

Cross-Reactivity of Rabbit Anti-Bovine Thrombin IgGs With Human α -Thrombin and a Recombinant Version of Human Thrombin (Recothrom)

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It has been reported that patients exposed to topical bovine thrombin preparations may develop antibodies against bovine thrombin, factor V or various other proteins found in these preparations. Such antibodies can cross-react with human endogenous coagulation proteins and may lead to alterations in the coagulation laboratory parameters, hypersensitivity reactions, and severe bleeding or thrombosis. The underlying mechanisms for the coagulopathy are not fully understood yet. To better understand the cross-reactivity of anti-bovine thrombin antibodies with human corresponding coagulation proteins, bovine crude thrombin, and its purified versions, thrombin 4A (the old version of Thrombin-JMI before year 2008) and thrombin 4B (the current version of Thrombin-JMI on market), were used to generate relevant anti-bovine thrombin immunoglobulin G (IgGs) in rabbits. Using Western blotting, the cross-reactivity of each IgG with human α -thrombin and a recombinant version of human thrombin (Recothrom) was investigated. The results indicated that no cross-reactivity with either human α -thrombin or

Recothrom was observed with both anti-bovine crude thrombin IgGs and thrombin 4B IgGs. However, anti-bovine thrombin 4A IgGs showed apparent cross-reactivity with human α -thrombin and Recothrom in a protein amount-dependent manner. Furthermore, the results revealed that the cross-reactivity of anti-bovine thrombin 4A IgGs with human α -thrombin and Recothrom was immunization time-dependent. The minimum concentration of 4A IgG required to exhibit cross-reactivity with human α -thrombin and Recothrom varied considerably among individual rabbits. These results indicate that rabbit anti-bovine thrombin IgGs can cross-react with human α -thrombin and Recothrom, suggesting that human antibodies against bovine thrombin may also cross-react with human recombinant thrombin. Thus, the patients who were previously exposed to bovine thrombin may also develop antibodies which can cross-react with human recombinant thrombin.

Keywords: bovine thrombin; human thrombin; Western blot; immunoglobulin G; topical hemostatic agents

Introduction

Although topical bovine thrombin preparations are widely used for surgical hemostasis with an estimated 500,000 patients exposed to these products

annually in the United States,^{1,2} there was a growing concern that antibodies against bovine thrombin and bovine factor V can develop from the innate human immune response to the α -Gal glycosylation moiety unique to lower mammalian species as well as other antigenic impurities in bovine thrombin preparations.² However, the improved purification of process reported by Terrab et al³ is now used for the manufacturing of a purified version of this product. The relative antigenic potential of this improved bovine thrombin is much lesser than the product

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Table 1. The Generation of Anti-Bovine Thrombin Antisera^a

Day of Schedule	Injection of Antigens	Bleed of Productions
Day 0	First injection of antigens	Bleed of preimmune serum
Day 21	Second injection of antigens	
Day 30		First bleed of antisera
Day 42	Third injection of antigens	
Day 50		Second bleed of antisera
Day 91	Fourth injection of antigens	
Day 105		Third bleed of antisera
Day 123	Fifth injection of antigens	
Day 137		Fourth bleed of antisera
Day 151	Sixth injection of antigens	
Day 165		Fifth bleed of antisera

^a The antigens used for the generation of antisera include bovine crude thrombin, thrombin 4A and thrombin 4B. Each of the antigens was injected into 3 individual rabbits to elicit the relevant antiserum.

used prior to this change. The currently marketed bovine thrombin product reflects the purified version of bovine thrombin which is devoid of the impurities that may have been present in the earlier products.

Patients exposed to topical bovine thrombin preparations may develop antibodies to bovine thrombin, factor V or various other proteins found in these preparations. In some cases, these antibodies, by cross-reacting with the corresponding human coagulation proteins and acting as acquired inhibitors of native human thrombin and factor V, cause a variety of complications ranging from laboratory abnormalities to life-threatening bleeding or thrombotic complications.⁴⁻¹⁶ In addition, the use of thrombin derived from plasma sources also carries a potential risk of transmitting blood-borne pathogens. Due to these safety concerns, a recombinant human thrombin, Recothrom (ZymoGenetics Inc; Seattle, Washington), which contains no human plasma components, was developed to provide an alternative to bovine thrombin. Recothrom was reported to be less immunogenic and relatively safer than the existing bovine thrombin preparations.¹⁷⁻²²

However, until now there is little understanding about the possible cross-reactivity of anti-bovine thrombin antibodies with Recothrom. To further evaluate the safety of Recothrom, this study was carried out to determine whether anti-bovine thrombin antibodies would cross-react with Recothrom.

