Thrombogenicity of small-diameter prosthetic grafts: Relative contributions of graft-associated thrombin and factor Xa

Boulos Toursarkissian, MD, Paul R. Eisenberg, MD, MPH, Dana R. Abendschein, PhD, and **Brian G. Rubin, MD,** *St. Louis, Mo.*

Purpose: **We evaluated the contributions of coagulation factors IIa (thrombin) and Xa to small-diameter prosthetic graft thrombogenicity in vivo.**

Methods: **Preclotted and nonpreclotted (collagen-coated) polyester grafts were studied before and 24 hours after implantation into pig femoral arteries. After incubation of explanted grafts was performed with plasma depleted of vitamin K-dependent coagulation factors by barium chloride adsorbtion (Ba-plasma), graft-associated thrombin activity was determined by radioimmunoassay for fibrinopeptide A. Fibrinopeptide A levels reflect thrombin-mediated fibrin formation. Factor Xa activity was characterized by measuring activation of prothrombin added to Ba-plasma.**

Results: **Thrombin and factor Xa were associated with the luminal surfaces of preclotted grafts before and 24 hours after implantation. Nonpreclotted grafts had negligible procoagulant activity before implantation. After 24 hours in vivo graft-associated factor Xa activity was similar in both nonpreclotted and preclotted grafts; however, more thrombin was bound to nonpreclotted coated grafts ($p < 0.01$).**

Conclusions: **The procoagulant activity of small-diameter prosthetic grafts persists for 24 hours after implantation and is attributable not only to graft-associated thrombin but also to de novo thrombin elaboration induced by factor Xa. Moreover, graft-associated procoagulant activity is not dependent on preclotting because it develops on nonpreclotted, collagen-coated grafts as well. Treatment strategies to attenuate graft thrombosis may require the inhibition of both thrombin and factor Xa. (J Vasc Surg 1997; 25:730-5.)**

The widespread use of small-diameter prosthetic grafts for distal lower extremity revascularization has been limited by their high early failure rates.[1] Although the graft material itself is inert,[2] the rapid accumulation of coagulation proteins on the luminal surface after implantation results in significant graft-associated procoagulant activity.[3,4] This has prompted the search for new strategies to reduce graft thrombogenicity. The development of novel therapeutic approaches is dependent on a detailed knowledge of coagulation protein deposition on prosthetic grafts. It is surprising that little information is available about the relative contributions of individual coagulation factors to graft procoagulant activity.

Blood coagulation is ultimately dependent on the activation of factor II (prothrombin) to factor IIa (thrombin), which cleaves soluble fibrinogen into insoluble fibrin, activates platelets, and potentiates procoagulant activity by activating factors V and VIII. Both thrombin and factor Xa/Va, the complex that activates prothrombin, bind to platelet-rich thrombi and may play a role in graft thrombogenicity. Our laboratory has previously shown that the procoagulant activity of whole blood clots is not solely due to thrombin but also to clot-bound factor Xa/Va.[5] Determining the relative contribution of each coagulation factor to graft thrombogenicity is important, because an anticoagulant active against one coagulation factor does not necessarily inactivate others. This study was designed to determine the specific contributions of coagulation factor Xa and thrombin to prosthetic graft thrombogenicity in vivo

From the Departments of Surgery and Medicine, Washington University School of Medicine.

This work was funded in part by a Lifeline Award Research Grant from the Society for Vascular Surgery/International Society for Cardiovascular Surgery and by a Monsanto-Washington University Biomedical Research Grant.

Reprint requests: Brian G. Rubin, MD, 216 South Kingshighway, Department of Surgery, Room 3510, St. Louis, MO 63110.

0741-5214/97/$5.00 + 0 **24/1/78149**

with small-diameter grafts implanted in the arterial circulation of pigs.

MATERIAL AND METHODS

Animal studies. Domestic pigs weighing 25 to 35 kg were anesthetized with an intramuscular combination of ketamine, xylazine, tiletamine, and zolazepam (5 mg/kg of each), intubated, and ventilated with oxygen-enriched room air for the duration of the surgery on a respirator. Blood gas analysis was used to adjust ventilation. Sedation was maintained with intermittent intravenous dosing of pentobarbital. Twenty milliliters of blood was drawn percutaneously and used for graft preclotting.

