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Comparison of Immunogenic Potentials of Bovine Thrombin Preparations

He Zhu, MD, Debra Hoppensteadt, PhD, Cafer Adiguzel, MD, Rodger L. Bick, MD, PhD, and Jawed Fareed, PhD

Using a membrane filtration step, bovine crude thrombin was purified into thrombin 4A and 4B preparations. The purpose of this study was to determine whether the improved purity of a bovine thrombin preparation can reduce its overall immunogenic potential and lower the risk of development of factor V antibodies. Bovine crude thrombin and its purified versions, thrombin 4A and 4B, were administered to individual groups of rabbits on days 0, 21, 42, 91, 123, and 151 using standard immunologic methods. Blood was drawn from each rabbit on days 30, 50, 105, 137, and 165, and the pooled antisera from individual groups were purified to obtain the Ig Gs using protein G affinity columns. Using Western blotting, the specificity of each immunoglobulin G collected at the first time point (day 30) and last time point (day 165) was determined. The results of Western blotting using the Ig Gs collected

on days 30 and 165 were consistent; both demonstrating that thrombin 4B has the least immunogenic potential among the 3 thrombin preparations tested. Compared with the immunoglobulin Gs collected on day 30, the Ig Gs from day 165 did not show obvious difference regarding their ability to detect antigens in bovine thrombin samples. Neither showed cross-reactivity with human coagulation factors nor the recognition of bovine factor Va antigens. These results suggest that despite the presence of a trace amount of bovine factor Va antigen in bovine thrombin preparations, these contaminants failed to elicit the generation of antibodies against factor Va light chain in rabbit

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

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} Bovine thrombin preparations commonly contain fibrinogen as well as small amounts of factor V and other proteins. Although topical bovine thrombin preparations have been used successfully in most patients, there are isolated reports of adverse events and deranged hemostasis resulting in severe or refractory bleeding and/or thrombosis.³ Patients exposed to topical thrombin preparations may develop antibodies to bovine thrombin, factor V, or various other

proteins found in these preparations.⁴⁻⁷ In some cases, these antibodies can, 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.^{4,8} The reported incidence of developing antithrombin and/or antifactor V antibodies in patients treated with topical bovine thrombin varies considerably from study to study. Ortel et al⁹ demonstrated, in a prospective series of 151 surgical patients, that more than 95% of patients developed antibodies to bovine thrombin or factor V and that approximately 50% of these patients had cross-reactivity to human coagulation factors. Streiff and Ness¹⁰ reviewed the published reports of factor V inhibitors and found that in adults, 40% to 66% of cardiac surgery patients and 20% of neurosurgery patients developed factor V antibodies after bovine thrombin exposure. Recently,

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Chapman et al¹¹ reported that 21.5% of patients receiving bovine thrombin developed antibodies to the product in a phase 3 trial comparing the efficacy and safety of recombinant human thrombin and bovine thrombin in surgical hemostasis. Similarly, Weaver et al¹² reported that 27% of patients in the bovine thrombin group developed antibodies to a bovine thrombin product in a study comparing recombinant thrombin to bovine thrombin as a hemostatic ancillary in patients undergoing peripheral arterial bypass and arteriovenous graft procedures.

Many case reports suggest that antihuman factor V antibodies elicited in response to exposure to bovine factor V, a contaminant present in bovine thrombin preparations, have played an important role in the development of coagulopathy.¹³⁻¹⁶ Factor V is synthesized as a 330-kd single-chain protein and undergoes several enzymatic cleavages to become activated factor V (Va), consisting of a 105-kd heavy chain and a 71-kd light chain noncovalently joined by calcium ions.¹⁷⁻¹⁹ With its heavy chain binding to prothrombin and light chain binding to platelet phospholipids and factor Xa, factor Va acts as a cofactor in the prothrombinase complex, which increases the efficiency of the conversion of prothrombin to thrombin by a factor of 300 000.²⁰ If an antibody-mediated inhibition/depletion of factor V occurs in a patient, that patient may develop a severe and refractory coagulopathy. Unlike reported cases of thrombin inhibitors where bleeding complications are unusual, the majority of the reported patients with factor V inhibitors have had bleeding complications, occasionally fatal.²¹⁻²⁵

