Association Between Acute-Phase Reactants and Advanced Glycation End Products in Type 2 Diabetes

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OBJECTIVE — Type 2 diabetes is associated with chronic low-grade inflammation, but the underlying mechanism(s) is not well understood. Because in vitro studies have shown that advanced glycation end products (AGEs) can trigger inflammatory responses, the present study has investigated whether serum concentration of AGEs is an important determinant of circulating levels of inflammatory markers, like C-reactive protein (CRP), in type 2 diabetic patients.

RESEARCH DESIGN AND METHODS — Diabetic patients ($n = 210$) and healthy control subjects ($n = 110$) of similar BMI were recruited. Serum AGEs were assayed by competitive enzyme-linked immunosorbent assay using a polyclonal rabbit anti-sera raised against AGE-RNase. Plasma high-sensitivity CRP was measured by an immunoturbidimetric assay and interleukin (IL)-6 by enzyme-linked immunosorbent assay.

RESULTS — Serum AGEs were increased in diabetic patients compared with control subjects (4.24 ± 0.88 vs. 3.15 ± 0.81 unit/ml, respectively, $P < 0.01$). Both plasma CRP (1.55 [0.81–2.95] vs. 0.88 mg/dl [0.51–1.89], respectively, $P < 0.01$; median [interquartile range]) and IL-6 (0.80 [0.68–0.97] vs. 0.69 pg/ml [0.48–0.84], respectively, $P < 0.01$) were also higher in diabetic patients than in control subjects. In the diabetic patients, log(CRP) correlated with AGEs ($r = 0.22$, $P = 0.002$) and with log(IL-6) ($r = 0.29$, $P < 0.001$). Forward stepwise linear regression analysis showed that BMI, log(IL-6), and AGEs were significant independent determinants of log(CRP) in the diabetic patients, accounting for 17, 12, and 10% of the variation in log(CRP), respectively.

CONCLUSIONS — Serum concentration of AGEs is increased in patients with diabetes and is an independent determinant of plasma CRP levels. Subclinical inflammation in these patients may therefore be partly due to activation of the inflammatory response by AGEs.

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Accumulating evidence suggests that type 2 diabetes is associated with chronic low-grade inflammation. Cross-sectional studies have shown that concentrations of inflammatory markers are elevated in patients with type 2 diabetes and in those with the metabolic syndrome. Abnormalities include small but definite increases of serum or plasma concentrations of several acute-phase proteins, including C-reactive protein (CRP), serum amyloid A, fibrinogen, α1-acid glycoprotein, and plasminogen activator inhibitor-1 (1–5). The underlying mechanism(s) for the augmented acute-phase response is not well understood, and the stimulus for this response is unknown. A number of hypotheses have been put forward, and these include obesity and insulin resistance, preexisting atherosclerosis and/or diabetic complications, and maladaptation of the normal innate immune system response to environmental threats (6–8). Immune abnormalities or chronic slow infections with concomitant immune activation may also occur as a result of metabolic disturbance in type 2 diabetes rather than representing its cause. It has, however, been suggested by Pickup and Crook (6) that hyperglycemia is unlikely to have a major effect on the acute-phase response in type 2 diabetes. This is based on their observations that there was a lack of correlation between serum sialic acid concentrations and glycemic control in type 2 diabetes (9) and that sialic acid concentration was normal in uncomplicated type 1 diabetic subjects with the same degree of glycemic control as type 2 diabetic subjects (10).

Hyperglycemia leads to nonenzymatic glycation of intracellular and extracellular proteins with the formation of advanced glycation end products (AGEs), a heterogeneous group of compounds that have been implicated in the pathogenesis of many of the complications of diabetes (11,12). Animal and in vitro studies have shown that AGEs affect cellular signaling, activation of transcription factors, and subsequent gene expression. Cytokines and adhesion molecule production are inducible by AGEs via reactive oxygen species production and nuclear factor-κB transcriptional activation (13–15). Because in vitro studies have shown that AGEs can trigger inflammatory responses and we have previously found (16) that serum concentration of AGEs is increased in patients with type 2 diabetes, the present study was performed to determine whether serum concentration of AGEs is an important determinant of circulating inflammatory markers like CRP in type 2 diabetic patients.

