

Detection of Ca^{2+} -dependent acid phosphatase activity identifies neuronal integrity in damaged rat central nervous system after application of bacterial melanin

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Abstract

The study aims to confirm the neuroregenerative effects of bacterial melanin (BM) on central nervous system injury using a special staining method based on the detection of Ca^{2+} -dependent acid phosphatase activity. Twenty-four rats were randomly assigned to undergo either unilateral destruction of sensorimotor cortex (group I; $n = 12$) or unilateral rubrospinal tract transection at the cervical level (C_{3-4}) (group II; $n = 12$). In each group, six rats were randomly selected after surgery to undergo intramuscular injection of BM solution (BM subgroup) and the remaining six rats were intramuscularly injected with saline (saline subgroup). Neurological testing confirmed that BM accelerated the recovery of motor function in rats from both BM and saline subgroups. Two months after surgery, Ca^{2+} -dependent acid phosphatase activity detection in combination with Chilingarian's calcium adenoside triphosphate method revealed that BM stimulated the sprouting of fibers and dilated the capillaries in the brain and spinal cord. These results suggest that BM can promote the recovery of motor function of rats with central nervous system injury; and detection of Ca^{2+} -dependent acid phosphatase activity is a fast and easy method used to study the regeneration-promoting effects of BM on the injured central nervous system.

Key Words: nerve regeneration; bacterial melanin; histochemical analysis; rubrospinal tract; sensorimotor cortex; Ca^{2+} -dependent acid phosphatase activity; rats; neural regeneration

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Introduction

Novel therapeutic strategies for neurodegenerative diseases mainly focus on preservation of neurons and suppression of microgliosis, inflammation, and oxidative damage. Neurobiologists have investigated the possibilities of applying physiologically active compounds to regulate the cascade of processes involved in nervous tissue regeneration (Brossmer and Schwab, 1996; Huebner and Strittmatter, 2009; Fakhoury, 2015). Different approaches have been used to test neuronal viability after injury or in the state of neurodegeneration and reveal axonal sprouting and the severity of gliosis. Various histochemical methods have been used in neuroscience to stain neurons, neuronal processes and neuroglia *in vitro* (Pilati et al., 2008). The Golgi's method, the most widely used silver staining technique, provides detailed information on neuronal morphology, but stains the neurons selectively with the majority of nerve cells unstained (Melendez-Ferro et al., 2007). Several modifications of the method have been introduced including alterations in solution composition and pH value (Van der Loos, 1956; Bertram and Ihrig, 1957; Morest and Morest, 1966; Stensaas, 1975; Gonzalez-Burgos et al., 1992; Angulo et al., 1994), replacement of embedding media (Blackstad

et al., 1984; Kolodziejczyk et al., 1990), use of microwaves (Armstrong and Parker, 1986; Berbel, 1986; Zhang et al., 2010) and vibratome, coating of brain blocks with egg yolk (Zhang et al., 2010), application of vacuum environment, and variation in incubation temperature (Angulo et al., 1994). The above mentioned modifications aimed to shorten the time required for the procedure, reduce precipitations, and result in a clear background and uniform impregnation and uptake of the Golgi stain in the nervous tissue. After long-term fixation, the nervous tissue gets brittle and sectioning is difficult. The deeply stained blood vessels appear with background interfered in deciphering the neuronal structures. In the present work, we applied a combination of two histochemical methods (Chilingaryan, 1986; Meliksetyan, 2007), which considerably shortens the tissue processing time and provides a clear picture of nervous tissue architectonics and well defined pattern of vascular network. The Nissl staining method, as another staining method for neurons, can label all neurons in the section but gives a very poor picture of neuronal morphology (Glaser and Van der Loos, 1981). Melanins are being actively studied and applied as medicinal preparations. Melanin metabolism disorders are involved in the etiology of such