The femoral bifurcations were isolated bilaterally with vertical groin incisions. The superficial femoral artery was replaced with a 4 cm long, 4 mm diameter segment of prosthetic graft implanted in an end-to-end fashion with running 6-0 polypropylene suture at both ends. No systemic anticoagulation was used. At the end of surgery the wounds were closed in layers, and the animals were allowed to recover. After 24 hours the animals were reanesthetized, and a midline abdominal incision was used to isolate the distal abdominal aorta. A cannula was placed in the distal aorta to irrigate both femoral grafts with 250 ml of phosphate-buffered saline solution at physiologic pressure. The grafts were then harvested with the adjacent 1 cm of native vessel at both the proximal and distal anastomoses. Each harvested unit was then divided into three 2 cm long segments to reflect the proximal anastomotic area, the midgraft, and the distal anastomotic area.

Grafts used in this study included knitted polyester grafts (Dacron, a gift of Meadox Medical, Oakland, N.J.) and collagen-coated knitted polyester grafts (Hemashield, a gift of Meadox Medical). Collagen-coated grafts are impervious to blood at arterial pressure and did not require preclotting before implantation. The noncoated polyester grafts were preclotted for 10 minutes with whole blood and were then mechanically stripped of gross clot before implantation. Preclotting of noncoated grafts is required to render the graft impervious to blood under arterial pressure and is standard clinical procedure.

We tested preclotted noncoated grafts that were not exposed to circulating blood and preclotted noncoated grafts that had been implanted into the femoral arterial circulation for 24 hours. Similarly, we tested collagen-coated grafts never exposed to blood, exposed to blood ex vivo for 1 minute, or implanted in vivo for 24 hours. Pigs were randomized to receive either two preclotted grafts, two collagen-coated grafts, or one of each. This procedure was done to minimize the effect of interanimal variability.

Preparation of reagents. Graft-associated thrombin activity was characterized by measuring fibrin formation induced by grafts incubated with recalcified plasma depleted of vitamin K-dependent coagulation factors (factors II, VII, IX, and X) by barium adsorbtion. Fibrin formation was characterized by measuring thrombin activity induced by activation of 0.9 μm prothrombin added to recalcified barium-adsorbed plasma incubated with grafts.

Adsorbtion of plasma by barium chloride (Ba-plasma). In brief, pooled frozen citrated human plasma was purchased from the American Red Cross (St. Louis, Mo.) and stir-mixed in a 9:1 ratio with 1 mol/L barium chloride in an ice bath for 30 minutes. The mixture was then centrifuged at 3000 rpm for 15 minutes, the supernatant collected, and the procedure repeated. The supernatant was then dialyzed in 25 mmol/L sodium citrate buffer containing 150 mmol/L chloride at pH 7.0 overnight to remove the barium chloride. At the completion of dialysis any remaining precipitate was centrifuged, and the plasma was stored at −70° C until use.[6]

Preparation of prothrombin. Human prothrombin was purchased from Hematologic Technologies Inc. (Essex Junction, Vt.) and treated with 5 mmol/L D-Phe-L-Pro-L-Arg-chloromethylketone (PPACK, a thrombin inhibitor) to remove any contaminating thrombin. The mixture was then dialyzed three times, 6 hours each time, against a 200 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid buffer containing 150 mmol/L sodium chloride to remove the PPACK before use. The lack of contaminating active thrombin was verified by the absence of FPA generation when the prothrombin was incubated with recalcified Ba-plasma.

Measurement of thrombin and factor Xa activities

Measurement of thrombin activity. Each 2 cm graft segment was closed at one end with a silk tie to allow the lumen to be filled with the testing solutions. Each segment was then filled with 150 μl of recalcified (25 mmol/L $CaCl_2$) Ba-plasma and incubated at 37° C for 15 minutes. The plasma was then removed, treated with 17 μl of an anticoagulant mixture (consisting of PPACK and aprotinin to inhibit thrombin and ethylenediaminotetraacetic acid), and immediately placed on ice to stop all coagulation reactions. The mixture was then stirred in a benton-

