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SPECTROSCOPIC STUDIES OF RAT MAST CELLS, MOUSE MASTOCYTOMA CELLS, AND COMPOUND 48/80—II.

THE SYNTHESIS AND SOME BINDING PROPERTIES OF SPIN-LABELED 48/80*

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Abstract—Compound 48/80 has been spin labeled (SL-48/80), purified and its interaction with mastocytoma cells studied. The labeled drug was similar to the unlabeled compound with regard to biological activity and binding to biological membranes. The electron spin resonance (E.S.R.) spectra of membrane bound SL-48/80 were indicative of a bound probe in an environment which is more restricted than that of bound stearic acid spin labels, since the low.field extremum was broad and the high field extremum was not discernible. Although ferricyanide ions broadened the E.S.R. signal of free SL-48/80, this agent did not affect the spectrum of SL-48/80 bound to mastocytoma cells. This would suggest that SL-48/80 binds in a region of the cell that is not in contact with the extracellular aqueous environment. Competition studies showed that SL-48/80 could be displaced by high concentrations of unlabeled 48/80 and polymyxin B.

The pharmacological action of compound 48/80 is well known. Stimulation of mast cells by concentrations of less than 1 μg/ml causes changes in the molecular properties of the plasma membrane that result in its sudden fusion with perigranular membranes [1]. The perigranular membranes also change, fusing with each other to form deep channels within the mast cell that communicate with the external environment. These events occur in less than 10 sec, releasing many potent pharmacological agents from the exposed granules. Compound 48/80 therefore initiates major molecular rearrangements within the plasma membrane that are continued beyond the point of initial stimulation. The nature of the initial stimulation and the sequence of events that culminates in rapid multiple membrane fusion are unknown.

Compound 48/80 was originally synthesized by Baltzly *et al.* [2] as a potent hypotensive agent. The basis for the hypotensive effect was subsequently found to be a noncytotoxic release of histamine from mast cells and basophils that is similar to the anaphylactic response. *In vitro* studies, however, showed that many aspects of the two reactions were different. Unlike the anaphylactic response, compound 48/80-induced mast cell degranulation does not require extracellular calcium [3], is not potentiated by phosphatidyl serine [4], and is not inhibited by low concentrations of disodium chromoglycate [5]. Furthermore, the 48/80-induced reaction is more rapid and efficacious and does not desensitize against the anaphylactic response [6]. It has been suggested, therefore, that 48/80-induced mast cell membrane activation is mediated via a separate pathway, although the final non-cytotoxic secretory process is probably the same.

The structure of the active constituent(s) of 48/80 has yet to be proven conclusively. The mixture of co-polymers is synthesized by condensing *p*-methoxy-*N*-methylphenethylamine with formaldehyde. In the crude product, the polymers range in size from dimer to heptamer or slightly larger. Only the large polymers are active and these usually constitute less than half of the crude product [7]. Recent studies using nuclear magnetic resonance have confirmed the earlier suggestion that the average molecular weight of active 48/80 is consistent with that of a hexamer [8].

At physiological pH, 48/80 is a highly ionized polycation which should bind predictably to many anionic sites both on the membrane surface and within the cell. Sialic acid-containing glycoproteins, acidic phospholipids, anionic proteins, nucleic acids, and the basophilic granular matrix of mast cells are all potential binding sites. Sullivan and Parker [9] have suggested that 48/80 may initiate degranulation by directly decreasing the cyclic AMP content of the perigranular membrane. Hino *et al.* [10], however, have shown that 48/80 bound covalently to Sepharose beads remains active. Based on this finding, they

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have postulated a plasma membrane binding site as the primary target. In a previous study, we have examined the interaction of compound 48/80 with rat peritoneal mast cells and mouse mastocytoma cells with the aid of spin-labeled analogs of stearic acid containing the nitroxide moiety [11]. We now wish to report the synthesis and biological properties of 48/80 which, itself, has been covalently spin labeled. Our results show the usefulness of this approach in studying the interaction of 48/80 with various cell systems.