Materials and Methods

Bovine Thrombin Preparations

Crude bovine thrombin was obtained through the activation of bovine prothrombin by a bovine lung thromboplastin activator. The crude thrombin was initially purified using ion-exchange chromatography to yield an intermediate sample referred to as thrombin 4A. Following elution, the chromatographically purified sample was filtered through an Omega 100K VR membrane filter resulting in thrombin 4B.³ The crude thrombin, thrombin 4A and thrombin 4B, preparations used in this study were kindly provided by King Pharma (Middleton, Wisconsin).

Human α -Thrombin and Recothrom

Human α -thrombin used in this study was obtained from Dr John Fenton of Albany College of Pharmacy, Albany, New York. Recothrom (Lot ZAC0712A, ZymoGenetics Inc, Seattle, Washington) was purchased from Loyola University Hospital. Both human α -thrombin and Recothrom were diluted with saline before loading.

Generation of Anti-Bovine Thrombin Antisera

To generate specific antisera in rabbits, the bovine thrombin preparations were provided to Lampire Biologics (Pipersville, Pennsylvania). Bovine crude thrombin, thrombin 4A and thrombin 4B, conjugated with keyhole lymphocyte hemocyanin (KLH) as a carrier were administered intravenously to individual groups of rabbits at a dosage of 100 μ g using standard immunologic methods. Preimmune blood and antiserum were collected from each rabbit in serial order as shown in Table 1. The antiserum from each rabbit and the pooled antisera obtained from 3 individual rabbits in each treatment group were stored at -80°C for further immunoglobulin G (IgG) isolation.

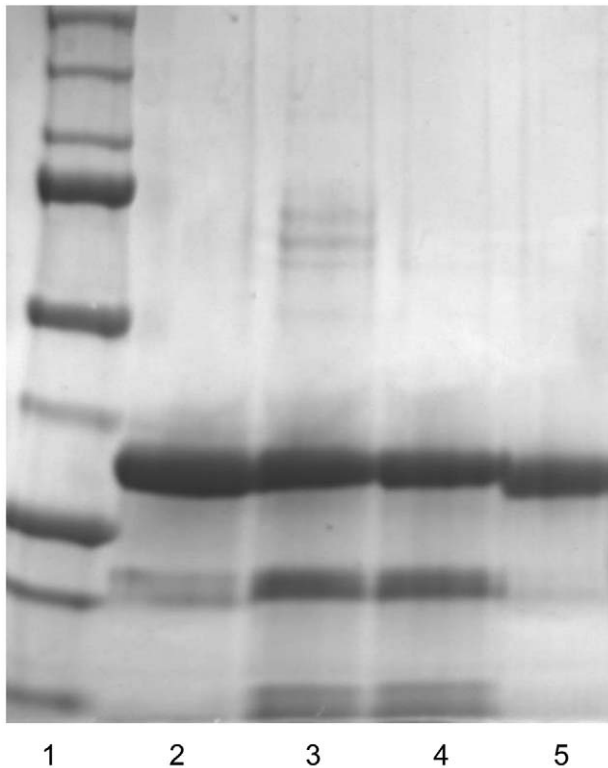


Figure 1. Gel electrophoresis analysis of Recothrom, human α -thrombin, and bovine thrombin 4A and 4B. Coomassie blue-stained gel showed molecular weight markers (lane 1), human α -thrombin (20 μ g, lane 2), bovine thrombin 4A (20 U, lane 3), bovine thrombin 4B (20 U, lane 4), and Recothrom (20 U, lane 5). From top to bottom, molecular weight markers represented protein bands of 250 kD, 150 kD, 100 kD, 75 kD, 50 kD, 37 kD, 25 kD, 20

Immunoglobulin G Isolations

HiTrap Protein G HP columns, 1 mL (GE Healthcare Bio-Science Corp, Piscataway, New Jersey) were used in this study to isolate IgG from rabbit anti-bovine antisera. The eluted IgG fractions were carefully collected and the concentration of harvested IgGs was adjusted with saline to 1 mg/mL based on the protein concentration determined using a modified Lowry's assay.²³ All IgGs were aliquoted and stored at -70°C .

