Two-Phase Antithrombotic Protection After Anti-Glycoprotein VI Treatment in Mice

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Objective—Collagen and thrombin are the strongest physiological platelet agonists, acting through different receptors, among which glycoprotein VI (GPVI) and protease-activated receptors, respectively, are the essential ones. In mice, targeting of GPVI with the monoclonal antibody JAQ1 induces depletion of the receptor from circulating platelets, resulting in abolished collagen responses and long-lasting antithrombotic protection.

Methods and Results—Mice were treated with JAQ1, and the early effects of this treatment were analyzed. In addition to the known abolition of the collagen reactivity, this treatment also affected platelet response to thrombin but not other agonists. In platelets from JAQ1-treated mice, thrombin-induced activation of integrin αIIbβ3, the surface expression of P-selectin, and the procoagulant activity were decreased on days 1 and 2, then progressively recovered and returned to normal on day 5. In parallel, the mice were transiently protected from lethal tissue factor–induced pulmonary thromboembolism (100% survivors versus 40% in control group), which appeared to be based on a decreased generation and activity of intravascular thrombin.


Key Words: collagen ■ GPVI ■ platelet ■ receptor ■ thrombin ■ thrombosis
such therapeutic considerations. The currently recommended compounds to prevent ischemic complications after percutaneous coronary interventions are the low–molecular weight GPIIbIIIa antagonists eptifibatide (integritin) and tirofiban and the mouse/human chimeric anti-GPIIbIIIa Fab fragment abciximab. Among them, only abciximab has been shown to markedly reduce the procoagulant activity of platelets. However, the anti-GPIIbIIIa treatment is associated with a highly increased bleeding risk throughout the therapeutic use and a short half life of the therapeutic compound, requiring infusions or repetitive injections. Other anticoagulant agents directly (hirudin and its analoga) or indirectly (heparin) inhibit active plasma thrombin in acute situations. But so far, no treatment has been established that targets the platelet thrombin receptors in vivo.

In previous studies, we demonstrated that treatment of mice with the anti-GPVI monoclonal antibody (mAb) JAQ1 results in long-term antithrombotic protection by in vivo depletion of the receptor. Here, we report that platelets from JAQ1-treated mice show a transiently reduced response to thrombin in terms of integrin activation, secretion, and procoagulant activity. In vivo, these GPVI-depleted mice were protected against tissue factor–induced thromboembolism.

Materials and Methods

Animals

For all animal experiments, which were approved by the local animal care and use committee, male NMRI mice (Charles River; Sulzfeld, Germany) were used at the age of 6 to 8 weeks.

Antibodies and Chemicals

Human thromboplastin (Thromborel; Dade Behring) was reconstituted according to manufacturer instructions. ADP, apyrase, high-molecular weight heparin, and procacyclin were from Sigma, α-thrombin and annexin V-Fluos from Roche Diagnostics, and collagen reagent “Horn” from Nycomed. The PAR4-specific peptide GYPGQV was kindly provided by S. Offermanns (University of Heidelberg, Germany). JON/A-phycoerythrin (PE), which preferentially binds to activated integrin αIIbβ3, and anti–GPIIIb-IX-V, integrin α2β1, were from Emfret Analytics. All other antibodies were from previously described sources.

Platelet Preparation and Aggregometry

Mice were bled under ether anesthesia from the retro-orbital plexus. Blood was collected in a tube containing 10% (v/v) 10 U/mL heparin, and platelet-rich plasma was obtained by centrifugation at 300g for 10 minutes at room temperature (RT). To determine platelet aggregation, light transmission was recorded by use of platelet-rich plasma (200 μL with 0.5 × 106 platelets/μL) in a Fibrinitter 4-channel aggregometer (APACT Laborgeräte und Analysensysteme).

Flow Cytometry

Washed platelets were diluted in modified Tyrode’s–hepes buffer containing 1 mmol/L CaCl2, and samples (105 platelets) were stimulated at RT with thrombin (0.001 to 0.1 U/mL) for 2 minutes, ADP (5 μmol/L) for 1 minute, or PAR4-specific peptide (65 or 250 μmol/L) for 2 minutes. Subsequently, platelets were stained with fluorochrome-labeled mAbs or annexin V-Fluos at saturating concentrations for 10 minutes at 37°C and directly analyzed on a FACScalibur (Becton Dickinson). Platelets were identified by FCS/standard saline citrate characteristics.

