott, Philadelphia, pp. 1-15.
2. Weiler, J. M., Gelhaus, M. A., Carter, J. G., Meng, R. L., Benson, P. M., Hottel, R. A., Schillig, K. B., Vegh, A. B., and Clark, W. R. (1985) *J. Allergy Clin. Immunol.*, **75**, 297-303.
3. Horrow, J. C. (1985) *Anesth. Analg.*, **64**, 348-361.
4. Lindblad, B. (1989) *Eur. J. Vasc. Surg.*, **3**, 195-201.
5. Rent, R., Ertel, N., Eisenstein, R., and Gewurz, H. (1975) *J. Immunol.*, **114**, 120-124.
6. Okajima, Y., Kanayama, S., Maeda, Y., Urano, S., Kitani, T., Watada, M., Nakagawa, N., and Ijichi, H. (1981) *Thromb. Res.*, **24**, 21-29.
7. Rosenberg, R. (1987) in *Hemostasis and Thrombosis: Basic Principles and Clinical Practice* (Colman, R. W., Marder, V. J., and Hirsh, J., eds.) 2nd Ed., J. B. Lippincott, Philadelphia, pp. 1373-1392.
8. Sela, M. (1969) *Science*, **166**, 1365-1374.
9. Cooper, H. N., and Paterson, Y. (1991) in *Current Protocols in Immunology* (Coligan, J. E., Kruisbbek, A. M., and Margulies, D. H., eds.) Green Publishing Associates and Wiley-Interscience, New York, pp. 2.4.1-2.4.7.
10. Hulin, M. S., Wakefield, T. W., Andrews, P. C., Wrobleski, S. K., Kadell, A. M., Downing, L. J., and Stanley, J. C. (1997) *Lab. Anim. Sci.*, **47**, 153-160.
11. Nell, L. J., and Thomas, J. W. (1988) *Diabetes*, **37**, 172-176.
12. Schick, B. P., Gradowski, J. F., San Antonio, J. D., and Martinez, J. (2001) *Thromb. Haemost.*, **85**, 482-487.
13. Shenoy, S., Harris, R. B., and Sobel, M. (1995) *Curr. Pharm. Res.*, **5**, 965-986.
14. Hulin, M. S., Wakefield, T. W., Andrews, P. C., Wrobleski, S. K., Stoneham, M. D., Doyle, A. R., Zelenock, G. B., Jacobs, L. A., Shanley, C. J., TenCate, V. M., and Stanley, J. C. (1997) *J. Vasc. Surg.*, **26**, 1043-1048.
15. Levy, J. H., Cormack, J. G., and Morales, A. (1995) *Anesth. Analg.*, **81**, 35-37.