Gel Electrophoresis and Western Blotting

Gel electrophoresis and Western blotting were carried out as previously described.²⁴ In brief, samples were subjected to electrophoresis through 4% to 20% gradient Tris-4-(2-hydroxyethyl)-1-piperazineethanesulfonic

acid (HEPES)-sodium dodecyl sulfate (SDS) polyacrylamide mini gels and then electrotransferred onto nitrocellulose membranes. The membranes were blocked with 5% milk and probed with anti-bovine crude thrombin IgG, antithrombin 4A IgG, and antithrombin 4B IgG, respectively. The membranes were then washed and incubated with horseradish peroxidase conjugated donkey anti-rabbit IgG (1:20,000 dilution). After extensively washing, immunoreactive bands were detected with SuperSignal West Pico Chemiluminescent Substrate followed by film exposure for 1 to 5 min.

Results

From our previous studies in which human α -thrombin was tested at a single amount of 0.2 μ g, no cross-reactivity of rabbit anti-bovine thrombin IgGs with human α -thrombin was observed.^{24,25} To explore the possibility of cross-reactivity of rabbit anti-bovine thrombin IgGs with human thrombin systemically, we have used serially diluted human α -thrombin up to 20 μ g instead of just one amount in the current study. As designed, the IgGs collected on day 30 (first time point) and day 165 (last time point) isolated from each pooled anti-bovine thrombin antisera were initially used to screen for the possibility of cross-reactivity. If any positive finding was found, the probing of human α -thrombin and Recothrom using the IgGs from all other time points (days 50, 105, and 137) was performed.

Gel electrophoresis analysis illustrated that human α -thrombin mainly consists of 2 protein bands, that is a strong band at 36 kD and a weaker band at 22 kD. Bovine thrombin 4A and 4B stained by Coomassie blue represented 3 thrombin-related protein bands (α -thrombin band at 36 kD, β -thrombin at 22 kD, and γ -thrombin at 17 kD). Besides these thrombin-related bands, thrombin 4A preparation also exhibited 4 extra faint protein bands with molecular weights ranging from 50 to 70 kD. The protein band pattern of Recothrom on Coomassie blue-stained SDS gels was similar to that of human α -thrombin, showing a strong and dark protein band at around 35 kD and a much weaker band at around 21 kD (Figure 1).

As shown in Figure 2, no visible cross-reactivity with human α -thrombin was observed with the anti-bovine crude thrombin IgGs collected from both days 30 and 165. The results from this experiment, in which the loadings of human α -thrombin

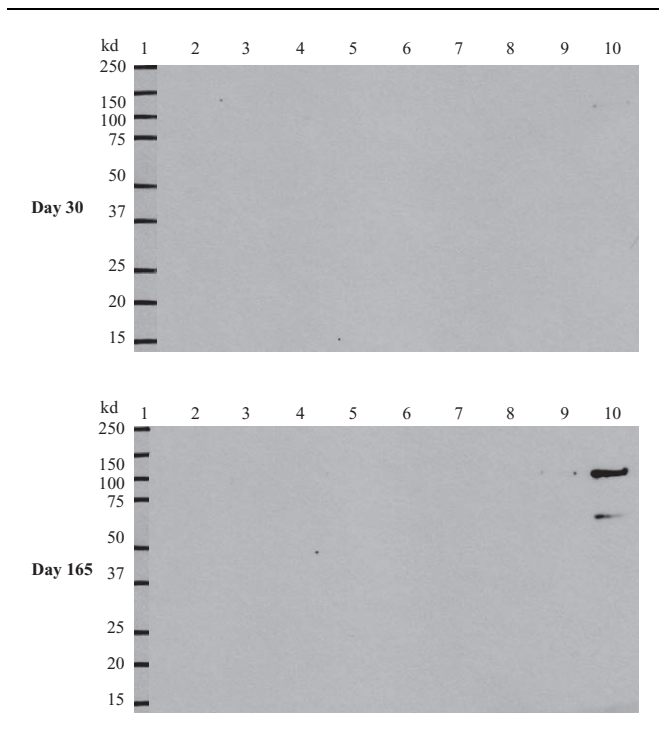


Figure 2. The determination of cross-reactivity of rabbit anti-bovine crude thrombin IgG with human α -thrombin and Recothrom. Serial amounts of human α -thrombin (5 μ g, 10 μ g, and 20 μ g, lanes 2-4), Recothrom (2.5 U, 5.0 U, and 10 U, lane 5-7), and bovine thrombin 4A (2.5 U, 5.0 U, and 10 U, lane 8-10) were probed with 2 μ g/mL of rabbit anti-bovine crude thrombin IgG collected on days 30 (top panel) and 165 (bottom panel), respectively. horseradish peroxidase (HRP)-linked donkey anti-rabbit immunoglobulin G (IgG) was used as the secondary antibody at a 1:20,000 dilution. Relative molecular weight markers, to the left of each panel, identified the size of immunoreactive bands.