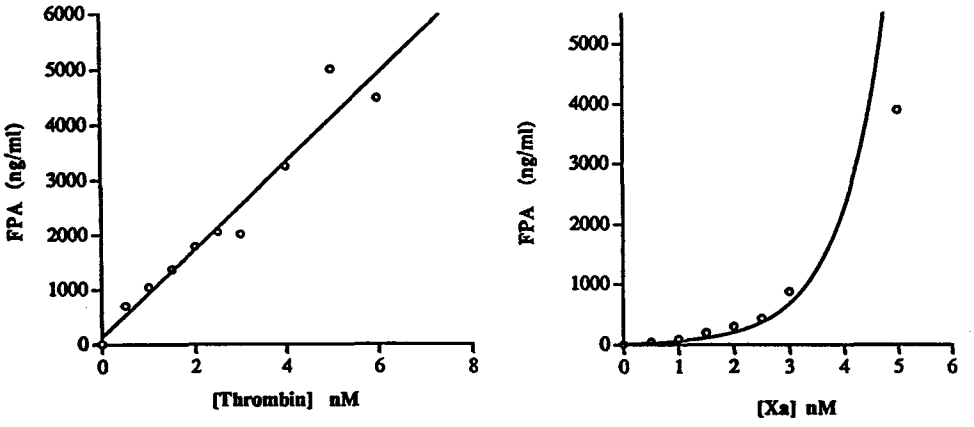

Fig. 1. Standard curves demonstrating amount of FPA generated by increasing concentrations of thrombin *(left)* or factor Xa *(right)*. Relationship between thrombin and FPA generation is linear, described by equation y = 798.946x + 131.888. Exponential equation y = (18.769) ($10^{0.519x}$) describes relationship between factor Xa concentration and FPA generation.

Table I. Increase in FPA (in ng/ml) attributable to thrombin and factor Xa on preclotted grafts

	0 hours	*24 hours*	*p value*
Thrombin	2448 ± 347	2134 ± 154	0.34
Factor Xa	4017 ± 1555	4815 ± 416	0.48

Results are reported as mean ± SEM.

ite slurry in a 1:2 volume ratio to precipitate any unused fibrinogen and was centrifuged at 2000*g* for 20 minutes. The supernatant was then collected, and its level of FPA was measured with a commercially available radioimmunoassay kit (Byk-Sangtec Diagnostica, Dietzenbach, Germany). The lower detection limit of this assay is 0.1 ng/ml. Because the Ba-plasma contains no thrombin, the FPA level measured directly reflects luminal graft-associated thrombin activity. This activity was quantified by comparison to standard curves.

Measurement of factor Xa activity. After assays were obtained for thrombin activity, the graft segments were washed thoroughly three times with large volumes of phosphate-buffered saline solution at 37° C. Each segment was then filled with 150 μl of recalcified (25 mmol/L $CaCl_2$) Ba-plasma, to which 0.9 mmol/L of purified thrombin-free prothrombin had been added. After a 15-minute incubation at 37° C plasma was removed and processed as described previously. Graft-associated factor Xa cleaved the added prothrombin into thrombin, which induced additional elaboration of FPA compared with that generated by graft-associated thrombin alone. The FPA levels measured by radioimmunoassay under these conditions reflected the graft-associated activities of both factor Xa and thrombin. By subtracting from the FPA generated by both factor Xa and thrombin activities that were due to thrombin alone, the FPA value attributable to factor Xa alone was calculated. Graft-associated factor Xa was quantified by comparison to standard curves.

Statistical analysis. Comparisons were performed with unpaired two-tailed Student *t* tests unless otherwise specified. The significance level was set at $p < 0.05$.

RESULTS

Six separate preclotted polyester grafts were studied before implantation. A total of seven preclotted polyester grafts were implanted into the femoral arterial circulation for 24 hours; thus 21 segments were studied. After preclotting alone FPA generation was measured, reflecting thrombin (2448 ± 347 ng/ml FPA) and factor Xa (4017 ± 1555 ng/ml FPA). After 24 hours of implantation in vivo significant FPA reflecting activities of both thrombin (2134 ± 154 ng/ml FPA) and factor Xa (4815 ± 416 ng/ml FPA) were tightly associated with all preclotted polyester grafts (Table I). FPA induced by thrombin and factor Xa after preclotting alone was not statistically different from values measured after 24 hours of implantation in vivo. Based on comparison with purified proteins added to the same plasma, bound thrombin and factor Xa concentrations after 24 hours of implantation were estimated to be 2.8 nm

Table II. Comparison of FPA (in ng/ml) generated by thrombin and factor Xa in different graft regions at 24 hours

	Proximal graft	*Mid graft*	*Distal graft*	*p value (ANOVA)*
Thrombin	2144 ± 221	2123 ± 327	2166 ± 236	0.99
Factor Xa	4239 ± 799	4985 ± 795	5219 ± 615	0.63

Results are reported as mean ± SEM, with n = 7 for all values.

and 4.6 nm, respectively (Fig. 1). This is equivalent to 7 ng of thrombin and 18 ng of factor Xa bound per centimeter of graft length.