Based on the assumption that impurities in bovine thrombin preparations could be responsible for severe adverse reactions in patients, efforts have been made to reduce or remove contaminants such as factor V from bovine thrombin products to improve the overall safety and efficacy of the products. A previous study has reported that a membrane filtration step performed to remove viruses from chromatographically purified bovine thrombin was also highly effective in reducing factor V or its fragments to undetectable levels.²⁶ To determine whether the improved purity of bovine thrombin can reduce its overall immunogenic potential and lower the risk of development of factor V antibodies following exposure, we performed a pilot experiment to compare the acute immunogenic potential of crude thrombin and its purified forms, thrombin 4A and thrombin 4B.²⁷ Our preliminary data obtained using the anti-bovine thrombin immunoglobulin Gs (IgGs)

collected on day 30 suggested that the reported purification process resulted in the removal of most of the antigens found in crude thrombin, and that none of the preparations tested generated any detectable anti-bovine factor Va antibodies in rabbits.²⁷ In this study, we repeated the Western blotting experiments using anti-bovine thrombin IgGs collected on day 165. The purpose of the present study was to confirm our previous findings by comparing the results obtained using anti-bovine thrombin IgGs collected on day 165 to those obtained previously from thrombin IgGs collected on day 30.

Materials and Methods

Bovine Thrombin Preparations

Crude bovine thrombin was obtained through the activation of bovine prothrombin by 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.²⁶ The crude thrombin, thrombin 4A, and thrombin 4B preparations used in this study were kindly provided by King Pharma (Middleton, Wis).

Bovine Plasma and Specific Human and Bovine Coagulation Factors

Bovine plasma was also provided by King Pharma and diluted with saline to obtain serial of dilutions ranging from 12.5% to 1.6%. Bovine factor Va light chain standard was purchased from Haematologic Technologies Inc (Essex Junction, Vt) and diluted serially with saline to the concentrations of 2.5, 5, and 10 µg/mL, before loading. All other human and bovine coagulation factors used in this study were obtained commercially from Enzyme Research Laboratories (South Bend, IN) and American Diagnostica (Stamford, CN) and diluted with saline accordingly before loading.²⁷ Molecular weight standards ranging from 10.0 to 250 kd were purchased from Pierce (Rockford, IL). These standards were used to cross-reference the molecular weight of various bands in the electrophoresis and Western blotting methods. Bovine alpha-thrombin was purchased from Enzyme Research Laboratories and transformed into beta and gamma forms by autolysis.

Generation of Anti-bovine Thrombin Antisera

To generate specific antisera in rabbits, the thrombin preparations were provided to Lampire Biologics (Pipersville, Pa). 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 µg using standard immunologic methods. Preimmune blood and antiserum were harvested from each rabbit in serial order as shown in Table 1. The antisera obtained from 3 individual rabbits in each treatment group were pooled and stored at -80°C for further IgG isolation. This study was approved by the Institutional Animal Care and Use Committee at Lampire Biologics.

Immunoglobulin G Isolations

HiTrap Protein G HP columns, 1 mL (GE Healthcare Bio-Science Corp, Piscataway, NJ), 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 from each group was adjusted with saline to 1 mg/mL based on the protein concentration determined using a modified Lowry's assay.²⁸ All IgGs were aliquoted and stored at -70°C .

Western Blotting

Western blotting was carried out as previously described.²⁷ In brief, samples were subjected to electrophoresis through 4% to 20% gradient Tris-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 (2 µg/mL). The membranes were then washed and incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (1:20 000 dilution). After extensively washing, immunoreactive bands were detected with Super-Signal West Pico Chemiluminescent Substrate followed by film exposure for 1 to 5 min. As a positive reference, bovine factor Va antigen was detected from bovine factor Va light chain standard using a monoclonal mouse anti-bovine factor Va antibody (Haematologic Technologies Inc). Horseradish peroxidase-linked goat antimouse IgG (Bio-Rad

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

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

Laboratories, Hercules, Calif) was used at a 1:10 000 dilution as the secondary antibody.

