Quantification of Human Cytomegalovirus DNA by a New Capture Hybrid Polymerase Chain Reaction Enzyme-Linked Immunosorbent Assay in Plasma and Peripheral Blood Mononuclear Cells of Bone Marrow Transplant Recipients

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Abstract

Objectives: **Quantitative monitoring of human cytomegalovirus infections is helpful in determining appropriate antiviral management in patients who receive bone marrow transplants. We sought to design and evaluate a new cytomegalovirus capture hybrid polymerase chain reaction enzyme-linked immunosorbent assay (PCR-ELISA) in plasma and peripheral blood mononuclear cells to monitor cytomegalovirus infection in bone marrow transplant recipients.**

Patients and Methods: **Twenty-six patients who received allogeneic bone marrow transplants, including 17 male patients and 9 female patients (9 adults, 17 children), were enrolled in this study. A total of 313 consecutive whole blood specimens, before and from 7 to 120 days after transplant, was evaluated in the study. A newly designed biotinylated probe-mediated quantitative competitive PCR-ELISA test was used to determine cytomegalovirus load in specimens of peripheral blood mononuclear cells and plasma.**

Results: **All 26 patients were cytomegalovirus seropositive before transplant. Capture hybrid PCR-ELISA of peripheral blood mononuclear cells detected cytomegalovirus DNA in 287 of 313 specimens (91.7%) even in cases with no active cytomegalovirus infection. in plasma, cytomegalovirus DNA was detected in 114 of 313 specimens (36.4%). Increasing titers of cytomegalovirus DNA were detected in 14 of 26 patients (53.8%).**

Conclusions: **The quantitative capture hybrid PCR-ELISA was able to diagnose and monitor cytomegalovirus infection in patients who received bone marrow transplants. Detection of cytomegalovirus DNA in plasma was more predictive of the onset of cytomegalovirus-related clinical symptoms, compared to detection in peripheral blood mononuclear cells.**

Key words: *Human cytomegalovirus, Capture hybrid PCR-ELISA, Bone marrow transplant, Viral load*

Human cytomegalovirus (CMV) is an opportunistic infection occurring in immunosuppressed patients, such as bone marrow transplant recipients, and may manifest as symptomatic end-organ dysfunction or CMV syndrome (1-3). Two approaches are currently used to prevent CMV disease in seropositive bone marrow or other organ transplant recipients. One approach involves the use of either ganciclovir, or

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less frequently, acyclovir, given prophylactically to all patients (4-6), despite their adverse effects. The other approach involves pre-emptive use of ganciclovir or foscarnet in patients presenting with laboratory and clinical data indicative of active CMV infection (7, 8). Because effective pre-emptive antiviral therapy depends on detecting CMV at an early stage of active infection, applying sensitive, specific, and accurate tests for monitoring patients at risk of developing the disease is an essential part of the management of these patients.

Qualitative and quantitative techniques, including assays for pp65 antigenemia, CMV DNA amplification in peripheral blood leukocytes and plasma, and CMV late messenger RNA (mRNA) amplification are sensitive and accurate means of determining CMV loads in different blood compartments (9, 10). The antigenemia assay is now widely recognized as a useful test for diagnosing and monitoring CMV disease in clinical situations (11, 12). The antigenemia assay is limited, however, because only viruses associated with cells are detectable, and viruses free in the plasma, urine, or cerebrospinal fluid can evade recognition by this assay. Furthermore, the antigenemia assay cannot be carried out when the patient's leukocyte count is low, as frequently is seen in bone marrow transplant patients. Traditional, qualitative polymerase chain reaction (PCR) assays are too sensitive to monitor CMV-related diseases, and they have some drawbacks in guiding when to start and terminate antiviral therapy. Sequential quantification of systemic CMV load during the subclinical early stage of infection may provide a sensitive and specific approach for predicting development of CMV disease in transplant recipients (13-15).

The aim of this study was to compare CMV load in plasma samples versus peripheral blood mononuclear cells of bone marrow transplant recipients by a newly designed in-house capture hybrid PCR enzyme-linked immunosorbent assay (ELISA) in a bone marrow transplant center. The results of this study could be used to guide decision-making with regard to the appropriate use of pre-emptive CMV therapy.