There were no differences in FPA generation reflecting thrombin or factor Xa activities between proximal anastomotic, mid-graft, or distal anastomotic graft regions with preclotted polyester grafts implanted for 24 hours (Table II).

To determine the contribution of the preclotting process to graft-associated procoagulant activity, collagen-coated grafts were tested. These collagen-coated grafts were not preclotted and were studied before any blood exposure (n = 6), after blood exposure ex vivo for 1 minute (n = 6), or after implantation into the femoral arterial circulation for 24 hours (n = 12). Grafts not exposed to blood exhibited essentially no thrombin or factor Xa activity, reflected by FPA values of 83 ± 31 ng/ml and 318 ± 252 ng/ml, respectively (Table III). In contrast, collagen-coated grafts exposed to blood for as little as 1 minute exhibited significant graft-associated thrombin (1897 ± 42 ng/ml FPA) and factor Xa (2943 ± 399 ng/ml FPA) activity. Moreover, although the FPA generated by thrombin (4098 ± 554 ng/ml FPA) and factor Xa (3372 ± 748 ng/ml FPA) activities at 24 hours were higher than at 1 minute, the differences were not statistically significant. Significantly more thrombin activity was present on the luminal surface of the graft after either 24 hours of exposure to arterial blood flow in vivo ($p < 0.0001$) or after 1 minute of exposure to whole blood ($p < 0.0001$) than was present before implantation. Also, significantly increased factor Xa activity was measured after both 1 minute ($p = 0.0007$) or 24 hours ($p = 0.01$) compared with preimplantation factor Xa activity.

At 24 hours after implantation preclotted polyester grafts trended towards increased factor Xa activity compared with collagen-coated grafts, although the difference was not statistically significant (FPA values of 4815 ± 416 and 3372 ± 748 ng/ml FPA, respectively, $p = 0.08$). However, graft-associated thrombin activity was significantly lower on preclotted polyester grafts compared with collagen-coated polyester grafts (FPA values of 2134 ± 154 and 4098 ± 554 ng/ml, respectively; $p < 0.01$). Finally, preclotted polyester grafts immediately before implantation were not different from collagen-coated, nonpreclotted polyester grafts that had been exposed to circulating blood for only 1 minute ($p = 0.31$ and 0.65 for thrombin and factor Xa activities, respectively).

Table III. FPA (in ng/ml) generated by thrombin and factor Xa on collagen-coated grafts (not preclotted)

	0 hours	*1 minute*	*24 hours*
Thrombin	83 ± 31	1897 ± 42	4098 ± 554
Factor Xa	318 ± 252	2943 ± 399	3372 ± 748

Results are reported as mean ± SEM.

DISCUSSION

Efforts at biochemically rendering grafts less than thrombogenic have been hampered by a lack of knowledge about the relative contributions of the coagulation factors to graft thrombogenicity. Most previous studies dealing with graft thrombogenicity have focused on the role of platelets in this process.[7,8] Two previous reports in the literature have attempted to assess the contribution of coagulation factors to graft thrombogenicity.[9,10]

In a study of left ventricular assist devices Wagner et al.[9] detected minimal thrombin activity both at the time of implantation and removal of Dacron graft connections. The binding of other coagulation factors were not evaluated. The grafts studied were large-caliber, unlike the ones used for lower extremity revascularization.

Kelly et al.[10] characterized bound coagulation factors in a baboon model of exteriorized high-flow Dacron segments in chronic arteriovirus shunts. Chromogenic substrates were used to characterize thrombin and factor Xa activity. Factor Xa activity, although present initially, was not detectable 1 hour after graft interposition into the shunt. Bound thrombin activity was detected immediately and did not decrease significantly over a 24-hour period.