Results

In this study, all the anti-bovine thrombin IgGs used were isolated from pooled antisera collected on day 165 from the rabbits that had been administered the relevant antigens 6 times before the antisera harvest (Table 1). The ability of the anti-bovine thrombin IgGs collected on day 165 to detect antigens in the crude thrombin samples was evaluated and compared with that of the anti-bovine thrombin IgGs collected on day 30. As shown in Figure 1, on blots identically loaded with serial amounts of bovine crude thrombin, rabbit anti-bovine IgGs from both time points were used at an identical concentration of 2 µg/mL to compare their detecting abilities for the antigens. When evaluated in terms of the lowest amount of crude thrombin sample that was detectable, no obvious differences were found between the thrombin IgGs from day 30 and the thrombin IgGs from day 165. In addition, among the 3 IgGs from day 165, the rank order of detecting ability for the antigens in the crude thrombin sample was the same as that revealed by the thrombin IgGs from day 30, that is crude

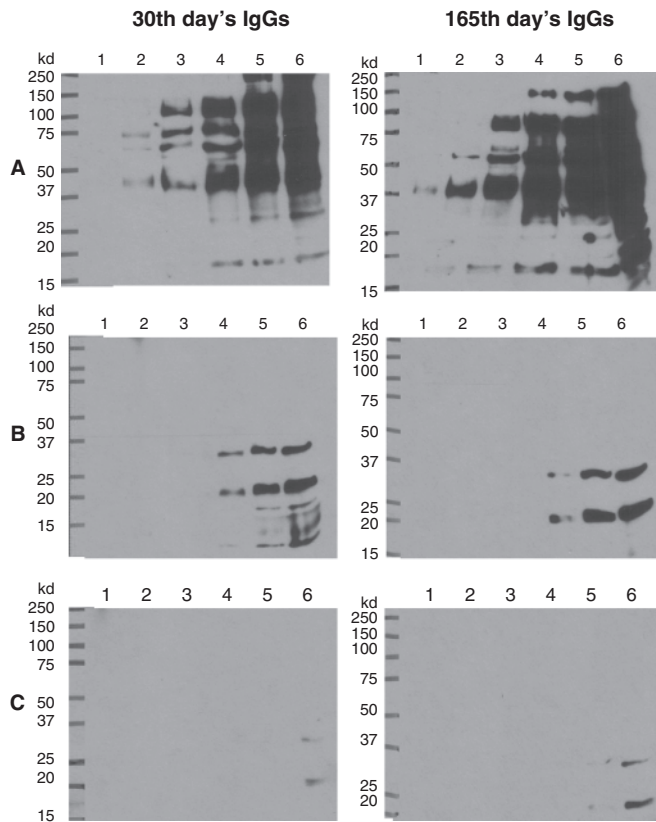


Figure 1. Western blot detection of antigens in a bovine crude thrombin by rabbit anti-bovine thrombin immunoglobulin Gs (IgGs) collected on days 30 (left column) and 165 (right column). Serially diluted bovine crude thrombin (0.062, 0.125, 0.25, 0.5, 1.0, and 2.0 U; lanes 1 through 6) was probed with 2 μ g/mL of crude thrombin IgG (panel A), 4A IgG (panel B), and 4B IgG (panel C). Relative molecular weight markers, to the left of each panel, identified the size of immunoreactive bands.

thrombin IgG > thrombin 4A IgG > thrombin 4B IgG. Crude thrombin IgG from day 165 could detect as low as 0.062 U of crude thrombin sample showing multiple immunoreactive bands, while the crude thrombin IgG from day 30 could detect as low as 0.125 U of crude thrombin showing a similar protein band pattern.