In Vivo Experiments

Mice were anesthetized by intraperitoneal injection of ketamine/xylazine (100 g/kg ketamine, Parke-Davis; 5 mg/kg xylazine, Bayer AG). For determination of bleeding times, 3 mm of tail tip was amputated with a scalpel. The tail was then blotted with filter paper every 15 s until the paper was no longer blood stained. Where necessary, bleeding was manually stopped after 20 minutes. In vivo thrombosis was induced by intravenous injection of thromboplastin (150 μL/kg) into the retro-orbital plexus at the indicated day after intraperitoneal pretreatment of the mice with either anti-GPVI mAb JAQ1 or irrelevant control IgG (20 μg/mouse in sterile PBS each plus additional 100 μg/mouse after 3 hours). Mice that did not die within 30 minutes were counted as survivors.

Plasma Levels of Thrombin-Antithrombin III Complex and GPV

Blood (50 μL) was drawn from the retro-orbital plexus into Tris buffered saline containing 10 U/mL heparin and centrifuged (13 000g for 5 minutes at RT) to obtain platelet-poor plasma. Plasma concentrations of thrombin-antithrombin III (TAT) were determined using a commercial ELISA kit (Enzygnost TAT; Dade Behring) according to manufacturer instructions. GPV levels were determined by an ELISA system as described previously.

Results

Reduced Thrombin Response After Anti-GPVI Treatment With JAQ1

In previous studies, mice treated with a 100 μg intraperitoneal bolus injection of JAQ1 antibodies showed decreased platelet counts for up to 72 hours. To achieve a less pronounced thrombocytopenia, here we first injected 20 μg/mouse JAQ1 and then another 100 μg per mouse after 3 hours. This regimen caused a transient reduction in platelet count to 17.7 ± 2.2% after 30 minutes, which rapidly reversed to 43.2 ± 9.2% after 3 hours and 68 ± 8.3% after 24 hours and returned to normal counts after 48 hours (Figure 1a). As described previously, JAQ1 treatment induced a rapid depletion of GPVI from circulating platelets, as examined by flow cytometry and Western blot analysis (data not shown); applying anti-GPVI mAb JAQ2, GPVI expression was not detectable on circulating platelets for ≥14 days and gradually returned thereafter (data not shown). The depletion of GPVI was rather specific because the surface expression of other major platelet receptors such as GPIIbIIIa (integrin αIIbβ3), GPIIb-IX-V, integrin α2β1, and CD9 were virtually unchanged (data not shown). As expected, in the absence of GPVI surface expression, ex vivo activation of platelets with the GPVI-specific agonist collagen-related peptide was abolished as shown by flow cytometric analysis of surface P-selectin expression and activation of GPIIbIIIa using the specific JON/A-PE antibody (Figure 1b). Contrarily, platelet activation in response to increasing concentrations of ADP was unchanged as shown by aggregometry and flow cytometry on day 1 (Figure 1c) and days 2 through 5 after injection (data not shown). Comparable data were obtained when the stable thromboxane A2 (TXA2) analog U46619 was used (Figure 1d). It is important to note that U46619 alone does only induce low levels of integrin αIIbβ3 activation under the experimental conditions used for flow cytometry (ie, diluted samples). Therefore, the experiments were performed in the presence of 5 μmol/L epinephrine, which acts on the Gz-coupled α2A adrenergic receptor, and does not on its own...
induce platelet activation but strongly enhances responses to other agonists. Together, these results indicated that the signaling pathways initiated through the activation of these G-protein–coupled receptors are not defective in those platelets.

However, on stimulation with thrombin, surprisingly, an up to 80% decreased platelet activation response was observed, becoming most evident with low concentrations like 0.001 U/mL. Compared with control platelets, GPIIbIIIa activation was reduced on days 1 through 3 and returned to normal on day 5 (Figure 2a). Similar results were obtained when degranulation was assessed by measuring the surface expression of P-selectin, which was markedly diminished on day 1 and then stepwise, returned to control level on day 5 after antibody treatment (Figure 2b). Both sets of experiments were performed with thrombin concentrations from 0.003 to 0.1 U/mL with comparable outcome (data not shown). In Figure 2c, representative dot plots obtained on day 1 after JAQ1 treatment are shown.