In this study we found significant and sustained thrombin and factor Xa activities at the time of implantation and after 24 hours. Our results may differ from those of Kelly et al. for several reasons. The chromogenic assay used by Kelly et al. to detect factor Xa activity may be less sensitive than the assay we used, which is based on measuring prothombin activation by the factor Xa/Va complex. Because of the amplification present at each step in the coagulation casacade, the technique used in our study enhances detection of factor Xa activity by elaboration of thrombin and generation of FPA. Because of this amplification small increases in factor Xa activity are reflected as large changes in FPA values (Fig. 1). In addition, the model we used has been reported to be significantly thrombogenic in other animals,[11] a fact possibly related to the small size of the grafted femoral artery or the significant outflow resistance of the lower extremity arterial bed. The model used by Kelly et al. involved high flow rates in an arteriovenous fistula and did not involve the creation of any surgical anastomosis at the time of graft interposition. We believe our model has a direct relevance to the human clinical situation, in which a distal prosthetic bypass graft is sutured to the donor and recipient vessels, graft flow rates and velocities are modest, and there is significant outflow resistance.

The deposition of thrombin and factor Xa activities on polyester grafts does not appear to be a unique consequence of the preclotting process, because collagen-coated grafts that were not preclotted exhibited similar procoagulant activity after even brief exposure to blood. The differences in thrombin activity observed at 24 hours between noncoated and collagen-coated grafts are of unclear biologic significance. Collagen-coated grafts at 24 hours exhibited higher levels of graft-associated thrombin activity than noncoated preclotted grafts at the same time point. This result could be related to additional coagulation factor or platelet activation by graft-bound collagen. However, the clinical importance of a modest increase in thrombin activity on collagen-coated grafts is uncertain, because these grafts are currently used only in large-diameter, high-flow arterial reconstructions. The observation that the proximal and distal segments contain only half the prosthetic graft length of the middle segment but generate equivalent amounts of FPA may be attributable to native vessel trauma and induction of procoagulant activity on the native vessel segment with anastomosis. Although direct comparisons are difficult because of differences in the models used, the amounts of thrombin and factor Xa activities seen in other studies[12,13] after vessel injury alone appear to be lower than those noted in this study.

Further studies are still required to determine thrombin and factor Xa activity after long-term graft implantation. A recent experimental study of Dacron grafts in the thoracic aorta of dogs suggested that gross thrombus formation on the luminal surface can continue to take place for years after implantation.[14]

Given our data it is no surprise that heparin does not passivate small-diameter prosthetic grafts, in view of its lack of effectiveness against clot-bound thrombin.[15] A variety of single agents have been studied in the experimental setting to decrease graft thrombogenicity, but none has achieved widespread clinical use. Our results suggest that a combination of anticoagulants targeting both factor Xa and thrombin perhaps in combination with antiplatelet agents might be required to effectively decrease the prothrombotic tendencies of small-diameter prosthetic grafts. The prothrombinase complex requires a phospholipid or platelet membrane to be active; thus inhibiting platelets may have the added benefit of further reducing thrombin and factor Xa activities. We have previously reported on the use of recombinant tissue factor pathway inhibitor, a natural inhibitor of tissue factor-VIIa and factor Xa, to decrease graft thrombogenicity in this model.[16] Although effective, recombinant tissue factor pathway inhibitor alone was not sufficient to achieve full passivation of grafts. A "cocktail" of inhibitors, for example hirudin (a thrombin inhibitor) plus tick anticoagulant peptide (a factor Xa inhibitor) plus a potent platelet inhibitor may be required to minimize graft thrombogenicity, although titrating combinations of drugs to achieve the desired therapeutic result while avoiding hemorrhage may prove challenging. Antithrombotic agents might also have a beneficial effect on the development of neointimal hyperplasia by inhibiting thrombin and factor Xa, both of which have been shown to be significant smooth muscle cell mitogens.[17,18] Indeed, when used after balloon angioplasty of atherosclerotic vessels, both thrombin and factor Xa inhibitors have been shown to reduce the restenosis rate.[19,20]

In summary, we have shown that thrombin and factor Xa are rapidly deposited on prosthetic grafts in biologically and clinically significant amounts and have demonstrated that procoagulant activity attributable to these factors is sustained for at least 24 hours.