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Abbreviations: AGE, advanced glycation end product; CRP, C-reactive protein; IL, interleukin; OD, optical density; RAGE, receptor for AGE.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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RESEARCH DESIGN AND METHODS

— Patients with type 2 diabetes ($n = 210$) were recruited from the diabetes clinics at Queen Mary Hospital, Hong Kong. Patients on insulin therapy were eligible if they had been previously managed by diet and with an oral agent at some point and had no known history of diabetic ketoacidosis. To rule out the confounding effects of preexisting atherosclerosis or renal impairment in causing an elevation of CRP, diabetic patients with clinical evidence of macrovascular disease, macroalbuminuria, and/or impaired renal function were excluded from the study. Macrovascular disease was defined as evidence of ischemic heart disease (according to clinical history and Minnesota coding of electrocardiogram), stroke, transient ischemic attack, or peripheral vascular disease. Diagnosis of retinopathy was based on fundoscopic finding by ophthalmoscopy (with pupils dilated to >3 mm in diameter) and peripheral neuropathy by assessment of vibration perception threshold. Microalbuminuria was defined as mean albumin excretion rate of 20–200 μg/min from two consecutive 12-h overnight urine collections. Patients on statins were eligible if the drug had been withdrawn for 8 weeks, as statins could lower CRP. Of these patients, 6% were on dietary therapy only, 78% of the patients were on oral hypoglycemic agents (11% on sulfonylurea monotherapy, 12% on metformin monotherapy, and the remainder on a combination of sulfonylurea and metformin), and the rest were on insulin therapy. None of the patients were on thiazolidinediones, and <2% of the patients were on aspirin therapy. The mean duration of diabetes was 9.2 ± 6.8 years. Of these patients, 26% had microalbuminuria, 12% had nonproliferative retinopathy, and 7% had neuropathy. We recruited 110 healthy control subjects from the community by advertisement. None of the women were on any form of estrogen therapy in either group. In all of the subjects, fasting blood samples were taken for the measurement of glucose, lipids, HbA_{1c}, interleukin (IL)-6, CRP, and serum AGEs. The study was approved by the Ethics Committee of the University of Hong Kong, and informed consent was obtained from all subjects.

Serum AGEs were measured by competitive enzyme-linked immunosorbent assay using a well-characterized polyclonal rabbit anti-sera raised against AGE-RNase as previously described (16). In brief, 96-well plates were coated with 50 μl/well of AGE-RNase (3.75 μg/ml). Serum (50 μl; 1:4 dilution) was added, followed by 50 μl of 1:500 diluted anti-AGE antibody. Alkaline phosphate-conjugated anti-rabbit IgG (1:2000) in dilution buffer was then added to each well and incubated for 1 h at 37°C. After washing, color was developed by addition of 100 μl pNPP substrate (Sigma). Optical density (OD) at 405 nm was determined by an enzyme-linked immunosorbent assay reader. Results were calculated as 1 – [(experimental OD − background OD)/(total OD − background OD)], and a 50% competition was defined as 1 unit of AGEs.

Plasma high-sensitivity CRP was measured by a particle-enhanced immunoturbidimetric assay (Roche Diagnostics, Mannheim, Germany) using anti-CRP mouse monoclonal antibodies coupled to latex microparticles. Plasma IL-6 was measured by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). Plasma total cholesterol and triglycerides were determined enzymatically on a Hitachi 912 analyzer (Roche Diagnostics, Mannheim, Germany). HDL cholesterol was measured using a homogenous method with polyethylene glycol-modified enzymes and α-cyclodextrin. LDL cholesterol was calculated by the Friedewald equation. HbA_{1c} was measured in whole blood using ion-exchange high-performance liquid chromatography with the Bio-Rad Variant Hemoglobin Testing System (Bio-Rad Laboratories, Richmond, CA).