sample was increased from 25 to 100 times higher than before, further confirm our previous finding that crude thrombin IgGs has no cross-reactivity with human α -thrombin.^{24,25} In addition, no recognition of Recothrom sample (up to 10U) by anti-bovine crude thrombin IgG collected on day 30 or on day165 was observed. Furthermore, in previous experiments, we have reported that anti-bovine crude thrombin IgGs could not reveal any protein band from bovine thrombin 4A sample (protein amount loaded was up to 2 U).^{24,25} The results obtained from this experiment were consistent with our previous findings, for example at an amount of 2.5 U, no immunoreactive band was revealed from the thrombin 4A sample by the crude thrombin IgGs tested. Crude thrombin IgG from day 30 was able to

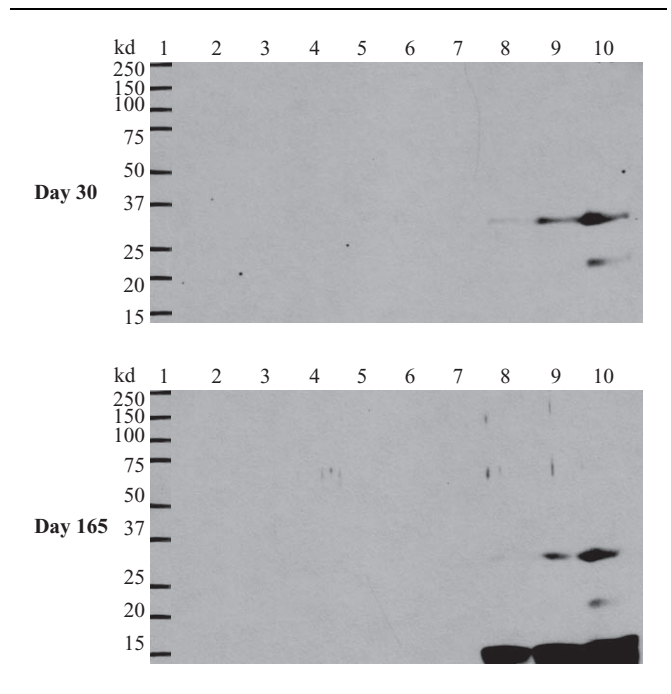


Figure 3. The determination of cross-reactivity of rabbit anti-bovine thrombin 4B immunoglobulin G (IgG) with human α -thrombin and Recothrom. Serial amounts of human α -thrombin (5 μ g, 10 μ g, and 20 μ g, lanes 2-4), Recothrom (2.5 U, 5.0 U, and 10 U, lane 5-7), and bovine thrombin 4A (2.5 U, 5.0 U, and 10 U, lane 8-10) were probed with 2 μ g/mL of rabbit anti-bovine thrombin 4B IgG collected on days 30 (top panel) and 165 (bottom panel), respectively. horseradish peroxidase (HRP)-linked donkey anti-rabbit IgG was used as the secondary antibody at a 1:20,000 dilution. Relative molecular weight markers, to the left of each panel, identified the size of

detect a very faint protein band at around 120 kd from the 10 U of thrombin 4A sample. The lowest amount of thrombin 4A which was detectable by crude thrombin IgG collected on day 165 was 5 U. From 10 U of thrombin 4A sample, crude thrombin IgG (day165) revealed a strong protein band at around 120 kd as well as a weak prothrombin-like band at around 70 kd.

In the next set of experiments, similar blots loaded with serially diluted human α -thrombin, Recothrom, and bovine thrombin 4A samples were probed with rabbit anti-bovine thrombin 4B IgGs collected on days 30 and 165. As shown in Figure 3, anti-bovine thrombin 4B IgG (day 30) could reveal an α -thrombin band at 36 kd and a β -thrombin band at 22 kd from the thrombin 4A sample. In addition to α - and β -thrombin bands, thrombin 4B IgG (day 165) could also detect a clear and strong γ -thrombin band at 17 kd from bovine thrombin 4 A sample.

