Peripheral blood leukocytes serve as a possible extrahepatic site for hepatitis C virus replication

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To study possible extrahepatic sites for the replication of hepatitis C virus (HCV), we examined fresh and cultured peripheral blood mononuclear leukocytes (PBML), as well as different subpopulations of PBML of HCV-infected patients, for the presence of viral genomic and antigenomic RNA. Sense and antisense oligonucleotide primers derived from HCV sequences were used for reverse transcription (RT) followed by an amplification with the polymerase chain reaction assay (PCR). Using antisense primers for RT, genomic viral RNA could be detected in serum, liver, total PBML and B lymphocytes of chronically infected patients. However, only liver tissue and PBML specimens were positive when a sense primer was used. To demonstrate further the specificity of these findings, total PBML were stimulated using pokeweed mitogen and synthesis of HCV RNA was determined by incorporation of [3H]uridine into nascent RNA molecules using a hybrid release assay. Additionally, total PBML from an uninfected person could be infected in vitro using an HCV RNA-positive serum. The PCR products obtained from serum, liver and PBML specimens of an HCV-positive individual were found to have nearly identical sequences. Our findings suggest that PBML could be a site for viral replication of HCV during the natural course of infection and may represent a reservoir for hepatitis C virions.

Introduction

Hepatitis C virus (HCV) is responsible for most cases of post-transfusional hepatitis (Alter et al., 1989; Choo et al., 1990), with a chronic course in up to 60% of individuals, leading to cirrhosis and eventual hepatocellular carcinoma in 20% of individuals (Sakamoto et al., 1988; Kiyosawa et al., 1990). Based on sequence analyses (Choo et al., 1991; Takamizawa et al., 1991), the genome of the virus is an ssRNA with positive polarity and contains about 9416 nucleotides. A single open reading frame encodes a precursor polyprotein of 3010/3011 amino acids. These characteristics as well as the genomic organization and sequence homologies suggest that HCV is related to flavi- and pestiviruses (Miller & Purcell, 1990). The virus is now classified in its own genus within the flaviviridae family (Wengler, 1991).

HCV can be detected in serum and liver using reverse transcription and subsequent amplification of the cDNA by polymerase chain reaction assay (PCR). In this study we used strand-specific oligonucleotide primers in the reverse transcriptase reaction to investigate genomic viral RNA and the presence of minus-stranded RNA of HCV in serum, liver tissue, total peripheral blood mononuclear leukocytes (PBML), and different lymphocyte subpopulations, including natural killer (NK) cells, B and T cells, as well as CD4 and CD8 subsets of T cells from chronically HCV-infected patients. Also, HCV-specific replication was demonstrated in vivo and in vitro infected total PMBL, employing a modified hybrid release assay detecting radiolabelled RNA.

Since graft-reinfection with HCV is common after orthotopic liver transplantation (OLT) in chronically HCV-infected patients (Read et al., 1991), it was the aim of the study to identify possible sites of extrahepatic replication which may serve as a source for virions causing reinfection.

Methods

Patients. Serum samples and PBML were obtained from patients with chronic HCV infection and also at regular intervals from patients who had undergone OLT for HCV-related end stage chronic liver disease. All patients were anti-HCV-positive in ELISA (2nd generation; Abbott) with an A > 2; they were positive for HCV RNA in the serum by PCR, and had elevated transaminases. Specimens of the explanted livers were immediately frozen in liquid nitrogen and stored at −70 °C. HCV-negative patients, who received transplants due to primary biliary cirrhosis, served as controls. Informed consent was obtained from all patients and the study had been approved by the ethical review committee of Heidelberg University.