MATERIALS AND METHODS

Spin-labeled probes and 48/80. Compound 48/80 was synthesized according to the procedure of Baltzly *et al.* [2], and used without further purification. Spin-labeled 48/80 was synthesized by dissolving compound 48/80 (0.35 g) in 30% aqueous acetone (15 ml) and adding IASL* (0.08 g) (Syva Research Chemicals, Palo Alto, CA) and K_2CO_3 (0.35 g). The suspension was stirred at room temperature for 48 hr, and then evaporated to dryness using a water pump. The residue was dissolved in 0.1 N HCl and then purified by gel permeation chromatography using a 100 cm × 4.5 cm column containing Sephadex G-25 (medium) suspended in 0.03 N acetic acid adjusted to pH 3.0 with HCl; it was eluted with the same solvent [7]. The effluent was monitored continuously at 280 nm and fractions were collected and lyophilized. The ratio of 48/80 monomer to spin label in the active fractions was determined to be 20:1 based on the absorbance at 280 nm and the concentration of spin label determined by E.S.R.

Incorporation of SL-48/80 into cells. The SL-48/80 was made up in Locke's solution to a concentration of 1 mg/ml. Aliquots of the stock solution were added to the cells at the concentrations indicated, and then the cells were centrifuged and resuspended in fresh Locke's solution prior to recording their E.S.R. spectrum.

Cell preparation. Purified rat mast cells and murine mastocytoma cells were obtained as described previously [11].

Histamine release. Histamine release was measured using unpurified rat peritoneal cells obtained from decapitated male Sprague–Dawley rats. Locke's solution was injected into the peritoneal cavity and reclaimed after 1 min of massage. The cells in this fluid consisted of 5–10% mast cells that contain virtually all of the histamine. The cells were incubated in SL-48/80 for 10 min at 25° and centrifuged for 10 min at 300 *g*. The per cent histamine released into the supernatant fraction was determined on the basis of that total originally present in the cells. Histamine was measured using the fluorometric method of Shore [12].

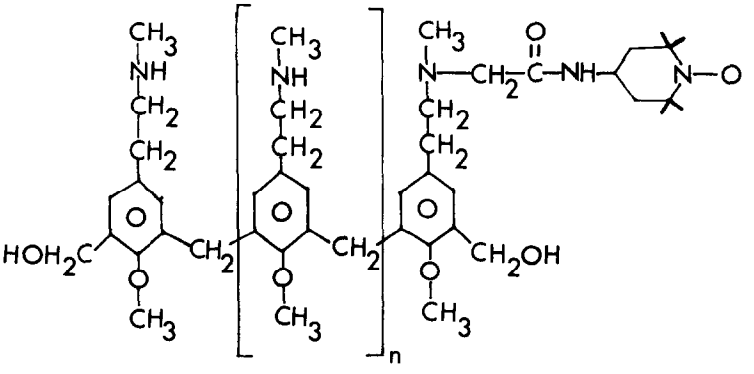
Spectral measurements. Electron spin resonance spectra were recorded using a Varian E-104 spectrometer or a Varian E-109 spectrometer equipped with a TM_{110} cavity as previously described [11]. A variable temperature apparatus was used to record the spectra in Fig. 3. The samples were contained in quartz aqueous sample cells.

RESULTS

Since the ratio of spin label to 48/80 monomer was 1:20 and the active fractions are thought to be hexamers [7, 8], most of the spin-labeled 48/80 polymers would have a single nitroxide moiety. The structure of the spin-labeled polymers is presumed to be that shown below although the exact position of the label is not known.

The elution profile of the spin-labeled 48/80, using Sephadex G-25 gel permeation chromatography, was similar to that previously obtained with unlabeled 48/80 [7]. The high molecular weight spin-labeled 48/80 released histamine in a dose-dependent manner (Fig. 1) similar to that observed for unlabeled 48/80, although it was somewhat less potent than the parent compound. In addition, mastocytoma cells were able to deplete the active SL-48/80 polymers from a solution of mixed polymers, indicating the presence of binding sites for active 48/80 on mastocytoma cells (Fig. 1).

The spectra from six representative column fractions of SL-48/80 were recorded using solutions con-



SL−48/80

* Abbreviations: IASL, 4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidinoxyl; SL-48/80, spin-labeled 48/80; and E.S.R., electron spin resonance.