Material and Methods

Peripheral blood samples were collected into EDTA tubes prospectively at least once a week for 16 weeks from 26 consecutive bone marrow transplant recipients at the department of hematology-oncology and bone marrow transplant in Namazi University Hospital, Shiraz, Iran. One sample was collected from each donor before donation of bone marrow, for determining CMV antibodies and performing PCR. The ethics committee of Shiraz University of Medical Sciences reviewed and approved the study protocol, and informed written consent was obtained from the patients and the guardians.

Ten milliliters of EDTA-treated peripheral blood was layered onto an equal volume of Lymphoprep (AXIS-SHIELD PoC AS, Oslo, Norway). The peripheral blood mononuclear cell layer was obtained by centrifugation at 800*g* for 45 minutes. The cells were washed twice in phosphate-buffered saline at pH 7.3, centrifuged at 600*g* for 20 minutes, and counted. Plasma was obtained from the supernatants of the undiluted upper layer of Lymophoprep density gradient centrifuge tubes. A second centrifugation was done at 800*g* for 10 minutes to remove cellular debris and remaining platelets.

DNA was extracted from 10^6 peripheral blood mononuclear cells or 100 μL plasma by standard procedures of proteinase K digestion, phenol-chloroform-isoamyl alcohol extraction, and precipitation with ethanol. Cytomegalovirus DNA was amplified by 22-bp oligonucleotides primers, CMV-B glycoprotein-F 5′-CGG TGG AGA TAC TGC TGA GGT C (CMV-UL55, upstream, 82 494 to 82 515) and CMV-B glycoprotein-R 5′-CAA GGT GCT GCG TGA TAT GAA C (CMV-UL55 downstream, 82 729 to 82 750), flanking a 257-bp segment of the B glycoprotein sequence of CMV. The reverse primer was 5′ end labeled with digoxigenin. The fragments were hybridized with a 5′ end-biotinylated (Bio), 24-bp probe, CMV-B glycoprotein-P (Bio-5′- ACA TTC CTC AGT GCG GTG GTT GCC [CMV B glycoprotein, 82 594 to 82 617]).

A 156-bp fragment of bacteriophage lambda genome was chosen and amplified using a primer pair containing CMV primer binding sites at 5' ends. The internal control F-primer sequence was 5′-CGG TGG AGA TAC TGC TGA GGT CTG CGT GTA GGC GAA TTT G (lambda genome, upstream, 40 954 to 40 971), and the sequence of reverse primer, internal control-R, was 5′- CAA GGT GCT GCG TGA TAT GAA CCC ACC GGA GAA ACT AAC GAC (lambda genome, downstream, 41 090 to 41 109). The product

amplicons were 200-bp and were hybridized with a 5′ end biotinylated 25-bp probe, internal control-P Bio – TAC AGA GCG TGG CGT TAA GTG GTC A. Primers and the biotinylated probes were provided by TIB Molbiol (Hamburg, Germany).

Two constructed plasmids (B glycoprotein plasmid and internal control plasmid), each separately containing 1 copy of the UL55 target sequence (B glycoprotein coding region) and 1 copy of the chimera 200-bp amplicon, respectively, were used as standards for CMV DNA quantification. To construct the B glycoprotein plasmid, DNA from an Iranian CMV strain (GenBank accession number: DQ089700) was amplified with primers CMV-B glycoprotein-F and CMV-B glycoprotein-R and cloned into the pTZ57R vector (Fermentas UAB, Vilnius, Lithuania). To construct the internal control plasmid, the chimera of the 200-bp amplicon was cloned into the pTZ57R vector. The concentrations of purified B glycoprotein and internal control plasmid DNA were determined with a spectrophotometer at 260 nm, and the corresponding copy numbers of the 2 plasmids were calculated.

Serial 10-fold dilutions of B glycoprotein-containing plasmid were coamplified with known amounts of internal control for determination of the standard curve. The experiments showed that 1000 internal control copies were the optimal number to be coamplified with serial dilutions of target CMV B glycoprotein-containing plasmid to reach a sensitive detection limit of 10 B glycoprotein DNA copies following coamplification (higher amounts of internal control caused a decrease in sensitivity of PCR) (16). When equal amounts of both plasmids were coamplified, equal amounts of amplicons were produced, indicating no difference between the amplification efficiency of the 2 DNA fragments for detection in PCR.