Cell preparation and flow cytometric analysis. A sample of 50 ml heparinized (30 units/ml) venous blood was used to obtain PBML in one or two successive Ficoll–Hypaque sedimentation gradients,

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Table 1. Nested primer sets used for amplification of HCV RNA

<table>
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<tr>
<th>No.</th>
<th>Sense</th>
<th>Position</th>
<th>Sequence (5'-3') (bp)</th>
<th>Product size (bp)</th>
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<td>1</td>
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<td>14 to 33</td>
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<tr>
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<td>5053 to 5076</td>
<td>ACTCTTGTCGCCAGCAAGCGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5549 to 5572</td>
<td>TCCCGTGCTGGTGTCGTGGC</td>
<td>519</td>
</tr>
</tbody>
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* The positions of the primers correspond to the prototype HCV-I sequence (Choo et al., 1991; Han et al., 1991).

centrifuged at 800 g for 20 min at 20 °C. Cells were collected and washed three times with RPMI 1640 medium containing 5% fetal calf serum (FCS), glutamine and antibiotics (penicillin, streptomycin; ICN).

Peripheral blood lymphocytes were purified from monocytes by plastic adherence. T and non-T cell fractions were enriched using the rosette formation method with sheep erythrocytes. T and non-T cells were separated by Ficoll-Hypaque gradient centrifugation. Sheep erythrocytes were lysed with 1 M-HCl. Finally, cells were washed twice in RPMI 1640 medium containing 10% FCS.

To separate lymphocyte subsets fluorescence-activated cell sorting (FACS; Becton Dickinson (BD)) was performed using mouse monoclonal antibodies (MAbs) to T helper cells (OKT4-FITC; BD), to T cytotoxic/suppressor cells (Leu-2a-PE; BD), to NK cells (Leu-11a-FITC) and B cells (Leu-16-PE; BD). The MAbs were conjugated either with fluorescein isothiocyanate or phycoerythrin. 1 x 10⁶ to 5 x 10⁶ cells were sorted per lymphocyte subset. The purity of the sorted cells was about 95% as evaluated by using a FACS-scan (BD).

Cell culture and stimulation of PBML. Total PBML were cultured in 24-well plates at 1 x 10⁶ cells per well or in 96-well U-bottomed plates at 2 x 10⁶ cells per well. RPMI 1640 served as growth medium containing 10% heat-inactivated FCS. Mitogen was added as cell culture initiation and maintained throughout the experiment [pokeweed mitogen (PWM) dilution 1:64; Serva]. Incorporation of [3H]uridine (10 µCi/ml; specific activity 25 to 30 Ci/mmol) was performed either with fluorescein isothiocyanate or phycoerythrin. 1 x 10⁶ to 5 x 10⁶ cells were sorted per lymphocyte subset. The purity of the sorted cells was about 95% as evaluated by using a FACS-scan (BD).

Hybrid release assay. A cloned PCR fragment (Apal- and NcoI-digested) of the HCV 5' untranslated and core region (map positions 83 to 465), or as control a plasmid containing the whole HBV genome, was fixed on nitrocellulose filters and [3H]uridine-labelled RNA from PBML was used as a probe. All procedures were done according to Maniatis et al. (1982). After hybridization and extensive washing procedures the dots were cut out and bound radioactivity was determined. Background radioactivity and non-specific binding to HBV DNA were subtracted.

Extraction of HCV RNA. Fresh and cultured PBML and subpopulations were washed three times with PBS. This procedure was sufficient to remove all free, serum-associated virion particles, as shown by the absence of viral RNA by PCR in the final wash supernatant. RNA was isolated by dissolving in 4 M-guanidinium isothiocyanate, 25 mM-sodium citrate, 0.5% sodium N-lauroylsarcosine, 0.1% 2-mercaptoethanol and by centrifugation through 5 M-sodium chloride. RNA was resuspended in diethyl pyrocarbonate (DEPC)-treated H₂O, precipitated with ice-cold ethanol, and resuspended in DEPC-treated H₂O containing 40 units of RNase inhibitor (Promega).

Serum samples (100 to 500 µl) were extracted by mixing with extraction buffer containing a final concentration of 4 M-guanidinium isothiocyanate, 25 mM-sodium citrate, 0.5% sodium N-lauroylsarcosine and 0.1 M-2-mercaptoethanol. Tissue specimens were homogenized in the same extraction buffer. The extract was laid on a 57 M-cesium chloride cushion and centrifuged in a Beckman SW55 Ti rotor at 20 °C for 6 h at 48000 r.p.m. The RNA pellet was resuspended in 200 µl DEPC-treated H₂O and precipitated as described above.