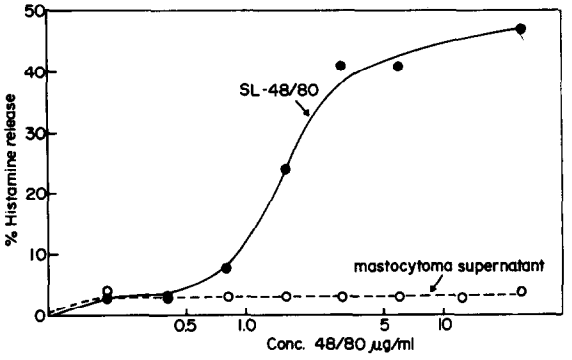


Fig. 1. Binding of active SL-48/80 to mastocytoma cells. Mastocytoma cells were extracted, washed, and incubated in SL-48/80 (fraction 53, 100 µg/ml) for 5 min. The cells were pelleted and the supernatant fraction was recentrifuged at 12,500 *g* for 30 min. It was then diluted in the same way as was the original SL-48/80 solution. The original and the supernatant SL-48/80 were then compared for their abilities to release histamine from rat peritoneal mast cells.

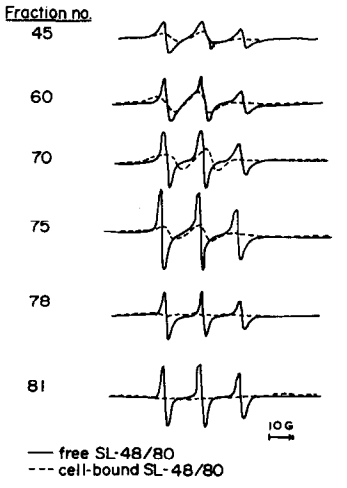


Fig. 2. Binding of various sized polymers of SL-48/80 to mastocytoma cells. Lyophilized SL-48/80 from representative column fractions (Fig. 1) was dissolved in Locke's solution at 100 µg/ml. After scanning the E.S.R. spectrum (solid lines), mastocytoma cells were added and incubated for 5 min. The cells were then spun down and resuspended in fresh Locke's solution. The E.S.R. spectrum of the cells was then scanned (dotted lines).

taining 100 µg/ml. These same solutions were then incubated with equal quantities of mastocytoma cells. The cells were pelleted, resuspended in fresh Locke's solution, and the E.S.R. spectrum of the cell-bound SL-48/80 was recorded. The spectrum of a rapidly tumbling nitroxide has narrow, sharp lines as seen in fraction 81 (Fig. 2). When the molecular motion of the nitroxide is decreased, the lines initially broaden, and then the outer extrema move further apart while their peak heights decrease. This can be seen in fractions 45, 60 and 70 where the nitroxide was more restricted because it was attached to larger polymers. When SL-48/80 was bound to the mastocytoma cells, the motion of the nitroxide was highly restricted and the high field extremum was lost altogether (fractions 45, 60, 70 and 75, Fig. 2). These spectral characteristics were similar for all of the fractions that bound to the cells. The low molecular weight polymers (fractions 78 and 81) did not bind to the cells. This was evident as cells that had been incubated in these fractions and washed had no E.S.R. signal. The E.S.R. spectrum of SL-48/80 bound to purified rat peritoneal mast cells was similar to that bound to the mastocytoma cells; however, it was harder to resolve, due to the difficulty in obtaining sufficient quantities of cells and to their rapid metabolism of the label.

Spectra of SL-48/80 in 50% glycerol were taken at temperatures ranging from 23 to −23°. The spectral shapes ranged from weakly immobilized, exhibiting Lorentzian line shapes, to highly immobilized, with distinct low and high field extrema (splitting = 70 G). Figure 3 shows the spectrum of SL-48/80 bound to mastocytoma cells and the spectrum of SL-48/80 in glycerol at −14°. The spectra are sufficiently similar to suggest that the spectrum of cell-bound SL-48/80 could be indicative of a single highly immobilized species.

To determine whether the SL-48/80 was exposed on the surface of the cells, the effect of ferricyanide ions was studied. When paramagnetic nuclei come within close proximity (4–6 Å) to a spin label, the signal is broadened [13, 14], often to the point where in can no longer be detected [15]. When ferricyanide was added to a solution of SL-48/80, the E.S.R.