The PCR assay was performed using the Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) in a final reaction volume of 50 μL. The reaction mixture consisted of 25 pmol of each primer (CMV-B glycoprotein-F and CMV-B glycoprotein-R); 200 μM each of deoxyribonucleotide triphosphate and 1.5 U *Taq* DNA polymerase, 1 × PCR buffer (consisting of 10 mM Tris–HCl, 50 mM KCl, pH 8.3), and 1.5 mM $MgCl_2$; and 10 μL DNA template. After denaturation at 94°C for 5 minutes, PCR was performed with thermal cycling parameters of 94°C for 45 seconds, 58°C for 1 minute, and 72°C for 1 minute for 35 cycles, followed by final extension at 72°C for 5 minutes.

Products of PCR were detected by using the PCR-ELISA Digoxigenin Detection kit (Roche, Germany). The manufacturer's procedure with slight modifications was followed. All buffers used were those supplied by the manufacturer. For 1 amplification tube, 2 hybridization processes were done: 1 for wild-type product and the other 1 for internal control product. Duplicates of 5 μL of a 1:10 dilution of an amplified mixture were mixed with 20 μL denaturation solution in 2 separated microcentrifuge tubes. After 10 minutes of incubation at room temperature, 220 μL hybridization buffer containing 8 pmol/mL of CMV-B glycoprotein P-biotin labeled probe or internal control P-biotin labeled probe was added. The hybridization step was carried out by adding 200 μL of the resultant mixtures into microtiter plate wells coated with streptavidin. The plates were incubated for 1.5 hours at 40°C on a shaker. The hybridization fluid was discarded, and the wells were washed 5 times with 300 μL washing buffer. The hybrid PCR products-probes were detected after the addition of 200 μL of 1:200 dilution anti-digoxigenin-peroxidase conjugate, incubation with shaking at 37°C for 30 minutes, and 5 washes. After 1 hour of incubation with 200 mL of 1 mg/mL ABTS (in the dark at room temperature), the optical density was recorded using a plate reader at a wavelength of 405 nm and a reference of 490 nm using a software program (Ascent Software, Version 2.6, Thermo Labsystems, Finland) that subtracted the optical density of the blank well from all wells and generated a standard curve (ratio of wild-type to internal control absorbance versus copies of plasmid amplified).

Values of experimental samples were determined from the standard curve, and subsequently divided by the initial amount of DNA amplified to yield copies per 10^5 peripheral blood mononuclear cells or per 100 μL plasma presented in each initial (pre-PCR) sample. The sensitivity of the PCR-ELISA assay was evaluated by using the B glycoprotein plasmid as a standard. One copy of the plasmid could be detected in 1 in 5 assays, while 10 plasmid DNA copies were detected with 100% sensitivity. Thus, the lowest limit for quantification was considered to be 10 copies of the target sequence. The specificity of the PCR-ELISA assay was tested by amplifying DNA from other herpesviruses: herpes simplex virus types 1 and 2,

varicella zoster virus, and Epstein-Barr virus (B95-8 cells). None of these samples yielded detectable amplification products (data not shown). The weekly results of the in-house PCR- ELISA test and quantitative Eclipse CMV DNA Detection Kit (IQ Products, The Netherlands) were compared for 16 weeks in 3 patients (47 specimens), and there were no significant differences between the results of the 2 tests (data not shown; $.9 \geq P \leq .109$).

Data are presented as mean ± standard deviation (SD) or median with range, as appropriate. Pearson analysis was used to compare the correlation between IgM seropositivity, age, sex, and underlying disease with CMV reactivation, and also to compare ganciclovir therapy with reduction in CMV copy number (as a qualitative variable). The K-independent test was used to compare the mean CMV DNA copy number in each group, and the chi-square test was used to compare the CMV DNA detection rate in each group. Differences in results of in-house hybrid capture PCR-ELISA and quantitative Eclipse CMV DNA detection kit were analyzed by using the Wilcoxon signed rank test. Values of $P < .05$ were considered statistically significant.