Preparation of HCV cDNA. HCV RNA and 50 pmol of antisense oligonucleotide primer (Table 1) were denatured at 70 °C for 5 min, annealed at 37 °C for 5 min, transcribed to cDNA by avian myeloblastosis virus (AMV) reverse transcriptase (5 units; Promega) in a final volume of 20 µl at 20 °C for 10 min, and then at 42 °C for 1 h (after 30 min another 5 units of AMV reverse transcriptase having been added), using cDNA buffer (50 mM-Tris-HCl pH 8.3, 50 mM-KCl, 5 mM-MgCl₂, 5 mM-DTT, 1 mM of each dNTP) and 1 unit/µl RNase (Promega). The reaction was stopped by heating to 95 °C for 10 min.

To detect minus-stranded RNA, cDNA synthesis was performed as described above using a sense oligonucleotide primer instead of an antisense primer. To exclude any contamination with plus-stranded RNA or resting reverse transcriptase activity, the reaction was stopped by heating, samples were then chilled on ice and RNase A (100 µg/ml), RNase H (100 units/ml) and RNase T1 (5000 units/ml) were subsequently added. Thereafter samples were incubated at 37 °C for 5 min. After phenol/chloroform extraction and ethanol precipitation, the cDNA was dissolved in PCR buffer (10 mM-Tris-HCl pH 8.3, 50 mM-KCl, 2.5 mM-MgCl₂, 0.1% BSA, 1% Triton X-100), containing the antisense primer.

Amplification of cDNA by PCR. Amplification of the HCV cDNA was performed in accordance with the method originally described by Saiki et al. (1988) with modifications. In short, 50 pmol of the sense (antisense for minus-stranded RNA) oligonucleotide primer, PCR buffer, and 1.5 units Taq DNA polymerase (Perkin-Elmer Cetus) were added to the heat-treated and chilled reverse transcriptase reaction to a final volume of 100 µl. Then 35 reaction cycles were carried out, each cycle including denaturation at 93 °C for 1 min, primer annealing at 50 °C for 1.5 min and primer extension at 72 °C for 3 min. The final step included an extension time of 30 min. To verify the first PCR product, a second PCR with nested primers (see Fig. 1) was performed using the same protocol as described above.

Analysis of amplified products and Southern blot hybridization. PCR products were treated for 30 min with 10 µg/µl RNase A at 37 °C, separated on a 1% agarose gel and revealed under u.v. light after staining with ethidium bromide. The gel was blotted on to a nitrocellulose filter and the filter was hybridized according to Maniatis et al., 1988; Maniatis et al., 1991; Han et al., 1991).
Replication of HCV in mononuclear cells

(a) 519 bp

(b) 355 bp

Fig. 1. Ethidium bromide-stained gel (a) and Southern blot analysis (b) showing the PCR product from genomic HCV RNA obtained with the nested primer set 2N derived from mononuclear leukocyte RNA of several anti-HCV negative (lanes 1 to 4) and anti-HCV-positive (lanes 5 to 9) patients.

Fig. 2. Ethidium bromide staining (a) and hybridization pattern (b) obtained after Southern blot analysis of HCV-specific PCR fragments. RNA was isolated from three different serum, PBML and liver samples (lanes 1, OLT 21; lanes 2, OLT 88; lanes 3, OLT 17). Synthesis of cDNA with antisense primers was done and nested PCR was performed using the outer primer set 1 and the inner primer set 1N. Hybridization was carried out with a 32P-labelled probe lacking the 5' and 3' primer sequences. Minus sign (−) denotes a negative control.

Results

Presence of HCV RNA in PBML of anti-HCV-positive patients

We selected two patients with post-transfusional hepatitis C and three patients who had undergone OLT. These latter patients were serum-positive for both anti-HCV antibodies and HCV RNA before OLT and experienced reinfection of the graft (Müller et al., 1992).

