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Medical Sciences

Human genetic deficiencies reveal the roles of complement in the inflammatory network: Lessons from nature

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ABSTRACT

Complement component C5 is crucial for experimental animal inflammatory tissue damage; however, its involvement in human inflammation is incompletely understood. The responses to Gram-negative bacteria were

here studied taking advantage of human genetic complement-deficiencies—nature's own knockouts—including a previously undescribed C5 defect. Such deficiencies provide a unique tool for investigating the biological role of proteins. The experimental conditions allowed cross-talk between the different inflammatory pathways using a whole blood model based on the anticoagulant lepirudin, which does not interfere with the complement system. Expression of tissue factor, cell adhesion molecules, and oxidative burst depended highly on C5, mediated through the activation product C5a, whereas granulocyte enzyme release relied mainly on C3 and was C5a-independent. Release of cytokines and chemokines was mediated to varying degrees by complement and CD14; for example, interleukin (IL)-1β and IL-8 were more dependent on complement than IFN-γ and IL-6, which were highly dependent on CD14. IL-1 receptor antagonist (IL-1ra) and IFN-γ inducible protein 10 (IP-10) were fully dependent on CD14 and inversely regulated by complement, that is, complement deficiency and complement inhibition enhanced their release. Granulocyte responses were mainly complement-dependent, whereas monocyte responses were more dependent on CD14. Notably, all responses were abolished by combined neutralization of complement and CD14. The present study provides important insight into the comprehensive role of complement in human inflammatory responses to Gram-negative bacteria.

Complement, an integral part of the innate immune system (1), has been described as a double-edged sword since it defends the host against infection (2), but can also cause harm when activated in an uncontrolled manner, as in sepsis (3). The anaphylatoxin C5a is thought to play an important role in these adverse clinical effects and particularly the development of the serious systemic inflammatory response syndrome associated with sepsis (4).

Important knowledge of the complement system has been gained from animal studies, in particular studies using knockout mice, and from the clinical phenotype of individuals with genetic deficiencies (5). Thus far, however, in vitro studies in humans have largely been limited to serum and isolated cells. We have developed a lepirudin-based model that allowed us to characterize the human-whole blood inflammatory response in vitro.

Lepirudin, unlike more commonly used anticoagulants, is inert with respect to complement activation (6). Thus, our whole-blood system allowed cross-talk to occur between complement and the remaining inflammatory network and made possible the recording and comparison of a number of read-outs of specific inflammatory responses to the same stimuli under identical conditions. The combination of the whole-blood method and naturally occurring human “knockouts” has afforded us an opportunity to directly assess the impact of complement on the inflammatory network, providing an overall picture of the comprehensive role of complement in human whole-blood inflammation induced by Gram-negative bacteria.

RESULTS

Characterization of the Complement Defects.

Both complement deficiencies were confirmed by genetic analyses and by structural and functional assays (Fig. 1). The mutation in the C2-deficient (C2D) patient was identified as a previously described 28-bp genomic deletion (7). Sequencing of the C5 cDNA revealed a previously undescribed C5 deficiency (C5D) with two aberrant mRNA products with deletions of exon 27 and exons 26 and 27, respectively. The C2 and C5 proteins were completely missing. Reconstitution with highly purified C2 or C5 completely restored functional activity. The C5D patient and the corresponding control individual displayed functionally equivalent genetic deficiencies in mannose-binding lectin (MBL) (Fig. 1*H*), whereas the C2D patient and corresponding control individual had normal MBL alleles.

Fig. 1.
Characterization of the complement deficiencies. (*A*) Molecular characterization of the C2 deficiency by gel electrophoresis of PCR fragments generated with primers flanking the 28 bp genomic deletion. A 174-bp fragment was generated in individuals without (more ...)

Inability of C5D Blood To Kill *E. coli*.