diseases as parkinsonism, senile macular degeneration, and senile deafness (Zecca, 2002). *Bacillus Thuringiensis*, a melanin-synthesizing bacterial stain, with a high level of pigment synthesis, was obtained by Institute of Biotechnology in Armenia (Popov, 2003; Azaryan et al., 2004; Aghajanyan et al., 2005). BM has been shown to facilitate the recovery of instrumental conditioned reflexes and paralyzed limb movements after unilateral destruction of substantia nigra pars compacta that had caused paresis of limbs in animal models of traumatic neurodegeneration (Petrosyan et al., 2014). There is evidence that BM can stimulate neuroregeneration after destruction of dopaminergic cells in substantia nigra pars compacta, suppress neuroinflammation, do not activate microglia, but increase capillary blood flow in the brain tissue (Petrosyan et al., 2014). Biotechnologically obtained water-soluble BM could be a potential biologic medical product for the treatment of neurodegenerative disorders (Parkinson's disease). Our previous studies have confirmed that BM promotes regeneration in damaged brain area, increases vascularization, dilates capillaries, stimulates axonal growth in damaged neurons and supports viability of cells after lesion (Gevorkyan et al., 2007a, b; Petrosyan et al., 2012, 2014). We have applied detection of Ca^{2+} -dependent acid phosphatase activity as a staining method for the central nervous system (CNS) tissue slices. The method provides overall labeling of neurons including the soma and processes of the cells. The method is very suitable for testing the viability of nerve cells in damaged CNS tissue or after neurodegeneration in different animal models. In the current study, we used a combination of detection of Ca^{2+} -dependent acid phosphatase activity and Chilingarian's calcium adenosine triphosphate method to study brain microvasculature. The histoangiological method provides a clear picture of vascular changes induced by the damage or severe hypoxia in animal brains. The purpose of the present study was (1) to show the effectiveness of Ca^{2+} -dependent acid phosphatase activity detection in analyzing the viability of neurons after brain lesion and (2) to confirm the effects of BM on axonal sprouting and neurologic function after destruction of sensorimotor cortex or transection of rubrospinal tract.

Material and Methods

Animals

Twenty-four experimental naïve Wistar male rats, aged 3–6 months, weighing 180–250 g, were included in this study. They were maintained on a standard light-dark cycle with food and water available *ad libitum* and cared and used in accordance with institutional guidelines and national and international laws and policies (EEC Council Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes). Rats were randomly allocated to undergo either unilateral destruction of sensorimotor cortex (group I, $n = 12$) or unilateral destruction of rubrospinal tract (group II, $n = 12$). In each group, six rats were randomly selected to undergo intramuscular injection of BM solu-

tion (BM subgroup) and the remaining six rats underwent intramuscular injection of saline and served as a control (saline subgroup). All efforts were made to minimize the number of animals used in this study and their suffering. The study was approved by the ethics committee of the Armenian National Academy of Science.

Surgical procedures

Destruction of sensorimotor cortex

Following anesthesia with Nembutal (40 mg/kg, intraperitoneal), unilateral ablation of sensorimotor cortex of the left hemisphere was performed in all rats. After craniotomy, a surface area, 2 mm lateral to, 3 mm caudal to, and 3 mm lateral to the "0" line of the coronal suture (bregma) was exposed and removed by suction through a fine glass pipette to the level of the white matter (Paxinos and Watson, 2005).

Transection of rubrospinal tract

Unilateral destruction of rubrospinal tract was performed at the C_{3-4} level (Gwyn, 1971; Murray and Gurule, 1979). A thin injection needle was fixed to the adjustable and movable holder of stereotaxic apparatus. After removing bones of the C_{3-4} cervical vertebrae, spinal cord was exposed and a needle was used to destruct the motor tract (Paxinos and Watson, 2005). The holder with the needle was positioned parallel to the posterior spinal artery. The dura mater under the needle was incised and the needle was shifted 1 mm to the left. Then the needle was moved down vertically for 1 mm, inserting it into the rubrospinal tract. A stereotaxy screw was used to help shift the needle to right for 0.5 mm and then back to the left, thus the soft, flexible fibers of rubrospinal tract were cut (Paxinos and Watson, 2005).

BM injection

Rats in the BM subgroup were administered BM solution (6 mg/mL, 170 mg/kg, intramuscular). The saline group rats identically underwent equal volumes of saline. All intramuscular injections were performed in femoral region on the second day after the operation.