To test whether the observed defect was based on reduced activity of PAR4 receptors, platelet responses to the PAR4-activating peptide GYPGQV were assessed. In response to 65 μmol/L PAR4 peptide, activation of GPIIbIIIa reached 55.9±18.8% and 76.2±23.8% of control level on days 1 and 2, respectively, with increasing activation levels on the following days (data not shown). The PAR4 defect became even more evident when the expression of P-selectin was determined. In response to 250 μmol/L PAR4 peptide, P-selectin surface expression reached only 38.0±4.2% and 58.5±9.2% of control on days 1 and 2, respectively. From day 3 on, P-selectin expression was comparable to controls (Figure 3a). Together, these results demonstrate that JAQ1 treatment not only results in depletion of the major collagen receptor but also evokes a transient defect in response to thrombin and PAR4 receptor–initiated signaling.

Because thrombin-induced platelet activation results in exposure of negatively charged PS, thereby inducing the formation of a procoagulant platelet surface, we were interested in whether PS exposure was altered after thrombin stimulation of platelets from JAQ1-treated mice (Figure 3b). In control platelets stimulated with 0.1 U/mL thrombin, binding of annexin V, which specifically recognizes surface-exposed PS, was detected in 30% of the platelets, whereas JAQ1-treated platelets exhibited a decreased PS exposure with 14.2±3.3%, 11.5±2.1%, and 16.0±4.6% annexin V–positive cells on days 1, 2, and 3, respectively. On day 5 after antibody treatment, annexin V binding (27.0±3.8%) almost returned to control level, indicating that the decrease in procoagulant platelet response follows a time course comparable to that found for reduced platelet response to thrombin.

In platelets, PAR receptors couple to Gq, G12/13, and Gi, and the activation of all 3 G-protein pathways is required for full cellular activation and secretion. Principally, the observed reduction in thrombin responses of platelets from JAQ1-treated mice could be based on a general reduction in PAR receptor activity or, alternatively, a selective defect in one of these downstream pathways. To discriminate between
these 2 possibilities, platelets from JAQ1-treated mice (day 1) were stimulated with thrombin in the presence or absence of specific activators of the Gq (5-hydroxytryptamine; 10 μmol/L), Gi (epinephrine; 10 μmol/L), or Gq/G12/13 (U46619; 1 μmol/L) pathways, and their activation was determined by flow cytometry. All of these agonists partly, but not fully, restored the defect in thrombin-induced P-selectin expression (Figure 3c) and GPIIbIIIa activation (data not shown) compared with control platelets, which were almost maximally activated by thrombin alone. These results show that none of the G-protein signaling pathways was maximally stimulated by thrombin in platelets from JAQ1-treated mice, suggesting a general reduction in PAR receptor activity rather than a selective defect in 1 pathway.

Figure 2. Altered thrombin-induced platelet activation after JAQ1 treatment. a through c, Washed platelets from both groups were stimulated at the indicated days after antibody injection with 0.001 to 0.1 U/mL thrombin and subsequently incubated with JON/A-PE or anti-P-selectin–FITC as indicated. Samples were analyzed by flow cytometry, and results (mean fluorescence intensity) are depicted as percentage of control (6 mice per group; a and b) or as representative dot plots from day 1 (c).

Figure 3. Reduced PAR4 activity after JAQ1 treatment. a, Washed platelets from both groups were stimulated at the indicated days after antibody injection of 250 μmol/L PAR4 peptide and subsequently incubated with JON/A-PE or anti-P-selectin–FITC as indicated. Samples were analyzed by flow cytometry, and results (mean fluorescence intensity) are depicted as percentage of control (ctrl; 6 mice per group). b, To determine exposure of PS, washed platelets were stimulated with 0.1 U/mL thrombin, subsequently incubated with annexin V–Fluos, and analyzed by flow cytometry. Shown are annexin V–binding cells±SD (n = 10). c, Washed platelets from control (black bars) or JAQ1-treated (gray bars) mice were stimulated on day 1 (d1) after antibody injection with 0.01 U/mL thrombin in the presence or absence of the indicated agonists and subsequently incubated with anti-P-selectin–FITC. 5-HT indicates 5-hydroxytryptamine. Samples were analyzed by flow cytometry, and results are shown as mean fluorescence intensity (mean±SD; 3 mice per group). Similar results were obtained when GPIIbIIIa activation was determined (data not shown).
To test whether the observed changes in platelet responses also affect hemostasis, the tail bleeding times of JAQ1-treated mice were determined on days 1, 3, and 5 after antibody injection (Figure 4). In correlation with the reduction in thrombin responses at early time points, mice on day 1 consistently displayed bleeding times ≥1200 s (untreated controls 302±113 s), whereas on day 3, bleeding times were moderately increased (718±172 s) and with 343±131 s, comparable to control on day 5, the latter confirming previous results.\(^\text{14}\)