Two sets of outer oligonucleotide primers and two inner primer sets were used for PCR and nested PCR respectively (Table 1). Most of the amplifications were performed with primer sets 1 and 1N (1, 2) derived from the well conserved 5' non-translated and core region. To test for HCV strain variability we decided to synthesize a second set of primers (2/2N) in the more heterogeneous NS3/NS4 region.

Amplification products of serum RNA obtained with these primers were cloned, sequenced, and compared to the American prototype HCV sequence ([HCV-1] Choo et al., 1991; Han et al., 1991) and to a Japanese isolate ([HCV-BK] Takamizawa et al., 1991). For HCV-1 we found a similarity of about 81 to 83% for nucleotides and 90% for amino acids and for HCV-BK 91% and 97%, respectively. It is noteworthy that the German HCV sequences appear to be more closely related to the Japanese isolates than other European isolates reported so far (Cariani et al., 1991; Kremsdorf et al., 1991).

No products could be detected on ethidium bromide-stained agarose gels after amplification of HCV RNA extracted from PBML of four anti-HCV-negative and
five anti-HCV-positive patients with primer set 2. However, a second PCR with a nested primer pair revealed a positive result in three of five anti-HCV-positive patients (Fig. 1). Patients with sporadic hepatitis, demonstrated by anti-HCV and HCV RNA in their serum, were not positive for HCV RNA in PBML even after long-term exposure of the autoradiogram (Fig. 1, lanes 5 and 8).

Detection of minus-stranded RNA in liver and mononuclear leukocytes

Hepatitis C virus contains an ssRNA molecule with positive polarity. In infected cells, RNA molecules with negative polarity representing replicative intermediates indicate active replication of the viral genome. To test for the presence of plus-stranded and minus-stranded RNA molecules, we analysed RNA extracted from serum and equal amounts of total RNA extracted from PBML and liver in a slot blot assay (data not shown). Anti-HCV-positive samples showed the presence of plus- and minus-stranded RNA both in PBML and liver. No minus-stranded RNA was detected in the serum samples. For further investigation, we chose three positive specimens and their corresponding sera. Nested PCR was performed and the amplified products were separated on an agarose gel (Fig. 2a). Southern blot hybridization analysis (Fig. 2b) demonstrated that the tested samples showed the expected product. In this experiment cDNA synthesis was carried out with antisense primers.

RNA extracted from PBML was also transcribed into cDNA with sense primers. The opposite primer was added for PCR amplification with primer set 1 followed by nested PCR with primer set 1N1. In Fig. 3, an HCV-specific amplification product with sense primers in the reverse transcriptase reaction (lanes 1 to 3, s) could be clearly detected in a Southern blot hybridization analysis. Lanes 1 to 3 a show amplified products derived from PBML RNA transcribed with the antisense primer. In this case detection was possible in both the ethidium bromide-stained gels and the Southern blot hybridization.

It is noticeable that detection of plus-stranded HCV RNA was much easier than detection of minus-stranded RNA. One possibility is that the latter exists as a replicative intermediate in a much smaller amount than plus-stranded RNA. Also, it may be that plus-stranded RNA is packed into the core immediately after synthesis and no longer subjected to degradation by cellular RNases. Therefore, predominantly plus-stranded RNA is detected. It should also be noted that no amplification product was found in the final wash supernatant of the PBML preparation, ruling out the possibility of contamination with free, serum-associated viral particles (data not shown).

Incorporation of exogenous uridine into HCV RNA in mitogen-induced PBML culture

To confirm that the data shown in Fig. 1 and 2 resulted from an infectious event rather than a non-specific adsorption of HCV virions on blood cells, active replication in these cells should be demonstrated. For
Table 2. Incorporation of $^3$H-labelled uridine into RNA after PWM stimulation of PBML

<table>
<thead>
<tr>
<th></th>
<th>HCV*</th>
<th>HCV-</th>
<th>HBV</th>
<th>HBV</th>
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<tr>
<td>Day 2</td>
<td>191.5</td>
<td>5.0</td>
<td>33.2</td>
<td>17.2</td>
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<tr>
<td>Day 3</td>
<td>69.0</td>
<td>15.0</td>
<td>23.7</td>
<td>18.7</td>
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<tr>
<td>Day 4</td>
<td>115.5</td>
<td>16.0</td>
<td>24.0</td>
<td>20.0</td>
</tr>
</tbody>
</table>