Results are expressed as mean and SD or as median and interquartile range if the distribution of the data was found to be skewed. Data that were not normally distributed were logarithmically transformed before analyses were made. Subjects with plasma CRP levels >10 mg/l (six in the diabetic group and five in the control group), indicating clinically relevant inflammatory conditions, were excluded from the analysis. Comparisons between the two groups were done using independent sample t test, and Pearson's correlations were used to test the relationship between variables. Multiple stepwise linear regression analysis was used to assess the relationships between CRP and different variables simultaneously.

Table 1—*Clinical characteristics, serum AGEs, plasma IL-6, and CRP in control and diabetic subjects*

	Control subjects	Diabetic patients
n	105	204
Men/women (%)	51/49	51/49
Age (years)	51.0 ± 7.5	53.2 ± 8.9*
BMI (kg/m^2)	25.2 ± 3.4	25.8 ± 3.5
Waist (cm)	81.9 ± 9.4	87.3 ± 8.5†
Waist-to-hip ratio	0.86 ± 0.05	0.90 ± 0.06†
Smokers (%)	9.8	8.5
Fasting glucose (mmol/l)	5.1 ± 0.5	8.1 ± 2.0†
HbA_{1c} (%)	5.8 ± 0.5	7.9 ± 1.3†
AGEs (unit/ml)	3.15 ± 0.81	4.24 ± 0.88†
IL-6 (pg/ml)	0.69 (0.48–0.84)	0.80 (0.68–0.97)†
CRP (mg/l)	0.88 (0.51–1.89)	1.55 (0.81–2.95)†
Total cholesterol (mmol/l)	5.5 ± 0.8	5.7 ± 1.1
Triglycerides (mmol/l)	1.1 (0.8–1.5)	1.5 (0.9–1.9)†
LDL cholesterol (mmol/l)	3.5 ± 0.8	3.8 ± 1.0
HDL cholesterol (mmol/l)	1.3 ± 0.4	1.2 ± 0.3*
Systolic blood pressure (mmHg)	123 ± 19	131 ± 14*
Diastolic blood pressure (mmHg)	78 ± 10	77 ± 9

Data are means ± SD or median (interquartile range). *$P < 0.05$; †$P < 0.01$ vs. control.

RESULTS

— The diabetic patients had significantly higher serum AGEs, plasma IL-6, and CRP than the control subjects (Table 1), and the differences remained significant after adjusting for age, sex, and BMI. Patients without any evidence of diabetic complications still had higher AGEs (4.25 ± 0.86 vs. 3.15 ± 0.81 unit/

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Table 2—*Relationships between anthropometric indexes, HbA$_{1c}$, plasma lipids, and acute-phase reactants and AGEs*

	Control subjects (n = 105)			Diabetic patients (n = 204)		
	Log(IL-6)	Log(CRP)	AGEs	Log(IL-6)	Log(CRP)	AGEs
Age	0.36*	0.23†	0.05	0.12	0.01	0.23*
BMI	0.21†	0.30*	0.1	0.15†	0.30*	0.02
Waist	0.29*	0.32*	0.11	0.12	0.22*	0.09
Waist-to-hip ratio	0.18	0.24†	0.06	0.06	0.11	0.10
HbA$_{1c}$	0.09	0.09	0.15	−0.02	−0.05	0.13
Log(TG)	−0.01	0.18	−0.05	0.06	0.18†	−0.03
HDL cholesterol	−0.12	−0.16	−0.09	−0.04	−0.14†	0.03

*P < 0.01; †P < 0.05. TG, triglyceride.

ml, P < 0.001), IL-6 (0.81 [0.68–0.96] vs. 0.69 [0.48–0.84] pg/ml, P < 0.001), and CRP levels (1.63 [0.95–3.08] vs. 0.88 [0.51–1.89] mg/l, P < 0.001) than nondiabetic control subjects. Comparison of different antidiabetic treatment including the use of metformin showed no significant effect, and those subjects on diet and oral hypoglycemic agents had similar concentrations of AGEs, IL-6, and CRP as those on insulin therapy. In diabetic patients with good glycemic control (HbA$_{1c}$ <6.5%), inflammatory parameters (P < 0.05) and AGEs (P < 0.05) remained significantly higher than those of nondiabetic control subjects. Correlations between log(CRP), log(IL-6), AGEs, anthropometric indexes, HbA$_{1c}$, and lipids are shown in Table 2. In both the control and diabetic subjects, log(CRP) correlated with BMI and waist circumference. A weak correlation with waist-to-hip ratio was only seen in the control subjects. Log(IL-6) correlated with BMI and waist circumference in the control subjects and only with BMI in the diabetic patients. There were no associations between AGEs and BMI or HbA$_{1c}$ in the control subjects, whereas in the diabetic patients, there was a trend toward a correlation between HbA$_{1c}$ and AGEs (r = 0.13, P = 0.06). Plasma lipids did not correlate with inflammatory parameters or AGEs in the control subjects, whereas a weak correlation was found between log(triglycerides), HDL cholesterol, and log(CRP) in the diabetic patients.