The thrombin 4A IgG from both time points could detect as low as 0.5 U of crude thrombin sample, mainly revealing 2 clear bands (α -thrombin at 36kd and β -thrombin at 22kd). With the weakest ability to detect antigens in a crude thrombin sample, thrombin 4B IgG from both time points could only detect 2 U of crude thrombin sample showing 2 visible thrombin bands (α -thrombin at 36kd and β -thrombin at 22kd).

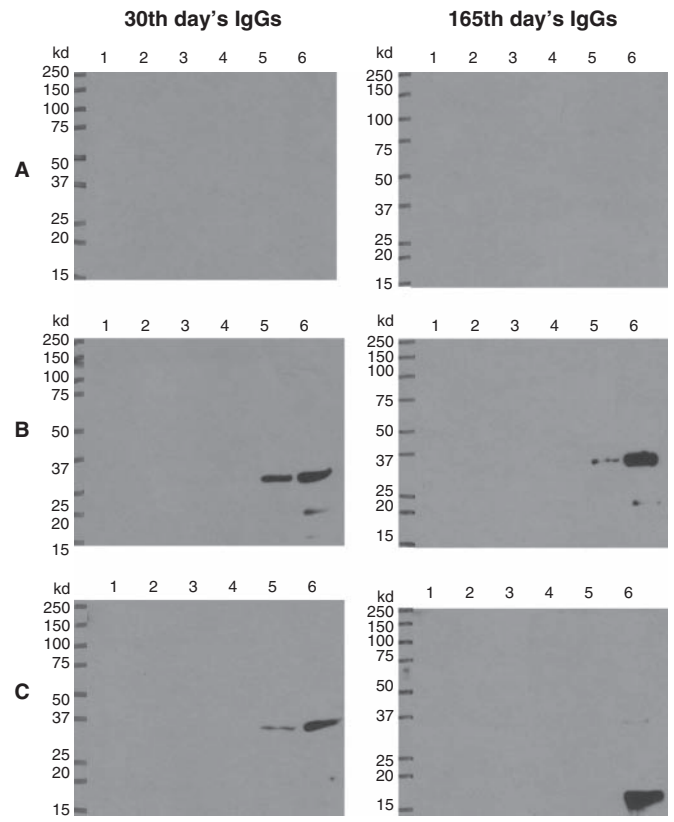


Figure 2. Western blot detection of antigens in a bovine thrombin 4A by rabbit anti-bovine thrombin immunoglobulin Gs (IgGs) collected on days 30 (left column) and 165 (right column). Serially diluted bovine thrombin 4A (0.062, 0.125, 0.25, 0.5, 1.0, and 2.0 U; lanes 1 through 6) was probed with 2 μ g/mL of crude thrombin IgG (panel A), 4A IgG (panel B), and 4B IgG (panel C). Relative molecular weight markers, to the left of each panel, identified the size of immunoreactive bands.

Similar comparisons were made between the anti-bovine thrombin IgGs from day 30 and the anti-bovine thrombin IgGs from day 165 using blots loaded with serial dilutions of bovine thrombin 4A (Figure 2). As with the day 30 IgG, crude thrombin IgG from day 165 failed to detect any protein bands in the thrombin 4A sample. The thrombin 4A IgGs from both time points could detect the same lowest amount of thrombin 4A sample (1.0 U). Using the thrombin 4A sample, thrombin 4A IgG from both time points revealed a similar immunoreactive band pattern. However, compared with thrombin 4B IgG from day 30, which could detect 1 α -thrombin band at 36kd in 1 and 2 U of thrombin 4A sample, the thrombin 4B IgG from day 165 had a weaker detecting ability for antigens in thrombin 4A sample revealing