Morphohistochemical study

Two months after injury, *i.e.*, when rat motor function was completely recovered, all rats were decapitated under deep anesthesia with Nembutal (45–50 mg/kg) for morphological study of spinal cord section. The brains were removed and then fixed in 5% neutral buffered formalin (phosphate buffer pH 7.4). Sections, 50–60 μ m thick, were obtained for microscopy. The morphofunctional state of cellular structures in the midbrain was assessed by histochemical and histoangiological studies. A histoangiological method was used to identify the microcirculatory bed (Chilingaryan, 1986) and a modified histochemical method was used to identify acid phosphatase activity (Meliksetyan, 2007), providing not only a clear morphological picture, but also an assessment of the integrity of neurons.

The Meliksetyan's method was used to detect Ca^{2+} -depen-

dent acid phosphatase activity in the CNS tissue (Meliksetyan, 2007). A mixture, containing 20 mL of 0.38% lead acetate solution, 5 mL of acetate buffer (pH 5.6), and 5 mL of 2% β sodium glycerophosphate solution, was prepared, adjusted with 3% solution of calcium chloride (non-fused) to the 100 mL and then filtered. Brain tissue sections were transferred into the prepared mixture, incubated in a thermostat at 37°C for 1–3 hours, rinsed with distilled water 3–5 times for 5 minutes each, developed in the sodium sulphide solution, washed repeatedly, and finally mounted in the balsam.

The Chilingaryan's calcium adenosine triphosphate method was applied to study brain microvasculature (Chilingaryan, 1986). The method is based on the selective deposition of phosphorus, cleaved from adenosine triphosphate by calcium ions and subsequently the reaction product is converted to black lead sulphide. The method provides clear, high-contrast selective detection of vascular and capillary network.

The mounted brain tissue sections were placed in saline and then transferred to the pre-incubation mixture containing 4 mL of 4 M 25% ammonia solution, 2 mL of 0.1 M calcium chloride solution, 2 mL of adenosine triphosphate solution (0.1 M freshly prepared solution of 5-disodium-adenosine-triphosphoric acid – “Reanal” company) and 12 mL of distilled water. Then they were incubated in the final volume for 30 minutes to 20 hours, rinsed in distilled water three times for 2–5 minutes each, immersed in a lead-replacing mixture (100 μ L of acetic acid was added to 100 mL of distilled water, then 2 g chemically pure lead acetate was dissolved in the same mixture, and 10 mL of 1 M acetate buffer (pH 6.2) and 15 mL of 8% solution of ammonium acetate were added. The mixture is stable and can be used repeatedly) for 10 minutes to 1 hour, rinsed in distilled water three times for 2–5 minutes each, immersed in a 20% solution of ammonium acetate for several minutes to 2 hours depending on the thickness of blocks, rinsed in distilled water three times for 2–5 minutes each, immersed in 2–5% solution of sodium sulfide for 5–10 minutes, rinsed in distilled water five times for 20 minutes to 1 hour each, mounted in Canada balsam, and finally observed using Carl Zeiss Microscope OPTON (Switzerland).

Neurological function assessment

Neurological examinations using a 6-point scale were performed every 2 days in all rats during 28 days of experiment. The detailed scoring of the 6-point scale is shown as follows (Li and Schluesener, 2006): 0: no neurologic deficit; 1, failure to extend left forepaw completely, indicating mild focal neurologic deficit; 2, circling to the left, indicative of a moderate focal neurologic deficit; 3, falling to the left, suggesting a severe focal neurological deficit; 4, cannot walk spontaneously and have a decreased level of consciousness; 5, death due to brain ischemia.

Statistical analysis

All data were statistically analyzed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA). Stu-

dent's *t*-test was used to assess the significant differences in morphometric data and neurologic function scores. $P < 0.05$ was considered statistically significant.

Results

Neurological testing scores

After unilateral destruction of sensorimotor cortex (left hemisphere), both saline- and BM-injected rats showed a deficit in motor function, presenting with paresis of the right hindlimb. Rat neurological function was assessed daily for 28 days. After 28 days, the neurologic test score was 2.5 ± 0.48 for the saline group and it was -1.3 ± 0.19 for the BM group, and there was significant difference between these two groups ($P = 0.0002$; **Figure 1**).