Killing of *Escherichia coli* (*E. coli*) was critically dependent on C5 (Fig. 2). No inhibition of bacterial growth was seen in C5D blood, whereas in control- and C5-reconstituted blood, *E. coli* was efficiently killed (Fig. 2*A Left*). No such difference was seen between the C2D and the C2-reconstituted blood (Fig. 2*A Right*), suggesting that the classical and lectin pathways are not essential

for killing of *E. coli*. In contrast, phagocytosis of *E. coli*, as determined by flow cytometry, was reduced in both the C2D and C5D samples, although not completely in the case of C2D (Fig. 2*B*). Reconstitution restored phagocytosis to levels similar to the controls. Phagocytosis in reconstituted and control blood was abrogated by a C5a receptor antagonist, supporting the notion of a crucial role for C5a-C5a receptor interaction in CD11b up-regulation as part of the phagocytic process.

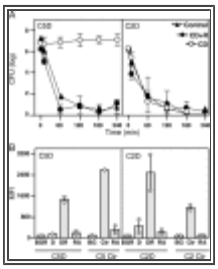

Fig. 2.
Bacterial growth and phagocytosis. (*A*) Growth of *E. coli* in complement-deficient (CD) (open circles), complement-reconstituted (CD+R) (closed circles) and control (closed triangles) blood. Live *E. coli* (1×10^5/mL) was added to whole blood and (more ...)

Complement-Dependent Tissue Factor Expression.

Monocyte tissue factor (TF) expression is a well recognized mechanism of disseminated intravascular coagulation in sepsis (8, 9). *Neisseria meningitidis* (*N. meningitidis*) induced monocyte TF expression in a complement-dependent manner (Fig. 3 *A* and *B*). Deficient blood challenged with bacteria expressed TF only slightly. Reconstitution with C2 or C5 restored TF expression, whereas exposure to the complement inhibitor compstatin or the C5a receptor antagonist reduced the expression level to that of unreconstituted blood. Collectively these data show that complement is responsible for >50% of *N. meningitidis*-induced TF expression.

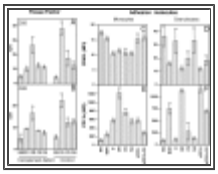

Fig. 3.
Leukocyte expression of tissue factor and adhesion molecules. (*A* and *B*) Expression of tissue factor (TF) on monocytes as indicated by median fluorescent intensity (MFI) in flow cytometry. Blood was challenged with *N. meningitidis* 5×10^6/mL or (more ...)

Differential Complement-Dependent Effects on Cell Adhesion.

CD62L on monocytes from the C5D patient was shed upon exposure to *N. meningitidis*. The shedding was not influenced by C5 reconstitution, but apparently mediated through CD14 (Fig. 3*C*). In contrast, CD62L on granulocytes was shed only after reconstitution with C5 and completely restored by addition of the C5a receptor antagonist (Fig. 3*D*), consistent with an essential role for C5a in the shedding of granulocyte CD62L. This

shedding occurred after reconstitution with C5 even in the absence of bacteria, consistent with the spontaneous activation of C5 under basal conditions and the induction of complement activation by certain plastic surfaces (10). Analogous results were obtained for C2D (Fig. S1). *CD11b* was expressed on monocytes from the C5D patient upon exposure to *N. meningitidis*, and this expression was markedly enhanced by reconstitution with C5 (Fig. 3*E*). Compstatin and the C5a receptor antagonist reduced the expression to levels of C5D blood, indicating that the complement effect was mediated by C5a. Approximately half of the bacteria-induced CD11b expression was mediated through complement and the other half through CD14. The combination of compstatin and anti-CD14 reduced expression of CD11b to background. Granulocytes from the C5D patient expressed CD11b upon exposure to *N. meningitidis* only after reconstitution with C5. The expression was abolished by the C5a receptor antagonist (Fig. 3*F*), consistent with a crucial role for C5 in granulocyte CD11b expression. In contrast to monocytes, the expression of CD11b on granulocytes was not inhibited by anti-CD14. Reconstitution with purified C5 increased the background CD11b expression on granulocytes, as described for CD62L (see above). Similar results were obtained for C2D (Fig. S1). The patterns for CD62L and CD11b were similar for both bacteria, and the response of the control individual was identical to that of the deficient patient after reconstitution with C2 and C5. Collectively, the data indicate that C5 is essential for Gram-negative bacteria-induced changes in CD62L and CD11b on granulocytes; CD14 apparently contributes equally to the monocyte expression of CD11b, whereas the shedding of CD62L from monocytes is complement-independent and relies on CD14.