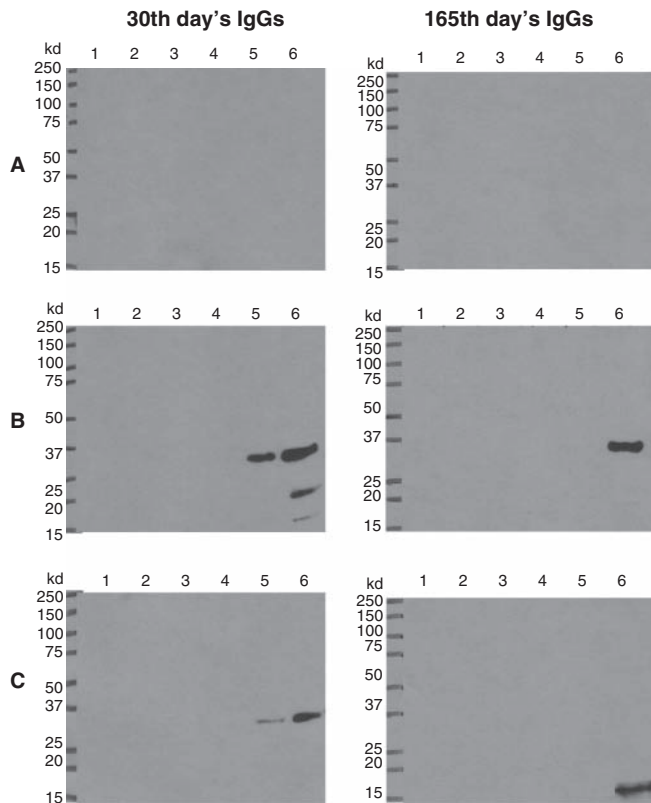


Figure 3. Western blot detection of antigens in bovine thrombin 4B with rabbit anti-bovine thrombin immunoglobulin Gs (IgGs) collected on days 30 (left column) and day 165 (right column). Serially diluted bovine thrombin 4B (0.062, 0.125, 0.25, 0.5, 1.0, and 2.0 U; lanes 1 through 6) was probed with 2 μ g/mL of crude thrombin IgG (panel A), 4A IgG (panel B), and 4B IgG (panel C). Relative molecular weight markers, to the left of each panel, identified the size of immunoreactive bands.

only 1 γ -thrombin band at 17kd in 2 U of thrombin 4A sample.

Figure 3 shows a comparison between the anti-bovine thrombin IgGs from day 30 and the anti-bovine thrombin IgGs from day 165 using blots loaded with serial amounts of bovine thrombin 4B. The crude thrombin IgGs from both time points could not detect any visible protein band in thrombin 4B sample. Compared with the day 30 IgGs, the thrombin 4A and 4B IgGs from day 165 demonstrated slightly weaker detecting abilities for the antigens in thrombin 4B sample.

Identical blots loaded with serial dilutions of bovine plasma were used to further compare the difference between the anti-bovine thrombin IgGs collected on day 30 and the anti-bovine thrombin IgGs collected on day 165. As shown in Figure 4,