* PWM stimulation of HCV-infected PBML in culture. Replicative activity and HCV specificity were determined by incorporation of $[^3]$H]uridine (c.p.m.) and hybridization of newly synthesized and labelled RNA to a PCR fragment derived from HCV RNA. Absolute c.p.m. values are given for the hybrid release assay from HCV-negative and HCV-positive PBML RNA hybridized to HCV and HBV fragments.

To define the existence of antigenomic RNA molecules in the stimulated PBML, cDNA was synthesized from total cellular RNA with strand-specific primers and then amplified. As is shown for viral genomic RNA in Fig 4(a) and (b), minus-stranded RNA was also detected in stimulated PBML, but only after long exposure of the autoradiograph from a Southern blot hybridization experiment (data not shown).

B lymphocytes are susceptible to HCV infection

To clarify further which subsets of peripheral leukocytes are infected in vivo, total PBML of isolates from four chronically HCV-infected patients were separated. Extracted RNA from each subset, including NK cells, B and T lymphocytes and CD4 and CD8 subsets, was transcribed into cDNA using strand-specific primers to detect viral genomic (plus-stranded) and antigenomic (minus-stranded) RNA. The average purity of each subset amounted to about 95%. Reverse transcriptase assay and PCR were performed using primer sets 1 and 1N2. HCV RNA was detected only in B lymphocytes (Fig. 5, lanes 2), and not in the T lymphocyte subsets (lanes 3 and 4) nor in NK cells (lanes 1; see Discussion). An amplified product due to viral genomic RNA could be seen in an ethidium bromide-stained agarose gel after nested PCR (Fig 5a, lanes 2); minus-stranded antigenomic RNA could be detected only by Southern blot hybridization (Fig 5b).

In vitro infection of PBML

As we had shown that PBML and especially B lymphocytes were infected in vivo during the natural course of chronic hepatitis C, it was possible that PBML could be infected in vitro. Therefore, PBML from a healthy donor were isolated, cultured in 24-well plates and incubated with an anti-HCV-positive serum for
Fig. 5 (a). Ethidium bromide-stained agarose gel of HCV-specific products after nested PCR with primer set 1N2. Different subsets of mononuclear leukocytes, i.e. NK cells (lanes 1), B lymphocytes (lanes 2), T helper cells, CD4⁺ (lanes 3) and T cytotoxic/suppressor cells, CD8⁺ (lanes 4) of four HCV-positive patients (B to E) and one HCV-negative control person (A) were examined to detect HCV RNA. (b) Autoradiography after Southern blot analysis of PCR products from B lymphocytes of HCV-positive patients (lanes B to E) and an HCV-negative patient (lane A). cDNA synthesis was carried out using strand-specific primers to detect minus-stranded RNA. Lanes M, DNA size markers.

Fig. 6. [³H]Uridine incorporation into HCV RNA of normal PBML infected in vitro (solid line) and non-infected control PBML (dashed line), as measured by the hybrid release assay. The c.p.m. values represent the newly synthesized HCV RNA, minus background and nonspecific HBV binding.

Discussion

Several reports have demonstrated by PCR the existence of HCV viral RNA in serum and plasma (for review see Houghton et al., 1991), liver tissues (Han et al., 1991; Sherker et al., 1991), peripheral blood mononuclear cells (Zignego et al., 1992; Li et al., 1992) and salivary glands (Takamatsu et al., 1992). In this report, we present evidence that HCV RNA can be found in PBML, and especially in B lymphocytes.
HCV is thought to have a strong tropism for hepatic tissues. However, for HBV and related animal hepatitis viruses it has been shown that not only viral DNA (Korba et al., 1988a; Hosada et al., 1990; Yoffe et al., 1990) but also replicative intermediates are found in extrahepatic tissues, suggesting active viral replication in these cells (Yoffe et al., 1986; Korba et al., 1988b).