Inability of C5D To Induce Oxidative Burst.

E. coli did not cause any increase in the granulocyte oxidative burst in the C5D patient (Fig. 4*A*). Reconstitution, however, led to a marked increase that was completely reversed by compstatin and the C5a receptor antagonist. In the C2D patient, the oxidative burst increased modestly and further increased after reconstitution (Fig. 4*A*). This increase was also abolished by complement inhibition. Anti-CD14 caused an additional reduction in burst in both individuals (Fig. 4*A*), but notably, in the absence of C5, no burst was obtained. Similar data were obtained for the oxidative burst in monocytes. Taken together, these data indicate that complement C5 and the

engagement of the C5a receptor are essential for the oxidative burst.

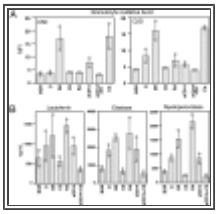

Fig. 4.
Activation of granulocytes. The designations given for the columns at the bottom of the panels are as described in Fig. 3. The “Ctr” column in panel *A* is deficient blood reconstituted with purified complement factor and challenged with (more ...)

Granulocyte Enzyme Release Depends on C3.

Release of the granulocyte specific enzymes lactoferrin, elastase, and myeloperoxidase (MPO) was examined. *E. coli* and *N. meningitidis* induced similar responses, and C2D and C5D responded similarly, but not identically. The data for *E. coli* in C2D blood are presented here (Fig. 4*B*).

The corresponding C5D data are shown in Fig. S2. All enzymes increased upon exposure to bacteria and further increased after reconstitution with C2, but hardly after reconstitution with C5. The enzyme release was apparently highly dependent on C3, since compstatin, but not the C5a receptor antagonist, completely blocked the increase. Anti-CD14 inhibited the release, but was less important than C3, since the combination of anti-CD14 and compstatin was barely more efficient than compstatin alone. These data indicate that complement C3, but not C5/C5a, is essential for granule release. This last observation is a finding in a human system and is in direct contrast to all of the other readouts in this study, in which the complement effect was mainly due to C5/C5a. To confirm these highly unexpected findings, supplementary experiments measuring release of MPO under identical conditions were performed in blood from five healthy blood donors. C3 inhibition by compstatin significantly reduced *E. coli*-induced MPO release close to background levels, whereas the C5a receptor antagonist had no effect (Fig. S3).

Complex Interplay Between Complement and CD14 in Cytokine Induction.

The primary role of complement in the induction of cytokines was investigated using a multiplex assay. Nine of 27 mediators increased after bacterial challenging with similar patterns for *E. coli* and *N. meningitidis*. The C5D data with *E. coli* are presented here. Similar results were obtained using C2D samples (Fig. S4). Similar results were also obtained for *N.*

meningitidis. Responses enhanced by complement: in the complement deficient samples, the cytokines were generally more dependent on CD14 than on complement, and the level of complement-dependence varied substantially among various cytokines (Fig. 5*A*). IL-1β and IL-8 were markedly dependent on complement, MIP-1α and TNF-α were slightly complement-dependent, IFN-γ was hardly influenced by complement, and IL-6 was completely complement-independent and fully dependent on CD14. MIP-1β displayed a pattern like that of MIP-1α. Notably, the release of all these cytokines and chemokines was abolished by the simultaneous inhibition of complement and CD14 (Fig. 5*A*). Responses inhibited by complement: in contrast to the cytokines describe above, the chemokine IP-10 and the anti-inflammatory mediator IL-1ra, increased substantially in the C5D blood after challenge with *E. coli*, and a remarkable inhibitory effect was observed after reconstitution with C5 (Fig. 5*B*). Furthermore, this effect could be reversed by the addition of complement inhibitors. These findings suggest that active complement normally inhibits the release of IP-10 and IL-1ra, an effect that has not previously been reported for human complement. The release of IP-10 and IL-1ra relied heavily on CD14 (Fig. 5*B*). The remainder of the cytokines did not increase upon incubation with *E. coli*, as shown for IL-10 (Fig. 5*C*). Collectively, these data strongly suggest that complement differentially affects the various components of the network, having a significantly enhancing effect on IL-8, IL-1β, and MIP-1α release, a minor effect on TNF-α and IFN-γ, no effect on IL-6, and a significant inhibitory effect on IP-10 and IL-1ra release.