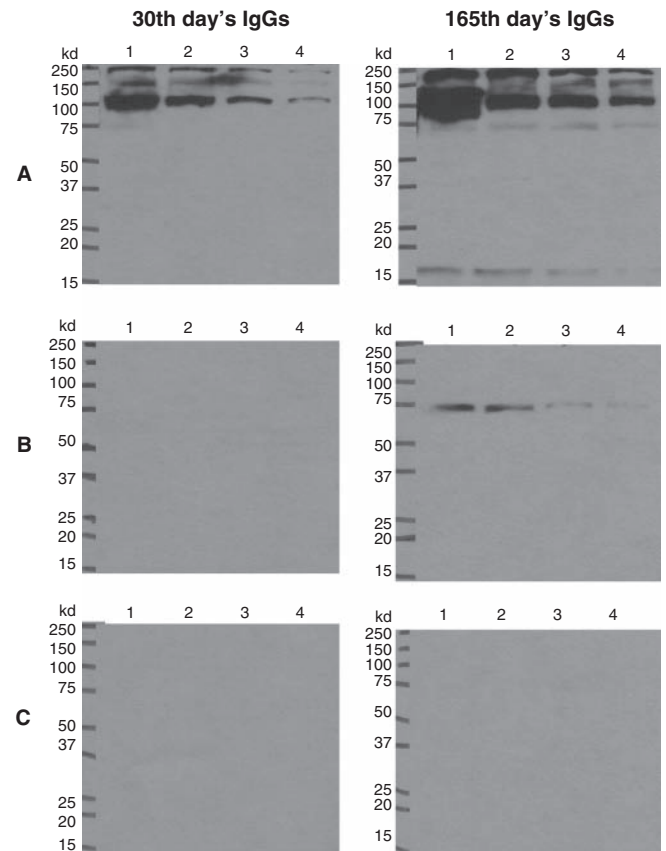


Figure 4. Western blot detection of antigens in bovine plasma by rabbit anti-bovine thrombin immunoglobulin Gs (IgGs) collected on days 30 (left column) and 165 (right column). Serially diluted bovine plasma (12.5%, 6.25%, 3.12%, and 1.56% in saline; lanes 1 through 4) was probed with 2 μ g/mL of crude thrombin IgG (panel A), 4A IgG (panel B), and 4B IgG (panel C). Relative molecular weight markers, to the left of each panel, identified the size of immunoreactive bands.

anti-bovine crude thrombin IgG from day 30 could detect multiple high-molecular-weight protein bands ranging from 80 to 250 kd with a protein concentration-dependent pattern from bovine plasma sample diluted from 12.5% to 1.6% with saline. However, both thrombin 4A IgG and 4B IgG from day 30 failed to detect any visible immunoreactive band from bovine plasma samples. In comparison to the IgGs from day 30, anti-bovine crude thrombin IgG from day 165 could detect multiple high-molecular-weight protein bands ranging from 17 to 250 kd including a band at 72 kd (prothrombin) and a band at 17 kd (γ -thrombin) from bovine plasma samples. The thrombin 4A IgG from day 165 also revealed a similar prothrombin-like immunoreactive band at 72 kd. However, as with the

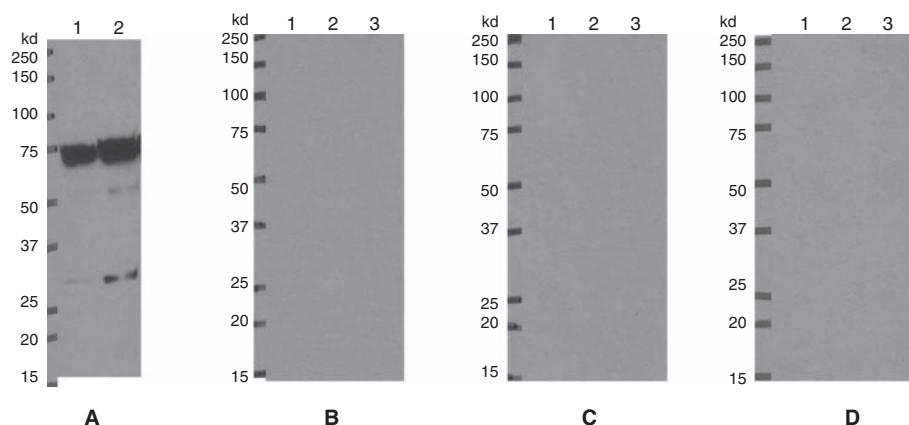


Figure 5. Western blot detection of bovine factor Va light chain standard with mouse anti-bovine factor Va antibody or rabbit anti-bovine thrombin immunoglobulin Gs (IgGs) collected on day 165. Serial amounts of bovine factor Va light chain (0.05, 0.1, and 0.2 µg, lanes 1 through 3) were probed with 1 µg/mL of mouse anti-bovine factor Va antibody (panel A), 2 µg/mL of rabbit anti-bovine crude thrombin IgG (panel B), 4A IgG (panel C), and 4B IgG (panel D). Either HRP-linked goat antimouse IgG or HRP-linked donkey antirabbit IgG was used as a secondary antibody at a 1:10 000 dilution following the incubation of relevant primary antibody. HRP = horseradish peroxidase.

day 30 IgGs, the thrombin 4B IgG from day 165 failed to detect any visible immunoreactive band from bovine plasma sample.