Results of histochemical study

Morphological study was conducted in all subgroups 2 months after the operation. All rats were decapitated under deep anesthesia. The location, extent, and depth of sensorimotor cortex lesions were evaluated in all rats. Brain sections from saline-injected rats (**Figure 2**) clearly showed the edges of destructed cortex area due to the formation of connective tissue scar. Scarring represents a powerful barrier for the migration of nerve and glial elements and is a blocking factor of axon growth pathways. Brain sections from BM-injected rats (**Figure 2B** and **D**) showed approximation of the lesion margins and fusion even appeared in some sections. The extent of damaged area with connective tissue infiltration differed significantly between BM and saline groups. There were more glial cell nuclei and fewer macrophage nuclei in the perilesional area in BM-injected rats than in saline-injected rats.

The microcirculatory bed examination revealed an increase in the degree of vascularization, which results from dilation of vessels, in the brains of both saline- and BM-injected rats. Morphometric study was performed in the sensorimotor area stained with Chilingaryan's method. The mean vascular diameter across 900 capillaries per section was $5.8 \pm 0.18 \mu\text{m}$ in saline-injected rats, while it was $6.3 \pm 0.16 \mu\text{m}$ in BM-injected rats. The mean vascular diameter in the BM-injected rats was increased by 8.7% compared to that in the saline-injected rats.

After unilateral destruction of rubrospinal tract, no matter rats treated with BM solution or those not treated with BM solution, exhibited a deficit in motor function as evidenced by paresis of the unilateral hindlimb. For 28 days after destruction, rat neurological deficit was daily evaluated, and there was significant difference in rat neurological score between BM- and saline-injected groups (2.11 ± 0.18 vs. 3.81 ± 0.44). For morphohistochemical study, longitudinal spinal cord sections were prepared (**Figures 3, 4**) to identify the course of damaged motor tract fibers. Progressive proliferation of glial cell nuclei and complete demyelination were revealed in sections harvested from saline-injected rats. The nerve cells on sections harvested from saline-injected rats were swollen with nuclei and axons not identified (**Figure 4A**).

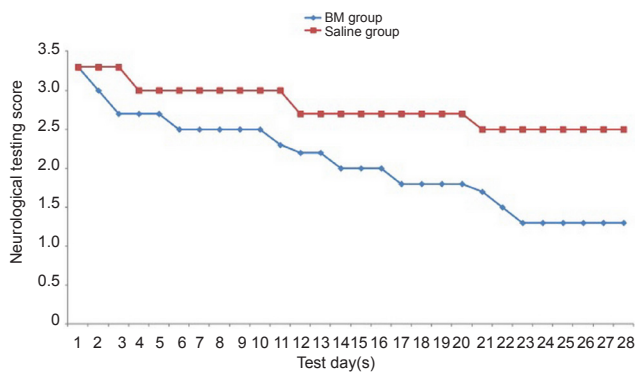


Figure 1 Neurological testing results for rats in the bacterial melanin (BM) and saline groups.

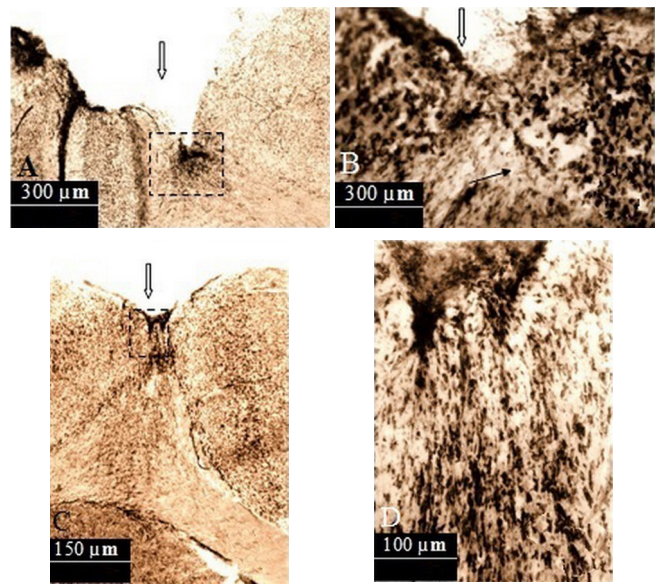


Figure 2 The destroyed area of rat sensorimotor cortex.