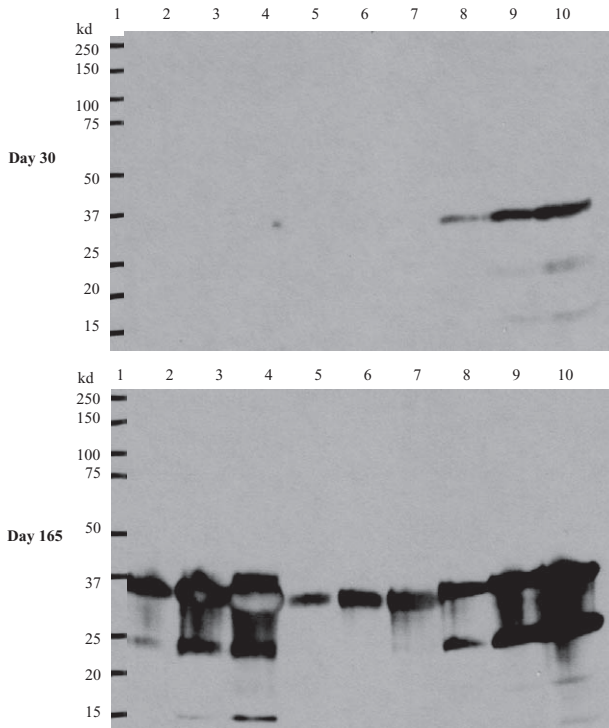


Figure 4. The determination of cross-reactivity of rabbit anti-bovine thrombin 4A immunoglobulin G (IgG) with human α -thrombin and Recothrom. Serial amounts of human α -thrombin (5 μ g, 10 μ g, and 20 μ g, lanes 2-4), Recothrom (2.5 U, 5.0 U, and 10 U, lane 5-7), and bovine thrombin 4A (2.5 U, 5.0 U, and 10 U, lane 8-10) were probed with 2 μ g/mL of rabbit anti-bovine thrombin 4A IgG collected on days 30 (top panel) and 165 (bottom panel), respectively. horseradish peroxidase (HRP)-linked donkey anti-rabbit IgG was used as the secondary antibody at a 1:20,000 dilution. Relative molecular weight markers, to the left of each panel, identified the size of

These results were consistent with our previous findings.^{24,25} However, anti-bovine thrombin 4B IgGs collected on days 30 and 165 did not show any visible cross-reactivity with either human α -thrombin or Recothrom (Figure 3).

Lastly, the cross-reactivity of anti-bovine thrombin 4A IgGs with human α -thrombin and Recothrom was explored using the same method as previously described above for the anti-bovine crude thrombin IgGs and 4B IgGs. Serial amounts of human α -thrombin and Recothrom were first probed using the IgGs from days 30 and 165 at a concentration of 2 μ g/mL, and if any cross-reactivity was observed, further probing using (1) the 4A IgGs from other time points; (2) the 4A IgGs obtained from individual rabbit would be performed. As shown in Figures 4

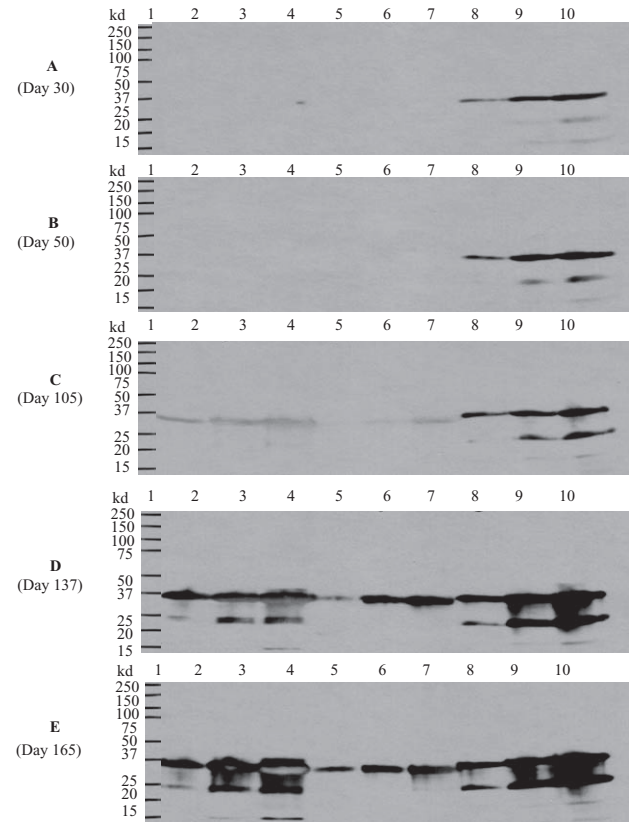


Figure 5. The determination of time-course of cross-reactivity of rabbit anti-bovine thrombin 4A immunoglobulin G (IgGs) with human α -thrombin and Recothrom. Serially diluted amounts of human α -thrombin (5 μ g, 10 μ g, and 20 μ g, lanes 2-4), Recothrom (2.5 U, 5.0 U, and 10 U, lane 5-7) and bovine thrombin 4A (2.5 U, 5.0 U, and 10 U, lane 8-10) were probed with 2 μ g/mL of rabbit anti-bovine thrombin 4A IgGs collected on days 30 (panel A), 50 (panel B), 105 (panel C), 137 (panel D), and 165 (panel E),