Results

A total of 313 clinical samples was collected from 26 bone marrow transplant patients. The duration of sampling for each patient was 7 to 120 days following transplant, unless for reasons such as death or moving to the place of origin. One sample was collected before transplant, and the rest were collected weekly afterwards. Five recipients died of causes other than CMV infections. Because in Iran, no postmortem autopsy is carried out, the exact causes of such deaths are unknown. Of the 26 patients, 17 were men (65.4%), and 9 were women (34.6%). The ages ranged from 5 to 42 years, with a median of 12 years. The reasons for bone marrow transplant were thalessemia in 14 patients (53.8%), acute myeloid leukemia in 8 patients (30.8%), anemia in 2 patients (7.7%), acute lymphoblastic leukemia in 1 patient (3.85%), and chronic myeloid leukemia in 1 patient (3.85%).

At the time of CMV infection, ganciclovir was administered at a dosage of 6 mg/kg twice daily for 2 weeks and then 5 mg/kg once daily for 5 days a week for an additional 4 to 5 weeks. For some patients the treatment extended to 11 weeks. All enrolled patients, whose diseases had been diagnosed at earlier times through increased load of CMV DNA copy numbers and followed by appropriate antiviral treatment, recovered from symptomatic CMV disease. It is worth mentioning that the initiation of therapy was based on the team of physicians' decision and clinical, paraclinical findings. The results of the quantitative analysis of both plasma and peripheral blood mononuclear cells of the patients along with the ganciclovir treatment status and posttreatment viral detection and loads are demonstrated in Table 1.

Cytomegalovirus load of 26 bone marrow transplant patients in 313 specimens was assessed by our established in-house hybrid capture assay. Capture hybrid PCR-ELISA of peripheral blood mononuclear cells detected CMV DNA in 287 specimens (91.7%); PCR-ELISA of plasma detected CMV DNA in 114 specimens (36.4%; $P = .001$). Mean copy number of CMV DNA in peripheral blood mononuclear cells in those with active CMV infection was significantly higher than in those without active infection (2175 ± 3512 vs 51 ± 53, respectively; $P = .001$). Of the 114 positive PCR-ELISA plasma specimens, only 6 did not belong to patients with active CMV infection, compared to 108 positive specimens from those with active infection ($P < .001$).

Of the 26 bone marrow transplant participants, CMV activation, that is, increasing titers of CMV DNA, was demonstrated in 14 of 26 patients (53.8%). All patients had CMV IgG and, in 6 patients, CMV IgM was also detectable. Except in 1 patient, all bone marrow donors (25 of 26, 96.2%) were CMV IgG positive, and CMV IgM was detectable in 2 bone marrow donors (7.7%). No significant correlation was found between the presence of CMV IgM antibodies in donors or recipients and CMV activation. In addition, a Pearson statistical analysis did not demonstrate any significant correlation between age, sex, and the nature of original diseases of the recipients and CMV activation. In 14 bone marrow transplant recipients, increasing loads of CMV were detected, leading to a spectrum of clinical presentations, ranging from fever with cystitis to severe gastroenteritis and pneumonitis (Table 1).

Two typical presentations of CMV disease after bone marrow transplant are described in detail (see Figures 1 and 2). In the first (patient No. 5 in Table 1; Figure 1), the recipient was an 8-year-old female primary school student who received a bone marrow

Table 1. Quantitative analysis of plasma and peripheral blood mononuclear cells of 26 bone marrow transplant recipients with their treatment status and clinical outcomes