The detection of HCV RNA in PBML appears to be a specific finding. It seems unlikely that detection of viral HCV RNA is an artefact of serum-associated viral particles and/or adhesion of circulating virions to the cells. In the experiments we report that PBML were washed extensively during preparation and, furthermore, the final wash supernatant was tested by PCR, giving a negative result. Also, the incorporation of exogenous [3H]uridine into in vivo and in vitro infected PBML could be used to demonstrate RNA synthesis conclusively.

One alternative explanation of our data could be the phagocytosis of viral particles released from infected hepatocytes by circulating monocytes. Several authors have described the detection of minus-stranded RNA in serum (Fong et al., 1991; Sullie et al., 1991) suggesting that this RNA can also be packed into viral particles. This, however, would not explain the de novo synthesis of viral nucleic acids in PBML.

The detection of minus-stranded RNA as an intermediate replicative form of HCV in B lymphocytes demonstrates active viral replication in these cells. In our laboratory, further studies using a larger number of patients demonstrated that not only B lymphocytes (10 out of 10 patients), but also T lymphocytes (six out of 10 specimens) were positive for HCV RNA by PCR (unpublished). However, it is not yet clear whether T lymphocytes are infected in vivo, and this result might also be explained by a cross-contamination of B lymphocytes in the T lymphocyte subset. Shimizu et al. (1992) recently reported an in vitro infection of a human T cell line (MOLT-4) containing minus-strand viral RNA. HCV replication, however, was demonstrated in a permanent T cell line and not in ‘normal’ T lymphocytes.

The fact that no amplification product can be detected in PBML of patients with sporadic hepatitis may be explained by observations in HBV-infected chimpanzees (Korba et al., 1986) and by the detection of HCV RNA sequences in peripheral mononuclear cells from patients with chronic HCV infection (Artini et al., 1991). It may be that the detection of viral RNA in PBML is related to a chronic carrier status. However, it is unknown at which time extrahepatic cells such as PBML are infected and whether they stay permanently infected. Theoretically, PBML of chronically infected patients could serve as a source of virus for the reinfection of hepatocytes.

The detection of minus-stranded HCV RNA in liver tissue indicates active replication in these cells. Although the presence of HCV RNA in lymphocytes has been observed by PCR (Zignego et al., 1992; Li et al., 1992; Shimizu et al., 1992), mitogenic activation of HCV replication in circulating PBML has not yet been reported. As well as serving as an extrahepatic pool of hepatitis C virions, PBML may also allow viral replication. It has not yet been shown, however, if the formation and release of infectious particles takes place from mononuclear leukocytes. No amplifiable HCV RNA could be detected in culture media of cultured PBML or B lymphocytes infected in vivo, suggesting that viral particles are not being released.

If monocytes contain HCV, one might argue that these cells may play a critical role when migrating to the liver and acting as an intermediate in transmitting the infection to hepatocytes (Zuckerman & Howard, 1978). The alteration of monocyte function could also have a marked effect on the immune response and play an important role in the pathogenesis of the HCV disease.

Another argument for susceptibility of B lymphocytes to HCV infection is that Epstein–Barr virus-transformed B lymphocytes derived from an HCV-infected patient showed specific amplification of HCV RNA by reverse transcriptase assay/PCR from day 14 onwards, with regard to the onset of transformation (unpublished).

HCV is related to flaviviruses and pestiviruses (Miller & Purcell, 1990), and both viruses infect lymphoid cell types (Susa et al., 1992). However, in contrast to a dramatic depletion of B lymphocytes in the end stage of, for example, lethal hog cholera virus infection in the natural host, chronically HCV-infected patients do not exhibit lymphocyte alterations (Prince & Fang, 1992). These findings do not indicate a chronic activation of the immune system by HCV.

HCV does not appear to be present in PBML of all HCV-infected patients. As we could show, viral RNA was detected only in PBML of chronic HCV carriers. All current data are consistent with the suggestion that PBML are sites of latent viral infection. More specific information is needed about the role of the PBML in the aetiology of the chronic stage of hepatitis C and on the kinetics of infection.

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References


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