and 5, anti-bovine thrombin 4A IgGs collected from all time points were able to reveal an α -thrombin band (36 kD), a β -thrombin band (22 kD), and a γ -thrombin band (17 kD) from thrombin 4A sample in a protein amount dependent manner. No cross-reactivity with either human α -thrombin or Recothrom was observed from rabbit anti-bovine thrombin 4A IgGs collected on days 30 and 50. However, when used at a concentration of 2 μ g/mL anti-bovine thrombin 4A IgGs collected on day 105 or later time points exhibited obvious cross-reactivity with human α -thrombin and Recothrom, that is the cross-reactivity with human α -thrombin and Recothrom started to appear from the IgG collected on day 105 and became more intensive on days 137 and 165. From human α -thrombin, 3 clear

immunoreactive bands with molecular weights at 36 kD, 22 kD, and 17 kD were revealed in a protein amount dependent manner. More interestingly, a distinctive immunoreactive band with a molecular weight around 35 kD was also revealed with Recothrom (Figures 4 and 5). These results suggested that the generation of cross-reacting antibodies to human α -thrombin and Recothrom in rabbit anti-bovine thrombin 4A IgGs is immunization time dependent, that is, the cross-reactivity may only be induced by repeated immunizations (at least >3-fold of antigen load) in an extended time interval (at least >50 days). Furthermore, followed individual rabbit anti-bovine thrombin 4A IgG studies revealed that minimum concentration of 4A IgG required to exhibit the cross-reactivity with human α -thrombin and Recothrom varied considerably among the 3 individual rabbits studied, ranging from 0.5 μ g/mL to 8 μ g/mL (Figures are not shown).

Discussion

Recombinant thrombin preparations are potential alternatives to plasma-derived thrombin preparations. Theoretically, because recombinant thrombin has the identical amino acid sequence as human thrombin, and is made from a genetically modified Chinese hamster ovary cell line,²⁶ it would provide a homologous protein that is safe and effective but without the attendant concerns of antibody production or viral transmission. Preclinical studies demonstrated that the application of Recothrom in animals was safe.¹⁷ Phase 2 trials also demonstrated that Recothrom was well tolerated and minimally immunogenic.¹⁸ In a phase 3 trial comparing the efficacy, safety, and antigenicity of Recothrom versus bovine thrombin, it was found that Recothrom was safe and as efficacious as bovine thrombin. However, in terms of antigenicity, 21.5% of the patients receiving bovine thrombin developed antibodies to the product, whereas only 1.5% of patients in the Recothrom treated group developed antibodies to recombinant thrombin.¹⁹ Recently, a companion study for the vascular subgroup, in the previously mentioned phase 3 trial, demonstrated the improved antigenicity profile for Recothrom versus bovine thrombin.²⁰ However, although there was a notably decreased rate of antigenicity associated with Recothrom, the safety profiles characterized by adverse events and laboratory evaluations, were similar between Recothrom and bovine thrombin treated groups.^{19,20} Moreover, the

plasma samples used to analyze the antibodies in both trials were obtained in the trial conducted by Chapman et al,¹⁹ which was completed in late 2006. Prior to the introduction of improved production process taken in 2007, the earlier bovine thrombin products were not as pure as the newer improved preparations.³