Lastly, the specificity of each IgG collected on day 165 was also determined using specific human and bovine coagulation factors.²⁷ Serial amounts of a bovine factor Va light chain standard were probed using the IgGs from day 165 to investigate whether there is any anti-bovine factor Va antibody in the anti-bovine thrombin IgGs. As shown in Figure 5, no immunoreactive band was detected from bovine factor Va light chain standard by any of IgGs collected from day 165. As with the IgGs collected on day 30, neither the cross-reactivity with human coagulation factors nor the recognitions of bovine factor Xa antigens were observed with any of the IgGs collected from day 165 (data not shown).

Discussion

The immunogens used to generate the antibodies (IgGs) in this study were bovine crude thrombin and thrombins 4A and 4B. Bovine crude thrombin was obtained through the activation of prothrombin by thromboplastin. Purification of crude thrombin by ion-exchange chromatography and filtration yielded thrombin 4A and thrombin 4B, respectively.²⁶ Compared with crude thrombin, thrombin 4A and thrombin 4B preparations represent highly purified and

improved thrombins with markedly reduced extraneous protein contaminants.²⁶

In this study, anti-bovine thrombin IgGs were isolated from pooled antisera collected on day 165 from rabbits challenged with bovine crude thrombin and purified 4A and 4B preparations 6 times during the study period. These IgGs were tested using bovine thrombin samples, bovine plasma samples, as well as specific human and bovine coagulation factors. When evaluated in the terms of the lowest amount of bovine thrombin samples that were detectable and the immunoreactive band pattern detected, the Western blotting results obtained from bovine thrombin samples probed with the IgGs from day 165 were consistent with those obtained using day 30 IgG. For example, thrombin 4B IgGs from both time points did not show any recognition for nonthrombin-related antigens. In addition, these IgGs demonstrated much weaker detecting abilities for antigens in crude thrombin sample in comparison to crude thrombin IgGs and thrombin 4A IgGs (Figure 1). In addition, at an identical concentration of 2 µg/mL, thrombin 4B IgGs detected less immunoreactive protein bands with weaker immunodensity from thrombin 4A and 4B samples when compared with thrombin 4A IgGs (Figures 2 and 3). Finally, thrombin 4B IgGs from both time points failed to detect any measurable protein bands from bovine plasma samples when used at the same concentration as the crude thrombin IgGs and thrombin 4A IgGs (Figure 4). All these results from IgGs collected on day 165, along with the data

obtained from the IgGs collected on day 30, suggest that the thrombin 4B exhibits the weakest immunogenic potential among the 3 thrombin preparations evaluated.

In a previous study on anti-bovine thrombin IgGs collected 30 days following the initial immunization, it was found that rabbit anti-bovine crude thrombin IgGs did not cross-react with bovine factor Va light chain.²⁷ This finding in rabbits is inconsistent with the data obtained from human patients following exposure to topical bovine thrombin during surgery.⁴⁻⁸ It was thought that the development of anti-bovine factor Va antibodies might have slower onset as compared to antithrombin IgGs in rabbit and therefore, a longer immunization time may be needed to generate anti-bovine factor Va antibodies in rabbits than in humans. To rule out this possibility, serial amounts of bovine factor Va light chain standard were probed in this study using the anti-bovine thrombin IgGs that were collected after 6 immunizations with relevant antigens. It was clearly demonstrated in Figure 5 that none of the IgGs could detect any visible protein bands from bovine factor Va samples. The same results were also obtained when the concentration of the anti-bovine thrombin IgGs was increased to 4 µg/mL (data not shown). Thus, it is clear from these results that despite the presence of detectable amount of bovine factor Va contaminant in crude thrombin preparations, this contaminant failed to elicit the generation of antibodies against factor Va light chain in rabbits. This inconsistency with the human case may be due to a difference in the immune responses among species, that is, unlike the immune response to bovine thrombin, rabbits and humans may have a different immune response to the bovine factor V/Va antigens. Bovine factor V/Va may be less immunogenic in rabbits than in humans.