Patient No.	No. of samples	No. of positive PCR (%)		Range of viral load		Ganciclovir treatment, weeks	Viral load after treatment		Clinical presentation †
		On 10^5 PBMCs	On 100 μL of plasma	Copies/ 10^5 PBMCs	Copies/ 100 μL of plasma		Copies/10^5 PBMCs	Copies/ 100 μL of plasma	
1	7	6 (85.7)	0 (0)	16-31	UD	NT	ND	ND	-
2	14	12 (85.7)	2 (14.3)	11-60	12-15	NT	ND	ND	-
3	7	4 (57.1)	0 (0)	23-38	UD	NT	ND	ND	-
4	7	6(85.7)	1 (14.2)	11-57	32	NT	ND	ND	-
5 *	15	15 (100)	8 (53.3)	10-4565	370-3240	9	446	BN	Gastroenteritis GVHD
6	14	14 (100)	1 (7.1)	12-70	60	NT	ND	ND	-
7 *	7	6 (85.7)	6 (85.7)	73-1328	800-1640	4	826	BN	Gastroenteritis hepatitis GVHD
8	9	9 (100)	0 (0)	11-75	UD	NT	ND	ND	-
9 *	12	11 (91.6)	6 (50)	44-459	270-897	7	181	BN	Renal involvement fever GVHD
10	16	11 (68.7)	0 (0)	10-42	UD	NT	ND	ND	-
11 *	13	13 (100)	11 (84.6)	73-10700	712-6845	NT	ND	ND	Gastroenteritis GVHD
12 *	16	15 (93.7)	10 (62.5)	13-1580	350-2800	8	98	BN	Pneumonitis
13 *	16	16 (100)	8 (50)	34-7111	348-6530	10	512	90	Gastroenteritis GVHD
14 *	11	11 (100)	8 (72.7)	39-13700	472-8937	6	7836	730	Pneumonitis
15	11	10 (90.9)	1 (9.0)	25-139	29	NT	ND	ND	-
16 *	13	12 (92.3)	7 (53.8)	15-1225	256-1028	8	856	BN	Gastroenteritis renal involvement
17	9	8 (88.8)	0 (0)	12-224	UD	NT	ND	ND	-
18 *	12	11 (91.6)	6 (50)	35-2523	740-3080	6	316	BN	Renal involvement GVHD
19	11	9 (81.8)	0 (0)	11-122	UD	NT	ND	ND	-
20 *	14	14 (100)	6 (42.8)	50-3163	304-3716	10	458	BN	Pneumonitis
21 *	16	16 (100)	8 (50)	18-3786	432-3225	10	274	BN	Renal involvement fever
22 *	13	12 (92.3)	5 (38.4)	72-836	351-1033	NT	ND	ND	Gastroenteritis renal involvement GVHD
23 *	16	16 (100)	9 (56.2)	59-6988	273-5640	11	ND	ND	Gastroenteritis renal involvement hepatitis
24	8	5 (62.5)	0 (0)	16-45	UD	NT	ND	ND	-
25 *	15	15 (100)	10 (66.6)	12-2401	384-2173	10	245	BN	Gastroenteritis GVHD
26	11	10 (90.0)	1 (9.0)	41-125	13	NT	ND	ND	-

*represent those with CMV active infection
†Only HCMV-linked clinical symptoms are cited in this table
Abbreviations: BN, became negative; GVHD, graft-versus-host disease; ND, not done; NT, not treated; PBMCs, peripheral blood mononuclear cells; UD, undetectable

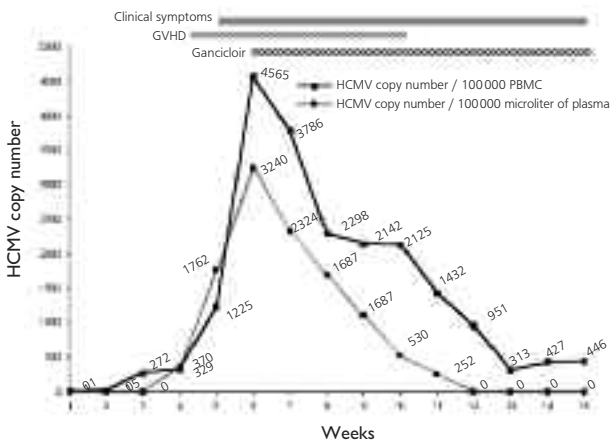

Figure 1. Follow-up of patients 5, time course of clinical disease associated with HCMV infection treated with ganciclovir and quantitative PCR results.