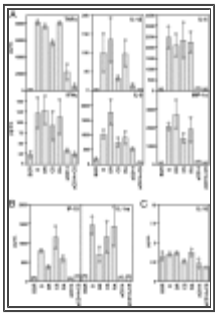

Fig. 5.
Release of cytokines. Results from the C5D patient are presented. Blood was challenged with *E. coli* at 1×10^6 or 5×10^6/mL in separate experiments on two consecutive days. Data are presented as mean and range of these two experiments. (more ...)

Verification of the Experimental Concepts by Complement Activation Assays.

Specific enzyme immunoassays for each of the pathways were used to analyze plasma samples from all of the experiments to confirm that the expected complement activation or lack of complement activation had indeed

taken place in deficient, reconstituted and complement inhibited samples. The results (Tables S1 and S2) support the validity of the data and the conclusions. In particular, there were no differences in activation products between the MBL-deficient individual and the MBL-sufficient control subject, or between the C2D and C5D patients after reconstitution.

DISCUSSION

Genetic deficiencies provide a unique tool for investigating the biological role of proteins in humans. The present study has explored the role of human complement in the inflammatory response by using fresh whole blood from individuals who were genetically deficient in specific complement proteins. We took advantage of a whole-blood model developed specifically for studying the mutual interaction between complement and the other pathways of inflammation (6). A comprehensive and representative panel of inflammatory reactions was examined, making it possible to simultaneously evaluate the relative role of complement in the different branches of the inflammatory network under identical conditions. As inducers of complement activation and inflammation we chose the Gram-negative bacteria *E. coli* and *N. meningitidis*, because both are clinically relevant and are known to induce a broad inflammatory response, including the systemic inflammatory response seen in sepsis (11).

Using this approach, we now provide evidence that, in humans, complement is crucial for several inflammatory reactions, including monocyte expression of TF, granulocyte expression of cell adhesion molecules, oxidative burst, and release of leukocyte enzymes. Furthermore, we observed a striking difference between C3 and C5. C5 was seen to be essential for bacterial killing and its main inflammatory split product C5a was responsible for phagocytosis, oxidative burst, and cell-surface expression of adhesion molecules. In contrast, leukocyte enzyme release was C5a-independent and highly dependent on C3. This finding was in fact highly surprising since this release reaction traditionally has been attributed to C5a. Previous studies, however, have been made with purified cells separated from whole blood and under experimental conditions where they most likely have been primed during the experimental procedure. This is in contrast to our model where the cells were investigated in fresh whole blood with all biological systems present and able to interact. To confirm these findings, we performed a

series of supplementary experiments using whole blood from normal blood donors, incubated with *E. coli* in the presence or absence of the C3 inhibitor compstatin, a C5a receptor antagonist, or a control peptide. The data unequivocally documented a critical role for C3, whereas C5a apparently had no effect on the release of MPO (Fig. S3). There is no evidence from our experiments to indicate that C3a is responsible for the granule release. In fact, it is at present tempting to speculate that contact between bacteria opsonized with C3b and granulocytes might induce enzymes release. Further studies are needed to explore these mechanisms in more detail.

We also identified inflammatory reactions that were less dependent on complement, including the release of several cytokines, and we indeed observed that the release of certain mediators was in fact inhibited by complement. This was the case for IL-1ra, an anti-inflammatory cytokine (12). These data are previously undescribed for humans, but consistent with a reported increase in IL-1ra when a complement inhibitor was used in mice (13). Thus, the activation of human complement may enhance inflammation not only by increasing the release of proinflammatory mediators but also by decreasing the release of anti-inflammatory mediators.

Inflammation is closely linked to coagulation (14, 15) and the expression of TF by monocytes is central to this process. We have now obtained direct evidence that monocyte TF expression is largely dependent on complement in humans. The effect was apparently mediated through C5a since, in reconstituted and normal blood, the C5a receptor antagonist was equally as efficient as compstatin in counteracting complement activation. Previous studies have indicated a relationship between C5a and TF expression on granulocytes (16). TF was not expressed on granulocytes in the present study, but transcriptional up-regulation of TF cannot be excluded since we measured only its membrane expression. Given the potent procoagulatory role of monocytes in disseminated intravascular coagulation (8), it is tempting to speculate that complement activation contributes appreciably to the disturbed hemostasis seen in human sepsis.