The relationships between log(IL-6) and log(CRP) in the control subjects and diabetic patients are shown in Fig. 1*A* and *B*, respectively. There were strong correlations between log(IL-6) and log(CRP) in both groups of subjects. On the other hand, no correlations were found between log(IL-6) and AGEs in either group. Serum concentration of AGEs correlated with log(CRP) only in the diabetic patients but not in the control subjects (Fig. 2*A* and *B*). Forward stepwise linear regression analysis including age, sex, BMI, smoking, AGEs, log(IL-6), and the presence or absence of diabetic complications showed that only BMI, log(IL-6), and AGEs were significant independent determinants of log(CRP), accounting for 17, 12, and 10% of variation in log(CRP), respectively, in the diabetic patients (R^2 of the model = 0.39, P < 0.001). Repeating the analyses with lipid parameters did not

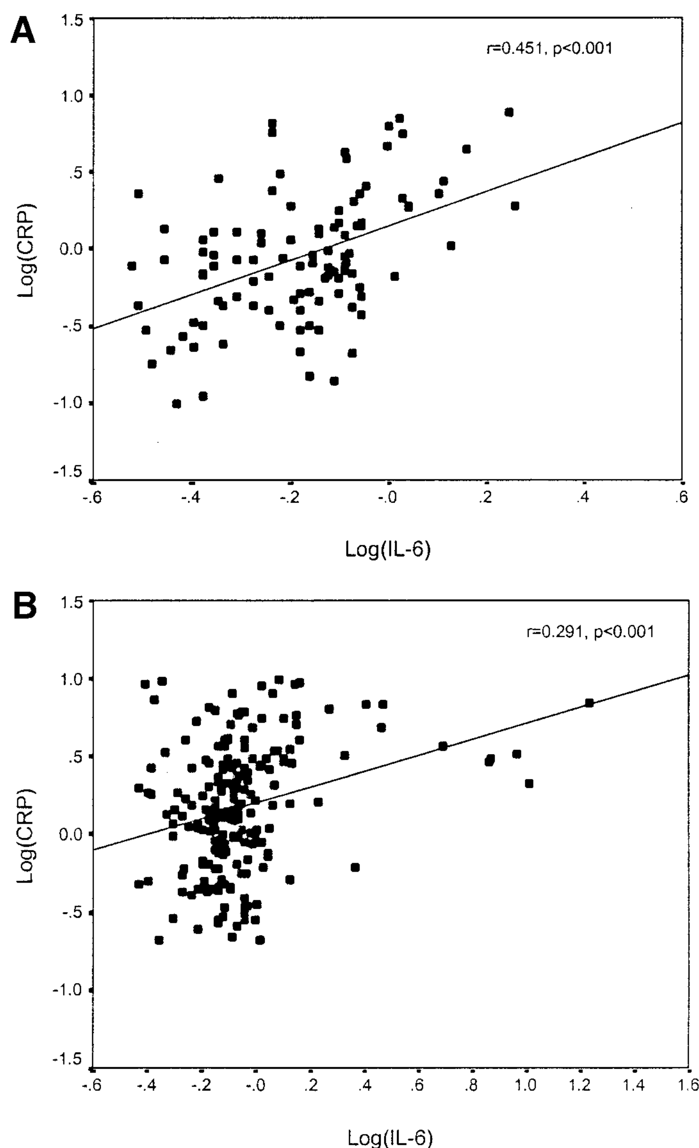

Figure 1—*Correlation between log(IL-6) and log(CRP) in control subjects* (A) *and diabetic patients* (B)*, respectively.*