(A, C) The destroyed sensorimotor cortex area (white arrow) in saline-injected rats. (B, D) The destroyed sensorimotor cortex area (white arrow) in rats injected with bacterial melanin solution (6 mg/mL). The destroyed area is filled with cells and neuronal staining is more intensive (detection of Ca^{2+} -dependent acid phosphatase activity; Carl Zeiss Microscope OPTON, Switzerland).

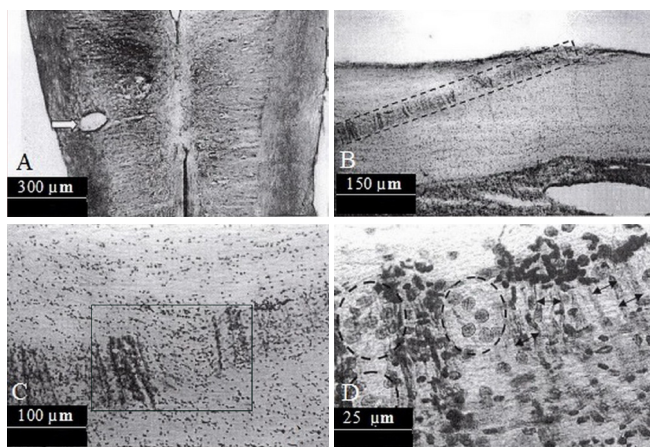


Figure 3 Longitudinal spinal cord sections harvested from saline (A)- and bacterial melanin (6 mg/mL, intramuscular)-injected rats (B, C) subjected to rubrospinal tract transection.

The white arrow in A indicates transection area and the dotted lines in B include the destroyed zone. (D) An enlarged fragment of the boxed area in C. The granular bodies found in the sections contain cell detritus (shown by circles) are identified, black arrows in this section indicate tract fibers (detection of Ca^{2+} -dependent acid phosphatase activity; Carl Zeiss Microscope OPTON, Switzerland).

In sections of BM-injected rats, a typical pattern, *i.e.*, absence of glial scar, was observed (Figure 3B, C). In transection area, empty space was revealed with a moderate proliferation of glial cell nuclei that have high enzymatic activity (Figure 4C, D). In the damaged area, regenerating fibers of transected axons were identified (Figure 4B, F, G). The shape of neurons, proximal to damaged axons, was preserved. The nuclei in the majority of neurons were not stained, granular precipitate was found in the cytoplasm containing cell debris, and the dendrites were not altered. The neuronal changes revealed in the sections from saline-injected rats could be described as central chromatolysis. In some sections of saline-injected rats, the remnants of neurons were not revealed. In spinal cord sections of BM-treated rats, increased vascularization was clearly identified in damaged area (Figure 4E), which provides with improved trophic support of nervous tissue, regeneration and suppression of the astroglial scar formation. The method also revealed intensity of collateral sprouting, proliferation, differentiation and formation of new cell elements.