REFERENCES

1. Veith FJ, Gupta SK, Ascer E, White-Flores S, Samson RH, Scher LA, et al. Six year prospective multicenter randomized

comparison of autologous saphenous vein and expanded polytetrafluoroethylene grafts in infrainguinal reconstructions. J Vasc Surg 1986;3:104-14.
2. Horbett TA. Principles underlying the role of adsorbed plasma proteins in blood interactions with foreign materials. Cardiovasc Pathol 1993;2(suppl):137S-148S.
3. Harker LA, Kelly AB, Hanson SR. Experimental arterial thrombosis in nonhuman primates. Circulation 1991; 83(suppl IV):41-55.
4. Nichter LS, Bindiger A. Improving microgratt patency. Microsurgery 1988;9:235-41.
5. Eisenberg PR, Siegel JE, Abendschein DR, Miletich JP. Importance of factor Xa in determining the procoagulant activity of whole blood clots. J Clin Invest 1993;91:1877-83.
6. Broze GJ Jr, Miletich JP. Human protein Z. J Clin Invest 1984;73:933-8.
7. Hamlin GW, Rajah SM, Crow MJ, Kester RC. Evaluation of the thrombogenic potential of three types of arterial graft studied in an artificial circulation. Br J Surg 1978;65:272-6.
8. Schneider PA, Kotzé HF, Du P, Heyns A, Hanson SR. Thromboembolic potential of synthetic vascular grafts in baboons. J Vasc Surg 1989;10:75-82.
9. Wagner WR, Johnson PC, Heil BV, Thompson KA, Kormos RL, Griffith BP. Thrombin activity resides on LVAD Dacron inflow and outflow grafts. ASAIO Journal 1992; 38:M634-7.
10. Kelly AB, Marzec U, White D, Hanson SR, Harker LA. The relative activity patterns of factor Xa and thrombin bound to arterial-type thrombus formed in vivo (abstract). Blood 1992; 80:324a.
11. Torem S, Schneider PA, Hanson SR. Monoclonal antibody-induced inhibition of platelet function: effects on hemostasis and vascular graft thrombosis in baboons. J Vasc Surg 1988; 7:172-89.
12. Dryjski M, Olsson P, Swedenborg J. Thrombin activity appearing on the vessel wall after trauma. Thromb Haemost 1985;54:773-5.
13. Ghigliotti G, Abendschein DR, Speidel CM, Eisenberg PR. Role of factor Xa/Va in the procoagulant activity on balloon-injured arteries (abstract). J Am Coll Cardiol 1996;272(Suppl A):64A.
14. Noishiki Y, Tomizawa Y, Yamane Y, Matsumoto A. The vicious cycle of nonhealing neointima in fabric vascular prostheses. Art Org 1995;19:7-16.
15. Weitz JI, Hudoba M, Massel D, Maraganore J, Hirsh J. Clot-bound thrombin is protected from inhibition by heparin-antithrombin III but is succetible to inactivation by antithrombin III-independent inhibitors. J Clin Invest 1990;86: 385-90.
16. Rubin BG, Toursarkissian B, Petrinec D, Yang LY, Eisengerg PR, Abendschein DR. Preincubation of Dacron graft with recombinant tissue factor pathway inhibitor decreases their thrombogenicity in vivo. J Vasc Surg 1996 (in press).
17. Fager G. Thrombin and proliferation of vascular smooth muscle cells. Circ Res 1995;77:645-50.
18. Gasic GP, Arenas CP, Gasic TB, Gasic GJ. Coagulation factors X, Xa, and protein S as potent mitogens for cultured aortic smooth muscle cells. Proc Natl Acad Sci U S A 1992; 89:2317-20.
19. Sarembock IJ, Gertz SD, Gimple LW, Owen RM, Powers ER, Roberts WC. Effectiveness of recombinant desulphatohirudin in reducing restenosis after ballon angioplasty of atherosclerotic femoral arteries in rabbits. Circulation 1991;84: 232-43.
20. Ragosta M, Gimple LW, Gertz SD, Dunwiddie CT, Vlasuk GP, Haber HL, et al. Specific factor Xa inhibition reduces restenosis after balloon angioplasty of atherosclerotic femoral arteries in rabbits. Circulation 1994;89:1262-71.

Submitted July 23, 1996; accepted Sep. 21, 1996.