To test whether the impairment of thrombin responses in platelets of JAQ1-treated mice was a GPVI-specific effect or mediated through other antibody-induced mechanisms, mice were injected with isotype-matched antibodies against GPV (DOM2; rat IgG2a) and GPIIIa (EDL1; rat IgG2a), and platelets were analyzed at different time points. GPVI expression and thrombin reactivity were not significantly altered at any tested time point (data not shown). We have shown previously that JAQ1 does not trigger downregulation of the receptor from the platelet surface in vitro.\(^\text{14,19}\) To test whether the thrombin activation defect can be induced by JAQ1 independently of GPVI downregulation, we incubated platelets with increasing concentrations of the antibody for 30 minutes and then stimulated the cells with thrombin (0.001 to 0.1 U/mL). JAQ1 had no significant effect on thrombin generation and P-selectin expression (data not shown). Together, these results show that the transient thrombin activation defect is a GPVI-specific effect and coincides with the downregulation of the receptor.

**Protection From Tissue Factor–Induced Thromboembolism**

The marked hemostatic defect at early time points after JAQ1 treatment suggested that the impaired thrombin-induced platelet activation and coagulant activity measured in vitro translates into a defective feedback loop of thrombin generation in vivo, as discussed previously for PAR4-deficient mice.\(^\text{7}\) To test this hypothesis, JAQ1-treated mice were subjected to a model of tissue factor–induced, thrombin-dependent, lethal thromboembolism (Figure 5a). For this, anesthetized control and JAQ1-treated mice were intravenously injected with 150 μL/kg of TP, which resulted in 60% lethality within 5 minutes in the control group pretreated with irrelevant IgG. In contrast, mice pretreated with JAQ1 1 or 2 days before TP challenge were completely protected from lethal pulmonary thromboembolism (100% survivors). As seen with the transiently defective thrombin response (Figure 2), the protective effect declined progressively and reached control level on day 5 (Figure 5a). In a parallel set of experiments, platelet counts were determined 1 minute after TP challenge in control IgG and JAQ1-treated mice (Figure 5b). In the control group, platelet counts sharply decreased from 1.05±0.15×10^6/μL to 0.16±0.1×10^6/μL, whereas the drop was markedly attenuated in JAQ1-treated mice on day 1 (0.81±0.13×10^6/μL to 0.7±0.26×10^6/μL) and day 2 (1.11±0.12 to 0.75±0.25×10^6/μL). These results suggested that the protection seen in JAQ1-treated mice was based on a selective defect in tissue factor–induced activation of circulating platelets but not on reduced platelet counts before challenge. To additionally assess the generation and proteolytic activity of thrombin, plasma levels of TAT and GPV, respectively, were determined 1 minute after TP injection. GPV (88 kDa) is noncovalently associated with GPIb-IX in the platelet membrane and efficiently cleaved by thrombin to release a 69-kDa fragment (GPVf1) that can be quantitated in plasma and may serve as an indirect measure of intravascular thrombin activity.\(^\text{21}\) In mice that had received JAQ1 1 or 2 days before TP injection, the TAT concentration was markedly reduced compared with TP-challenged control mice (Figure 5c), indicating a substantially lower generation of thrombin. A similar pattern was found for the plasma levels of GPV as determined by ELISA.\(^\text{22}\) The basal plasma level of GPV was 5-fold increased in TP-treated control mice, whereas in JAQ1-treated mice on days 1 and 2, the GPV levels were found to be only 2- to 3-fold, and they increased on days 3 through 5 to the levels observed in control mice (Figure 5d). Both findings demonstrated a markedly reduced

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**Figure 4.** Transient defect in hemostasis. Tail bleeding times of JAQ1-treated mice were determined at the indicated days (day 1 [d1], d3, and d5) after antibody injection. The experiment was stopped manually after 20 minutes. Each symbol represents 1 individual. Bleeding times on d1 and d3 are significantly (P<0.001) increased compared with the control.