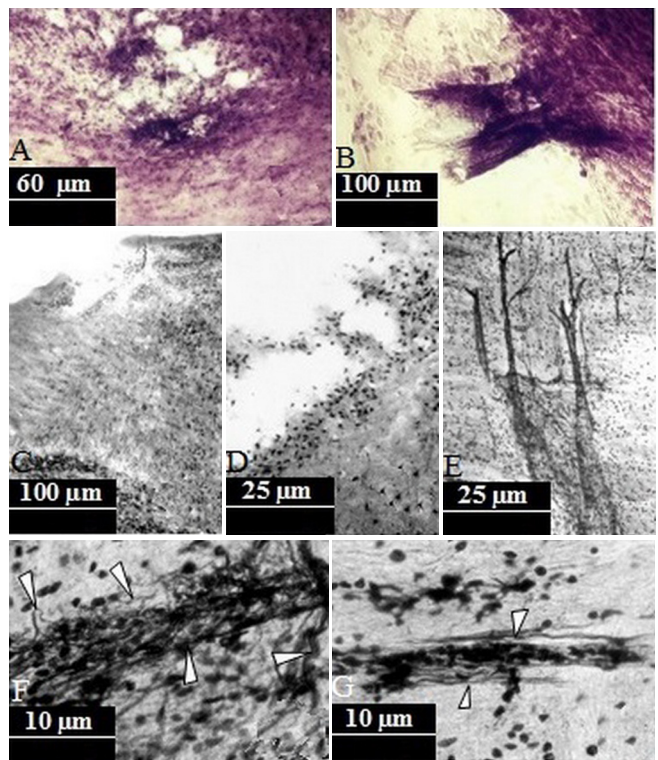


Figure 4 Longitudinal spinal cord sections prepared 2 months after rubrospinal tract transection harvested from saline (A, C, D) - and bacterial melanin (intramuscular, 6 mg/mL; B, E, F, G) -injected rats. (C, D) There was no intensive sprouting. (E) Dilated blood vessels were identified in spinal cord section of damaged area. (F, G) White arrows indicate regenerating fibers of damaged motor tract (detection of Ca^{2+} -dependent acid phosphatase activity; Carl Zeiss Microscope OPTON, Switzerland).

Discussion

Different doses of BM have been tested by us in previous series of experiments (Gevorkyan et al., 2007a). The dose 6 mg/mL calculated as 170 mg/kg was confirmed to be the most effective. BM at 6 mg/mL exhibited more favorable and highly expressed effects on nerve regeneration, sprouting, and functional recovery than BM at higher doses. BM at 6 mg/mL does not produce any toxic effect or cause microgliosis that initiates neuronal destruction. Injection of BM on the next day after surgery is an attempt to eliminate motor deficit and restore motor function in rats. BM supports the viability of neurons in the CNS after destruction. A large number of preserved neuronal bodies was revealed in spinal cord sections of rats injected with BM solution after sensorimotor cortex destruction. BM has been reportedly to promote the sprouting of nerve fibers in a series of experiments conducted in rats (Petrosyan et al., 2012).

In the present study, postoperative histochemical results regarding BM efficacy revealed great approximation of lesion area margins and absence of glial cell nuclei proliferation. These effects were not observed in spinal cord sections of saline-injected rats. There was considerable difference in the degree of glial cell infiltration in the damaged area between saline- and BM-injected groups. The glial scar was not observed in sections from BM-injected rats and the viability of neurons surrounding the damaged area was significantly higher in BM-injected rats than in saline-injected rats. The neuroprotective mechanism of BM is still unknown, and further studies involving molecular methods are needed to clarify the effects induced by BM.

The Golgi method for the study of nervous tissue has been used in a review by García-López et al. (2007). Meliksetyan's method is suitable to study the morphology of neurons, glial cells and neuronal processes in brain slices. The method is a fast and easy tool to study CNS pathology, and the functional status of brain structures, nerve fiber, or axonal sprouting. In the pathogenesis of neurodegenerative diseases, such as Parkinson's disease or Alzheimer's disease, the dendritic spines are largely affected in initial stages of the pathology (Fiala et al., 2002; Hill et al., 2006; Baloyannis et al., 2007). The Meliksetyan's method helps to clearly visualize changes in the number, size and shape of neuronal processes. The major disadvantage of the Golgi method is that the long period of fixation turns the tissue brittle creating problems in sectioning (Adams, 1979; Grandin et al., 1988; Melen-dez-Ferro et al., 2009; Ranjan and Mallick, 2010). The method of Ca^{2+} -dependent acid phosphatase activity detection allows differentiation of stained neuronal elements from other surrounding tissues and vessels. The short fixation time of this method reduces tissue shrinkage and makes sectioning easy and the staining of fine branches of neuronal processes possible. The described methodology needs no cryopreservation. The presented method is simple and needs no special instrumentations. In the present study, we also used the Chlingarian's method to evaluate microcirculation and diameter

change of blood vessels. Application of two different methods to assess nervous tissue and microcirculation separately allows for an effective evaluation on the influence of neuroprotective substances on regeneration process after damage. These methods can be effectively applied in different models that are aimed to study the neurotrophic action of various agents.

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Author contributions: *AST designed this study. TRP performed experiments, analyzed the data and drafted the paper. ASH provided technical and material support. All authors approved the final version of this paper.*

Conflicts of interest: *None declared.*

Plagiarism check: *This paper was screened twice using Cross-Check to verify originality before publication.*

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