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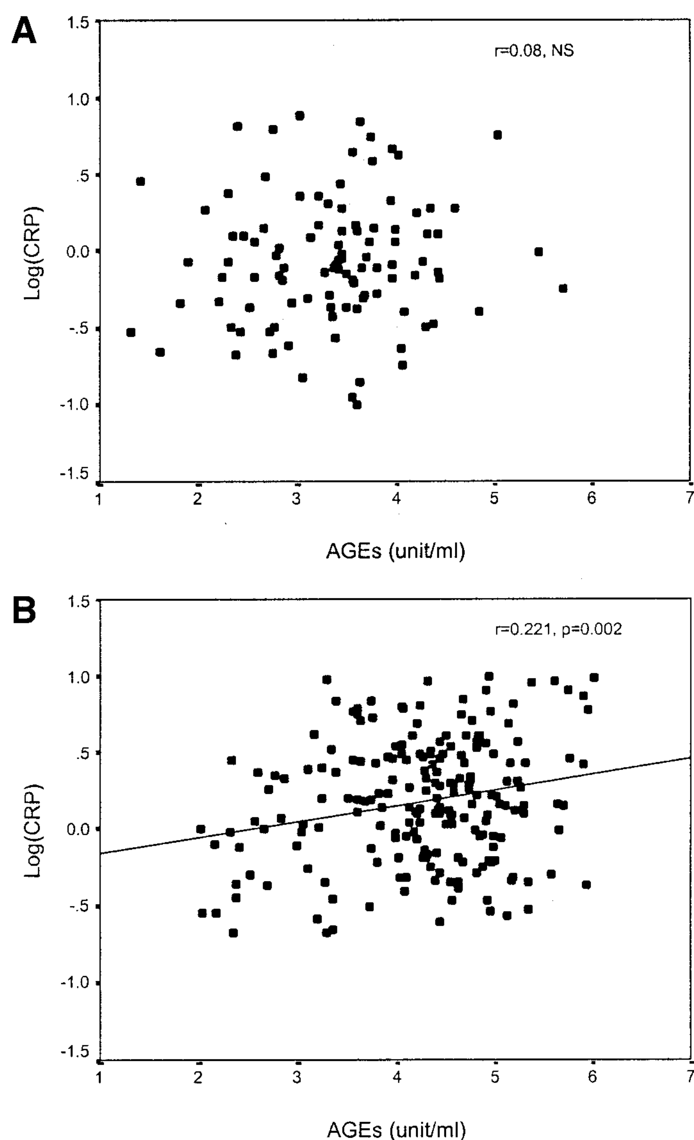

Figure 2—*Correlation between AGEs and log(CRP) in control subjects* (A) *and diabetic patients* (B), *respectively*.

change our results. In nondiabetic control subjects, log(IL-6) was the most important determinant of log(CRP), accounting for 21% of the variability, with BMI accounting for only 6% of the variability (R^2 of the model = 0.27, $P < 0.01$).