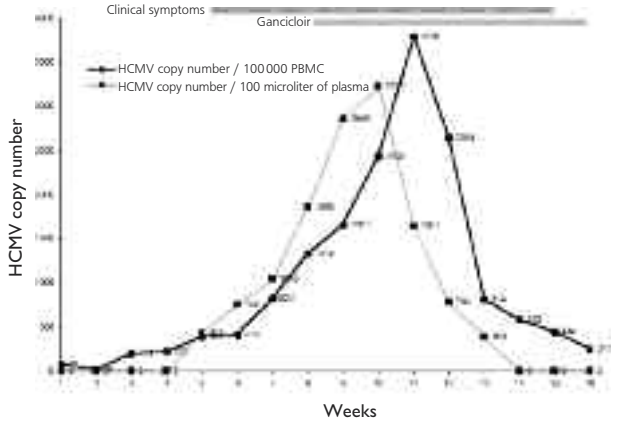

Figure 2. Follow-up of patient 21, time course of clinical disease associated with CMV infection treated with ganciclovir and quantitative PCR results.
Abbreviations: HCMV, human cytomegalovirus; PBMC, peripheral blood mononuclear cells.

transplant owing to thalassemia major. The donor was her 18-year-old sister. Both the donor and the recipient had CMV IgG and were negative for CMV IgM. The blood types in both cases were A+. Acute gastroenteritis was the main clinical disorder in this recipient. In addition, graft-versus-host disease began with skin manifestations, followed by systemic symptoms. Having used antiviral therapy with ganciclovir, the virus titer dropped significantly in 2 weeks and this trend continued until the 15th week (R = 0.866, P = .001) (Figure 1).

In the second, the recipient (patient No. 21 in Table 1; Figure 2) was a 21-year-old female student who received a bone marrow transplant to treat type 2 acute myeloblastic leukemia. The donor was her 15-year-old sister. Both the donor and recipient had CMV IgG and were negative for CMV IgM. The blood type in both cases was B+. The major clinical disorder in this recipient was acute hemorrhagic cystitis. Even 3 weeks after the start of antiviral therapy, the CMV load was increasing. After 16 weeks of antiviral therapy, the patient was recovered and discharged from the hospital. Figure 2 shows the viral load along with duration of clinical symptoms and the use of ganciclovir therapy.

Discussion

Several clinical studies have demonstrated the importance of CMV among immunocompromised patients such as bone marrow transplant recipients (17, 18, 19). Several effects have been linked to active CMV infection, including direct effects of CMV-related symptoms and organ dysfunction, and indirect effects owing to the immunosuppressive effect of CMV (20, 21). Cytomegalovirus also can be responsible for chronic graft rejection (22). Furthermore, the immunosuppressive effects of CMV can induce other complications, such as bacteremia and invasive fungal disease (23, 24). Therefore, the development of rapid diagnostic tests is of great clinical importance, to provide early detection of infection, differentiate CMV infection from other opportunistic infections or rejection, and guide the use of antiviral drugs as pre-emptive therapy (7, 25, 26, 27, 28).

As demonstrated in the results, more peripheral blood mononuclear cell specimens (91.7%) were positive for CMV DNA, compared to plasma specimens (36.4%). There was no correlation between the proportion of positive results in PCR-ELISA tests of peripheral blood mononuclear cells and reactivation of CMV in patients with active CMV disease, in comparison to patients who did not show symptoms of active CMV infection. However, the mean copy number of CMV DNA in peripheral blood mononuclear cells in those with active CMV infection was significantly higher than in those without active infection. In contrast, most positive PCR-ELISA plasma specimens occurred in patients with active CMV infection.

Cytomegalovirus DNA was still detected in peripheral blood mononuclear cells in some recipients without active infection. Monocytes have been implicated as a site of CMV latency or persistence, and they can disseminate CMV to other cell types permissive for virus replication (29, 30). As shown in Table 2, in all but 1 of the patients treated with ganciclovir, the virus could not be detected in the plasma by PCR-ELISA, while viral DNA could still be detected in several peripheral blood mononuclear cells of the same patients.

At our bone marrow transplant center, the treatment of active CMV infection was based on symptoms of active infection. When the viral load was significantly high, it took a longer period of antiviral therapy to clear the virus from patients. This can lead to unwanted medication adverse effects in the patients. By comparing the qualitative and quantitative results of CMV detection in plasma and peripheral blood mononuclear cells, both results could be used to determine the start of therapy. It can be recommended that the same results in plasma can help determine the end of thrapy. However, plasma analysis was more accurate for guiding decision-making in using pre-emptive therapy. This study has shown the potential of viral load testing by a quantitative capture hybrid PCR-ELISA for diagnosis and monitoring of CMV infections in bone marrow transplant recipients. This assay has the potential to facilitate pre-emptive antiviral therapy in this group of patients.

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