Bovine thrombin 4A is an intermediate product obtained from crude thrombin by the purification using ion-exchange chromatography. Then it undergoes further purification by filtration through an Omega 100K VR membrane filter, resulting in thrombin 4B.³ It is interesting to note that in the current study only anti-bovine thrombin 4A IgG, but not anti-bovine crude thrombin IgG and 4B IgG, displayed an apparent cross-reaction with Recothrom. The possible explanations for the phenomenon are as follows: (1) The protein structures of bovine thrombin were subtly changed or modified in the purification processes taken from crude thrombin to thrombin 4B. Thus, the epitopes on each thrombin product, which could be recognized by the relevant antibodies, were slightly different. It appears like that bovine thrombin 4A and Recothrom share some identical epitopes that can be specifically recognized only by rabbit anti-bovine thrombin 4A IgGs. (2) In the current study, no obvious cross-reactivity of anti-bovine crude thrombin IgG and 4B IgG with either human α -thrombin or Recothrom was observed. However, we cannot rule out the possibility that due to some possible changes in protein structures and/or physical and chemical qualities, the generated anti-bovine crude thrombin IgGs/4B IgGs may still possess a much weaker binding affinity for both human α -thrombin and Recothrom when compared with anti-bovine thrombin 4A IgG. Under the current experimental designs, only a fixed concentration of 2 μ g/mL was used to screen the possible cross-reactivity with human α -thrombin and Recothrom for all 3 anti-bovine thrombin IgGs studied. Therefore, the nonexistent cross-reactivity of anti-bovine crude thrombin IgGs and 4B IgGs with human α -thrombin and Recothrom may just result from an inadequate IgG concentration. Further experiments using higher concentrations of anti-bovine crude thrombin IgGs and 4B IgGs may help to clarify this issue. Moreover, variations in the antigenic responses are commonly observed in both animals and human populations. While the bovine thrombin 4B preparation did not generate cross-reactive antibodies in the 3 rabbits injected in this study, it may result in variable responses in larger group of rabbits.

As reported in the literature, human anti-bovine thrombin antibodies, in some cases, may cross-react with the corresponding human coagulation proteins, such as human thrombin and factor V, resulting in hemorrhagic complications.⁹⁻¹⁶ Because Recothrom has an identical amino acid sequence as human thrombin, theoretically there is a possibility that anti-bovine thrombin antibodies could also cross-react with Recothrom. So far, no systemically comparative clinical investigation or preclinical research has been done to clarify this issue. In the current study, by using Western blotting method, we have demonstrated that it is the case that rabbit anti-bovine thrombin IgG, in addition to recognizing its immunogen (bovine thrombins), could also cross-react with human α -thrombin and Recothrom. Based on the finding presented in this study, it is reasonable to predict that human anti-bovine thrombin antibodies may also be able to cross-react with Recothrom. Thus, the application of Recothrom may not be suitable for the patients who have been previously treated with/exposed to bovine thrombin preparations. It should also be noted that humans are constantly exposed to bovine proteins and beef products as a major dietary source. Therefore, the preexistence of anti-bovine thrombin-related antibodies in humans before a surgery is not unexpected, as this constant exposure to beef proteins may result in the generation of polyvalent antibodies in some of the individuals. In fact, the presence of anti-bovine thrombin antibodies has been previously reported in healthy individuals or bovine thrombin-naïve patients before a surgery.^{12,18-20} Consequently, caution should be taken when using Recothrom in such population. Moreover, population-based differences in the antibody generation should also be taken into account as additional immunogenic factors may contribute to the cross-reactivity of neoepitopes to antithrombin antibodies.

Conclusions

The results reported in this study indicate that rabbit anti-bovine thrombin IgGs can cross-react with Recothrom in an immunization time-dependent manner. Thus, the data strongly suggest that it is likely that human antibodies against bovine thrombin may also cross-react with human recombinant thrombin. Therefore, the application of Recothrom may not be recommended in the patients who have been previously treated with/exposed to bovine

thrombin preparations. Moreover, additional predisposing factors and population responses may also contribute to the interaction of anti-bovine thrombin antibodies with human recombinant thrombin.

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References

1. Schoenecker JG, Hauck RK, Mercer MC, Parker W, Lawson JH. Exposure to topical bovine thrombin during surgery elicits a response against the xenogenic carbohydrate galactose α 1-3galactose. *J Clin Immunol.* 2000;20(6):434-444.
2. Lawson JH. The clinical use and immunologic impact of thrombin in surgery. *Semin Thromb hemost.* 2006;32(S1):98-110.
3. Terrab A, Pawlak D, Spaay P, Hoppensteadt D, Fareed J. Further removal of factor V related antigen from bovine thrombin by utilizing a membrane-filtration step. *Clin Appl Thromb Hemost.* 2008;14(2):135-140.
4. Nelson PA, Powers JN, Estridge TD, et al. Serological analysis of patients treated with a new surgical hemostat containing bovine proteins and autologous plasma. *J Biomed Mater Res.* 2001;58(6):710-719.
5. Rapaport SI, Zivelin A, Minow RA, Hunter CS, Donnelly K. Clinical significance of antibodies to bovine and human thrombin and factor V after surgical use of bovine thrombin. *Am J Clin Pathol.* 1992;97(1):84-91.
6. Cmolik BL, Spero JA, Magovern GJ, Clark RE. Redo cardiac surgery: late bleeding complications from topical thrombin-induced factor V deficiency. *J Thorac Cardiovasc Surg.* 1993;105(2):222-228.
7. Chouhan VD, De la cadena RA, Nagaswami C, Weisel JW, Kajani M, Rao AK. Simultaneous occurrence of human antibodies directed against fibrinogen, thrombin, and factor V after exposure to bovine thrombin: effects on blood coagulation, protein C activation and platelet function. *Thromb Haemost.* 1997;77(2):343-349.
8. Winterbottom N, Kuo JM, Nguyen K, et al. Antigenic responses to bovine thrombin exposure during surgery: a prospective study of 309 patients. *J Applied Res.* 2002;2:1-11.
9. Ortel TL, Charles LA, Keller FG, et al. Topical thrombin and acquired coagulation factor inhibitors: clinical