**Figure 5.** Decreased tissue factor–induced thromboembolism. Mice treated with control IgG or JAQ1 were challenged by an intravenous bolus injection of TP (150 μL/kg) at the indicated time points. a, Shown are survival rates with numbers of participating mice given in brackets. b, Platelet counts were determined in diluted whole blood 1 minute after TP challenge. c and d, Plasma concentration of TAT complexes (c) and plasma levels of GPV (d) were determined in platelet-poor plasma obtained 1 minute after injection of TP. Results are expressed as mean±SD (n=12 to 20 per group).
presence of thrombin within the vasculature on TP injection at the early time points after JAQ1 treatment.

Discussion
Previously, we demonstrated that treatment of mice with an mAb directed against the collagen receptor GPVI (JAQ1) resulted in depletion of GPVI from circulating platelets. In the absence of GPVI, platelets were unresponsive to collagen, and mice were protected against collagen/epinephrine-induced thromboembolism.24

In the current study, the influence of JAQ1 treatment on thrombin-mediated platelet responses 1 to 5 days after antibody injection was investigated in vitro and in vivo. Platelet activation was markedly affected on days 1 through 3, as demonstrated by reduced αIbβ3 activation and P-selectin expression in response to this agonist (Figure 2a through 2c).

Importantly, this defect was selective for thrombin because responses to ADP and the TxA2 analog U46619 were normal at every tested time point after JAQ1 treatment (Figure 1).14 This suggests that a reduced expression or function of ≥1 thrombin receptor(s) rather than a general failure in G-protein–mediated signaling pathways is responsible for the observed defect. This assumption is confirmed by the diminished activity of the PAR4 receptor at early time points after JAQ1 treatment, as assessed with a PAR4-activating peptide (Figure 3a). In addition to the PAR receptors, thrombin also binds to GP Ibα on platelets,23 and this interaction is of major importance for thrombin-induced activation and procoagulant activity of the cells.24 Although we found no significant effect of JAQ1 treatment on GP Ibα surface levels at any time point, we cannot totally exclude the possibility that the antibody induced a functional defect in this receptor, especially, as Arthur et al25 recently demonstrated, an association between GPVI and GPIb on the platelet surface.

Although our results demonstrate that JAQ1 treatment selectively affects thrombin responses in platelets (at least partly) through an effect on PAR4, we were unable to assess this effect directly because no functional antibodies or other tools are available at present to analyze mouse PAR4. Our attempts to detect the receptor in mouse platelets by immunoprecipitation or Western blot analysis with different commercially available polyclonal rabbit anti-PAR4 antibodies failed (data not shown). Therefore, it was not possible to determine whether the reduced PAR4 activity is based on changes in the expression levels of the receptor (eg, by proteolytic cleavage or internalization) or other mechanisms. It will be important to generate high-affinity antibodies against mouse PAR4 to address this question directly.