Initial events in leukocyte activation include rolling and firm attachment where the adhesion molecules CD62L and CD11b are important (17). A differential dependence of complement on monocyte and granulocyte expression of CD11b has previously been demonstrated (18). Similarly, our

data indicated that CD62L shedding was completely C5-independent and CD14-dependent in monocytes, whereas complement and CD14 contributed equally to monocyte CD11b up-regulation. These findings were in sharp contrast to those for granulocytes, in which both CD62L shedding and CD11b up-regulation were virtually completely dependent on C5a. A major strength of the present model, supporting the validity of the different patterns observed for monocytes and granulocytes, is that these cell populations could be clearly separated and simultaneously studied under exactly the same conditions without manipulation of the cells as part of a purification process.

The granulocytes have developed several strategies to fight microbes (19), including the oxidative burst and granule enzyme release, which may prove harmful also for host tissue. Our findings indicated that C5/C5a was highly critical for the oxidative burst. CD14 was also involved, but only in the context of an intact C5-C5aR axis. Our data support the view that the C5aR is crucial for CD11b up-regulation, which is a prerequisite for phagocytosis and subsequent release of toxic oxygen species. In sharp contrast is the fact that release of the enzymes lactoferrin, elastase, and MPO from granulocytes appeared to be completely C5a-independent; rather the effect was solely dependent on C3, as discussed above. Inhibition of CD14 also reduced the enzyme release, but it was particularly noteworthy that inhibition with compstatin alone was virtually as efficient as combined inhibition of complement and CD14. These data are consistent with a partially redundant CD14-dependent mechanism that can trigger some degree of enzyme release in the absence of complement.

The dissociation of C3 and C5 with regard to the different leukocyte responses has not previously been described in a human setting and emphasizes the value of the current model as a tool for dissecting complement functions at different levels of the cascade. The data presented here might have consequences for the design of potential complement-inhibitory therapeutic strategies for treatment of sepsis, since inhibition at the level of C3 vs. C5 will have different effects on inflammation.

CD14 is an important recognition molecule (20) and together with MD-2 and TLR4, it constitutes the LPS receptor (21). CD14 has also been associated with the functions of TLR2 (22, 23) and TLR3 (24). Thus, both complement

and CD14 act upstream in innate immunity. The data obtained in the present study support our hypothesis that many of the inflammatory mediators induced by Gram-negative bacteria are complement-mediated and that the remaining effects can largely be attributed to CD14. Thus, although they are partly redundant, these systems seem to be the two main initial pathways responsible for the inflammatory reactions induced by *E. coli* and *N. meningitidis*. Our findings document which of the inflammatory reactions would be attenuated by inhibiting either complement or CD14, and they indicate that a combined inhibition of complement and CD14 may be an efficient anti-inflammatory therapeutic regimen, as has recently been proposed by our group on the basis of inhibitory studies of cytokine release (10).

It should be noted that the sample size in our investigation was limited by the extreme rarity of these naturally occurring deficiencies. Genetic C5 deficiency has only been reported in approximately 40 individuals worldwide (25). The genetic basis of the deficiency described here is unique, and the patient is the only C5-deficient individual known in Norway. We demonstrate that C5, but not C2, is crucial for bacterial killing. This result is in contrast to our observations regarding phagocytosis, which was compromised both in C2D and C5D blood. Thus, even though C2D blood lacks the ability to phagocytose *E. coli*, most likely because of insufficient C3 opsonisation, the bacteria are still efficiently killed. C5D blood, however, lacked both the ability to phagocytose and to inhibit bacterial growth, functions that are ascribed to C5a and C5b-9, respectively. The C5-deficient patient was also MBL-deficient, but by introducing an equivalent MBL-deficient control, we were able to verify that the MBL-dependent complement pathway did not play a significant role in the responses we studied.