CONCLUSIONS — Acute-phase reactants like CRP are primarily produced by hepatocytes, and their chief inductor is the proinflammatory cytokine IL-6. There is also a contribution by other inflammation-associated cytokines such as tumor necrosis factor-α or IL-1 (17). Functionally, CRP provides a downstream integration of overall cytokine activation. Unlike upstream cytokines, CRP has a long half-life, affording stability of levels with no observable circadian variation, and has proved to be a very useful marker of inflammation in clinical and epidemiological studies (18,19). Circulating levels of CRP have been consistently shown to be increased in patients with type 2 diabetes (3–5), but the underlying mechanism(s) is not fully understood. This is the first clinical study to show that chronic subclinical inflammation as indicated by elevated plasma CRP levels is associated with increased serum concentrations of AGEs in patients with type 2 diabetes. Our results may lend support to the hypothesis that low-grade inflammation associated with diabetes and its complications might be mediated in part by AGEs. Many of the biological effects of AGEs, including their proinflammatory action, are receptor dependent, and several receptors have been identified (11). Of these, the best characterized is the receptor for AGE (RAGE), which is expressed by multiple cell types, including endothelium and mononuclear phagocytes (20). Engagement of RAGE by AGEs activates key transduction pathways, such as p21 ras, extracellular signal–related kinases 1 and 2, and nuclear factor-κB, and this cascade of events leads to enhanced expression of proinflammatory mediators (21,22). Macrophages release IL-6, tumor necrosis factor-α, and IL-1β upon stimulation with AGEs (13). The stimulation of monocyte/macrophage by AGEs might therefore be an initial signal of an inflammatory cascade leading to CRP production in the liver. Whether AGEs may also directly induce CRP production in hepatocytes and what type of AGEs may be involved needs further investigation. Although it has been shown that AGEs stimulated IL-6 production by macrophages, we did not find any association between plasma IL-6 and AGEs. This is probably because circulating IL-6 is also partly derived from adipose tissue (23). We also did not find a significant relationship between AGEs and HbA_{1c}. This lack of correlation between AGEs and HbA_{1c} has also been reported by other groups and may be caused by a different turnover and/or a time lag in AGE production and removal (24,25).

In addition to the association between AGEs and CRP, our data showed that BMI and IL-6 were also important determinants of circulating CRP. This is in keeping with previously reported data (26,27). IL-6 is a primary stimulant for the hepatic acute-phase response and is capable of inducing all acute-phase proteins involved in the inflammatory response. A number of cell types may be induced to release IL-6, including activated macrophages as well as T- and B-cells, endothelial cells, and smooth muscle cells. Recent evidence suggests that adipose tissue is also a major source of plasma IL-6. Adipose tissue synthesizes cytokines such as tumor necrosis factor-α and IL-6 (28), and Mohamed-Ali et al. (23) have shown that ~20–30% of the total circulating concentration of IL-6 originates from subcutaneous adipose tissue in healthy adults. Obesity itself can

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promote inflammation and abdominal obesity, and various components of the insulin resistance syndrome, including insulin resistance itself, have been related to increased levels of circulating inflammatory proteins (29–31). Whether chronic inflammation leads to a condition of insulin insensitivity and associated disease or whether insulin insensitivity associated with obesity and atherosclerosis brings about a condition of chronic inflammatory stress is still unclear. We also found a strong correlation between age and inflammatory markers in the control subjects, whereas such a relationship did not exist in the diabetic patients. This may be because factors other than age, like hyperglycemia and the increased formation of AGEs, play a more important role in causing subclinical inflammation in diabetic patients.

A number of factors can potentially confound our results and need to be addressed. The relationship between the quality of diabetes control, CRP, and AGEs may have been biased by multiple drugs and comorbidities that affect CRP levels. In our study, inflammatory parameters in diabetic subjects with good glycemic control remained significantly increased. This is in keeping with a recent study (32) showing that a large number of type 2 diabetic subjects maintained high CRP levels despite achieving glycemic control. Metformin has been reported to lower CRP levels (33), and we have also analyzed the possible modulating effect of antidiabetic drug therapy on cytokine and CRP concentrations but did not find any significant effect. With the development of diabetic complications, a substantial rise of systemic IL-6 has been described (34). Analyses of the associations between IL-6, CRP, and AGEs have to deal with diabetic complications as a possible confounder; therefore, we have included the presence or absence of diabetic complications as a covariate in our model. Another potential confounding factor is the presence of cardiovascular disease. Because individuals with diabetes are at increased risk of atherosclerosis, the higher CRP level can also reflect, in part, the inflammatory component of atherosclerotic process that is so prevalent among patients with diabetes. We have therefore excluded diabetic patients with overt cardiovascular disease in our study. However, some of our subjects might have undiagnosed atherosclerosis, and we did not perform any surrogate measures of atherosclerosis, such as carotid intima-media thickness. Our study is also limited by its cross-sectional design, and relationships between AGEs and CRP cannot therefore be deemed causal in nature.

In summary, serum concentration of AGEs is increased in patients with diabetes and is an independent determinant of plasma CRP levels. Subclinical inflammation in patients with diabetes may therefore be partly due to activation of the inflammatory response by AGEs.

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