In this study, the specificity of anti-bovine thrombin IgGs collected on day 165 from rabbits administered with relevant antigens 6 times over the study period was evaluated and compared with that of anti-bovine thrombin IgGs collected on day 30 (2 immunizations with the antigens). Some minor differences were observed when comparisons were made based on the immunoreactive bands that were detectable. For example, besides the recognition of multiple high-molecular-weight bands that were detectable by crude thrombin IgG from day 30, anti-bovine crude thrombin IgG from day 165 could also detect a prothrombin-like band at 72 kd and a γ -thrombin-like band at 17 kd from bovine plasma. The thrombin 4A IgG from day 165 revealed a

prothrombin-like immunoreactive band at 72kd, which was undetectable to the thrombin 4A IgG from day 30. It was also found that instead of recognition of α -thrombin band at 36 kd, which was detectable by the thrombin 4B IgG from day 30, the thrombin 4B IgG from day 165 detected 1 strong γ -thrombin band at 17 kd from bovine thrombin 4A and 4B samples. The presence of these differences may be caused by slower onsets of some relevant antibodies in the IgGs. The nature and significance of these findings are unknown.

As stated in the introduction section, the reported incidence and significance of development of antithrombin and/or antifactor V antibodies in patients treated with topical bovine thrombin varies considerably from study to study.⁹⁻¹² The conflicting nature of these reports as well as the issues of trying to draw conclusions from nonrandomized controlled studies is evidenced by the conclusion by Ortel et al that observed adverse clinical complications that arose in the postoperative period were not associated with elevated antibody titers to either bovine or human coagulation factors.⁹ Furthermore, postoperative elevated antibody levels to two or more bovine proteins were found to be associated with a statistically significant decrease in the risk of an adverse event.⁹ In addition, 2 blinded, randomized controlled trials have not demonstrated a significant difference in adverse events between either human recombinant thrombin or bovine plasma-derived thrombin, despite greater elevations in antiproduct antibodies observed in the patients treated with bovine thrombin.^{11,12} Of note is the observation that approximately 5% of the patients randomized to bovine thrombin in the Chapman study had preexisting anti-bovine thrombin antibodies.¹¹ It was not clear whether these patients had previous exposure to bovine thrombin or whether these antibodies arose from environmental exposure. It is therefore imperative to demonstrate the role of bovine thrombin preparations in antibody formation in a bovine protein naive model.

Because rabbit feed does not include any beef proteins, rabbits could be used as a bovine protein naive model. The data from our initial study had shown that preimmune IgG obtained from the rabbits did not demonstrate any recognition of epitopes in bovine crude thrombin, thrombin 4A, thrombin 4B, and factor Va light chain.²⁷ The results from this study clearly demonstrated that factor V/Va contaminants in bovine thrombin preparations do not appear to generate an immune response in a naive rabbit model even after repeated exposure. Therefore, this

observation points to a possibility that the reported presence of anti-bovine factor Va antibodies in some patients exposed to bovine crude thrombin may be caused by an immune response to the preexisting bovine immunogens in these patients.

Conclusion

The results presented in this study confirm our previous findings that the purified bovine thrombin preparations such as the 4A and 4B are less immunogenic in comparison to the crude thrombin preparation. In particular, the thrombin preparation 4B exhibits the least immunogenic potential among the 3 thrombin preparations tested. Furthermore, it is clearly proven from our results that despite the presence of a detectable amount of bovine factor Va/Va fragments in crude thrombin and 4A preparations, these contaminants fail to elicit the generation of antibodies against factor Va light chain in rabbits.

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