In parallel with the reduction in platelet activation, the thrombin-induced surface exposure of procoagulant PS was decreased (Figure 3b). The in vivo significance of this result was then confirmed in a model of acute tissue factor–induced thromboembolism. Here, injection of TP, which mimics intravascularly exposed tissue factor, triggers the coagulation cascade finally resulting in thrombin generation, platelet consumption, and lethal pulmonary thromboembolism.26 On challenge with TP, JAQ1-treated mice were protected at the before-mentioned early time points (Figure 5a), demonstrating that the residual platelet thrombin response and procoagulant activity found ex vivo were not sufficient to induce platelet aggregation and consequent fatal pulmonary thromboembolism in vivo. We cannot definitively rule out that the observed protection on day 1 was in part attributable to a reduced platelet count, but the fact that counts were normal on day 2 (Figure 1a) and at the same time survival was still 100% (Figure 5a) suggests a mechanism independent of platelet numbers. The reduced TAT and GPV plasma levels in those mice (Figure 5c and 5d) revealed a markedly reduced concentration and activity of thrombin after TP injection, respectively. These results show that a partial defect in thrombin-induced activation responses in platelets can result in a markedly reduced thrombotic activity in vivo. This is in line with studies in mice deficient in the ADP receptor P2Y1, in which platelet activation in response to thrombin is not markedly decreased/affected, whereas they are largely protected from tissue factor–induced thromboembolism.27 Similarly, mice deficient in Gr13, which show only partial defects in response to thrombin and TxA2, are profoundly protected from injury-induced arterial thrombosis and have massively prolonged bleeding times.28 A comparable bleeding time prolongation can also be observed in JAQ1-treated mice at early time points after antibody injection, which parallels the defect in thrombin responses and is presumably related to it. Deficiency in the GPVI/Fc receptor–chain complex only results in a very mild bleeding tendency in humans and mice,14,29–31 showing that alternative agonist-induced pathways must be operative to activate platelets during hemostasis. We recently provided evidence that TxA2 is one of these agonists,32 and the data shown here indicate that thrombin is another one. In fact, it may not be surprising that hemostasis is severely impaired in individuals with absent/reduced platelet responses to the 2 most potent physiological agonists: collagen and thrombin, respectively. However, it is important to note that there is no clear correlation between the bleeding time and bleeding risk,33 making it difficult to predict the actual risk of clinical hemorrhage in anti-GPVI–treated humans.

The lifespan of circulating platelets in mice is ~5 days,34 which correlates well with the time required for normalization of the thrombin activation defect and the protection from tissue factor–induced thromboembolism in JAQ1–treated mice. In contrast, the antibody-induced GPVI deficiency lasts ≥2 weeks (ie, 3 generations of platelets).14,19 This strongly suggests that the mechanisms by which JAQ1 induces GPVI downregulation on platelets circulating in the blood stream at the time of antibody injection and those being generated later must be different. Recent evidence suggests that there could indeed be different pathways to downregulate GPVI. We have shown that in circulating platelets, the GPVI/JAQ1 complex can, at least in part, become internalized and degraded.14 Subsequently, different groups demonstrated that GPVI can also be cleaved from the surface of human and mouse platelets by a metalloproteinase–dependent mechanism in vitro,35,36 indicating that such a mechanism could also contribute to GPVI downregulation in vivo. Because JAQ1 not only binds to circulating platelets in mice but also to bone marrow and splenic megakaryocytes,14 it appears likely that downregulation of the receptor also occurs there, which
would explain the long-lasting GPVI deficiency in antibody-treated animals. Based on this assumption, one might speculate that JAQ1-induced downregulation of GPVII in megakaryocytes does not affect the activity of thrombin receptors, whereas it does so in circulating platelets. But clearly, the mechanistic basis for this difference is unclear at present, and further studies will be required to test this hypothesis.

GPVI-deficient patients with highly specific antibodies against the apparently absent receptor have been reported who do not experience major bleeding, and their platelets display normal responses to agonists other than collagen, including thrombin. This shows that the long-term exposure of humans to anti-GPVI antibodies, and presumably also other to anti-GPVI agents, produces a phenotype similar to that observed in JAQ1-treated mice on day 5 or later. However, the data presented here indicate that the first treatment of a patient with a GPVI-depleting agent may transiently result in very strong platelet inhibition, which then gradually turns into a partial (collagen-specific) but long-lasting antithrombotic effect. Although one may speculate that such a “2-phase” antithrombotic therapy might be advantageous in the prevention or treatment of acute cardiovascular events, it may not be desired for other patients. However, under any condition, it will be important to avoid the transient but severe drop in platelet counts in anti-GPVI-treated individuals. Therefore, further investigations are needed to identify the mechanisms underlying the transient thrombin activation defect and thrombocytopenia in anti-GPVI–treated mice and to assess the relevance of these findings for the treatment of humans. The latter is particularly important because in human platelets, PAR1 (which is not expressed in mouse platelets) but not PAR4 is the major thrombin receptor, making it difficult to extrapolate the data obtained in mice to the human situation.

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Disclosures

None.

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