- spectrum and laboratory diagnosis. *Am J Hematol*. 1994;45(2):128-135.
10. Zehnder JL, Leung LLK. Development of antibodies to thrombin and factor V with recurrent bleeding in a patient exposed to topical bovine thrombin. *Blood*. 1990;76(10):2011-2016.
11. Lawson JH, Lynn KA, Vanmatre RM, et al. Antihuman factor V antibodies after use of relative pure bovine thrombin. *Ann Thorac Surg*. 2005;79(3):1037-1038.
12. Ortel TL, Mercer MC, Thames EH, Moore KD, Lawson JH. Immunologic impact and clinical outcomes after surgical exposure to bovine thrombin. *Ann Surg*. 2001;233(1):88-96.
13. Sands JJ, Nudo SA, Ashford RG, Moore KD, Ortel TL. Antibodies to topical bovine thrombin correlate with access thrombosis. *Am J kidney Dis*. 2000;35(5):796-801.
14. Sarfati MR, Dilorenzo DJ, Kraiss LW, Galt SW. Severe coagulopathy following intraoperative use of topical thrombin. *Ann Vasc Surg*. 2004;18(3):349-351.
15. Savage WJ, Kickler TS, Takemoto CM. Acquired coagulation factor inhibitors in children after topical bovine thrombin exposure. *Pediatr Blood Cancer*. 2007;49(7):1025-1029.
16. Streiff MB, Ness PM. Acquired FV inhibitors: a needless iatrogenic complication of bovine thrombin exposure. *Transfusion*. 2002;42(1):18-26.
17. Heffernan JK, Ponce RA, Zuckerman LA, et al. Preclinical safety of recombinant human thrombin. *Regul Toxicol Pharmacol*. 2007;47(1):48-58.
18. Chapman WC, Lockstadt H, Singla N, Kafie FE, Lawson JH. Phase 2, randomized, double-blind, placebo-controlled, multicenter clinical evaluation of recombinant human thrombin in multiple surgical indications. *J Thromb Haemost*. 2006;4(9): 2083-2085.
19. Chapman WC, Singla N, Genyk Y, et al. A phase 3, randomized, double-blind comparative study of the efficacy and safety of topical recombinant human thrombin and bovine thrombin in surgical hemostasis. *J Am Coll Surg*. 2007;205(2):256-265.
20. Weaver F, Lew W, Granke K, et al; Study Investigators. A comparison of recombinant thrombin to bovine thrombin as a hemostatic ancillary in patients undergoing peripheral arterial bypass and arteriovenous graft procedures. *J Vasc Surg*. 2008; 47(6):1266-1273.
21. Greenhalgh DG, Gamelli RL, Collins J, et al. Recombinant thrombin: safety and immunogenicity in burn wound excision and grafting. *J Burn Care Res*. 2009;30(3):371-379.
22. Singla NK, Ballard JL, Moneta G, Randleman CD Jr, Renkens KL, Alexander WA. A phase 3b, open-label, single-group immunogenicity and safety study of topical recombinant thrombin in surgical hemostasis. *J Am Coll Surg*. 2009;209(1):68-74.
23. Sandermann H Jr, Stromiger JL. Purification and properties of C 55-isoprenoid alcohol phosphokinase from *staphylococcus aureus*. *J. Biol. Chem*. 1972;247(16): 5123-5131.
24. Zhu H, Hoppensteadt D, Cunanan J, et al. Reduced immunogenic potential of membrane filtered bovine thrombin preparations for hemostatic application. *Clin Appl Thromb Hemost*. 2008;15(1):32-40.
25. Zhu H, Hoppensteadt D, Adiguzel C, Bick RL, Fareed J. Comparison of immunogenic potentials of bovine thrombin preparations. *Clin Appl Thromb Hemost*. 2008;15(1):41-49.
26. RecothromTM [Package insert]. Seattle, WA: Zymogenetics; 2008.