In conclusion, the strength of our investigation lies in its approach and the techniques used, namely: (*i*) the use of genetically complement-deficient fresh human blood, (*ii*) applied to a whole-blood model allowing inflammatory cross-talk by using complement-inert anticoagulation, (*iii*) activation with clinically relevant Gram-negative bacteria, and (*iv*) including a comprehensive panel of readouts representative of the inflammatory reaction. These studies have allowed us to delineate in humans the complement-dependence of each of the inflammatory responses investigated, as well as the relative importance of C3, C5, and CD14 in those

responses, as schematically illustrated in Fig. 6. Despite their being limited to the in vitro situation, our data add essential information regarding the role of complement in the pathophysiology of Gram-negative sepsis and should be taken into account when designing future anti-inflammatory therapeutic regimens for this condition.

Fig. 6.
The role of complement and CD14 in the inflammatory reactions induced by Gram-negative bacteria. Crucial dependence on C3 and C5 are shown in blue and red, respectively. CD14-dependence is shown in yellow, and the relative dependence on C5 and CD14 is (more ...)

MATERIALS AND METHODS

The study was approved by the regional ethics committee. Patients and controls gave their written, informed consent. Selected parts of the *Materials and Methods* section are described below. For further details, see *SI Text*.

Patients and Controls.

The patients had been diagnosed with complement defects. The C2-deficient (C2D) patient was an 18-year-old male who was referred to the hospital at the age of 9 because of recurrent infections, primarily of the airways. He had followed the normal Norwegian vaccination program without adverse reactions. The clinical investigation was normal, and laboratory testing revealed a normal white blood cell count and normal immunoglobulins, including IgG subclasses. No CH50 activity was detected for the classical pathway, whereas the CH50 for the alternative pathway was normal. Western blot analysis showed an absence of C2 (Fig. 1*B*). The subsequent genetic analyses that were performed are described below and in the legend of Fig. 1. The C5-deficient (C5D) patient, hitherto the only C5-deficient individual identified in Norway, was a 44-year-old woman who had suffered a total of four serious meningococcal infections, including meningitis and severe septicemia, between the ages of 7 and 42. Her sister died at the age of 3 as a result of a meningococcal infection. She had normal Ig levels, including IgG subclasses, and normal levels of complement factors C3 and C4, but no activity in hemolytic assays. Western blot analysis showed a complete absence of C5 (Fig. 1*E*; subsequent genetic analyses are described below and in Fig. 1). The bacteria from her first two

meningococcal infections were not serotyped, but serotypes C and Y, respectively, were identified from the last two infections. After her fourth infection she was vaccinated with the conjugated tetravalent meningococcal vaccine Mencevax against groups A, C, W135, and Y. In both patients, complement deficiency was confirmed in a commercially available ELISA that separately determines the activities of the classical, lectin, and alternative pathways (26). The C5D patient was found to be lectin-pathway deficient as well, with a very low serum concentration of MBL (< 50 μg/L) (Fig. 1*H*). For this reason, we chose as a control for the C5D patient an individual who was C5-sufficient but MBL-deficient, with an MBL genotype similar to that of the patient (Fig. 1 *F* and *H*). Thus, the differences observed between these two individuals could be clearly attributed to the C5 deficiency and not to the MBL-deficiency. Various MBL-deficiencies occur in a considerable proportion of the human population (27). The control individual for the C2D patient was selected on the basis of a having completely normal complement system including MBL.

Genetic Analyses.

These are described in detail in the legend of Fig. 1. The C2 deficiency has been published (7). Western blot analysis was performed according to Laemmli (28). Primers used for the genetic analyses are listed in Table S4. MBL (*MBL2*) genotypes were determined as previously described (29).

Data Presentation and Statistical Considerations.

The design of the present study precluded traditional statistical handling of the material. The data, however, speak for themselves and, according to a recommendation from a biostatistician they are therefore not interrupted with statistics (30).

SUPPLEMENTARY MATERIAL

Supporting Information

Click here to view.

ACKNOWLEDGMENTS.

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FOOTNOTES

Conflict of interest statement: J.D.L. is the inventor of patent applications related to the use of Compstatin and C5aR antagonist as therapeutic complement inhibitors. T.E.M. is an inventor of a patent application related to the use of combined inhibition of complement and CD14. The other authors have no competing financial interest to declare.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0903613106/DCSupplemental.

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