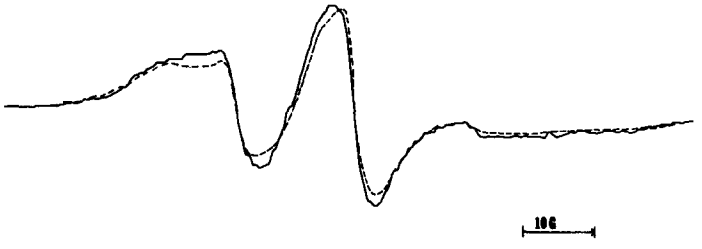


Fig. 3. SL-48/80, in glycerol at −14° and bound to mastocytoma cells. Solid line: SL-48/80 (500 µg/ml) was incubated with mastocytoma cells (10^7/ml) for 10 min and the cells were resuspended in fresh Locke's solution. The spectrum was recorded immediately. Dotted line: SL-48/80 (1 mg/ml) was suspended in 50% glycerol and the spectrum recorded at −14°.

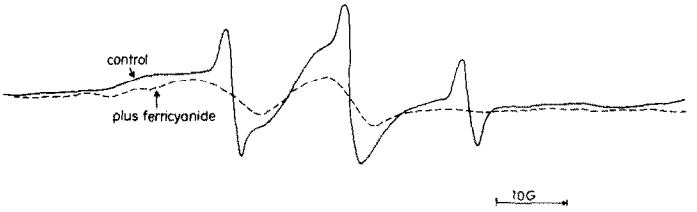


Fig. 4. Effect of ferricyanide ions on the E.S.R. signal from SL-48/80 bound to mastocytoma cells. Mastocytoma cells were suspended in SL-48/80 (100 μg/ml) and the E.S.R. signal was scanned. Several crystals of potassium ferricyanide were added and the signal was re-scanned immediately.

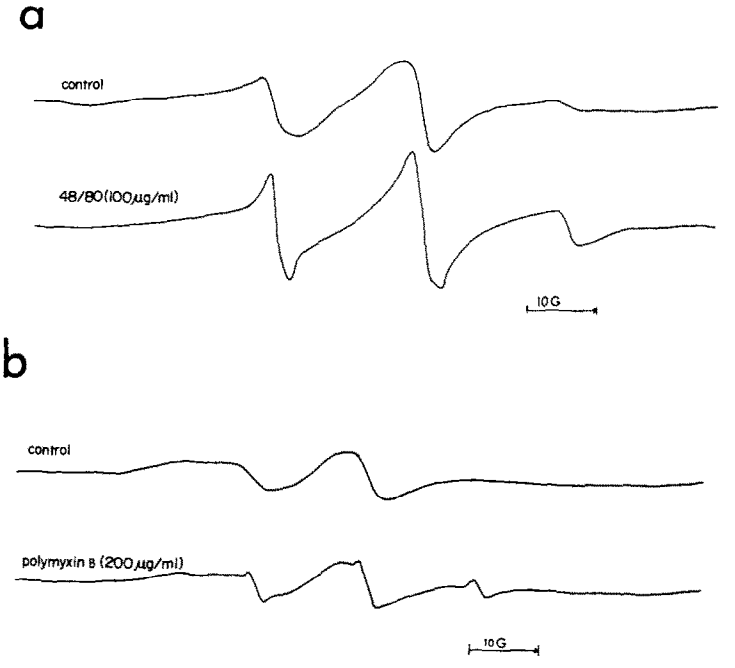


Fig. 5. Displacement of SL-48/80 from mastocytoma cells by 48/80 and polymyxin B. Mastocytoma cells from two mice were treated with SL-48/80 (100 μg/ml), centrifuged and resuspended in Locke's solution. Unlabeled 48/80 (panel a) or polymyxin B (panel b) was added and the spectrum was recorded immediately.

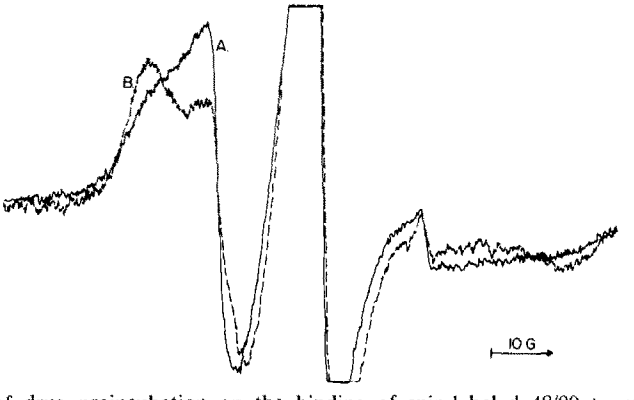


Fig. 6. Effect of drug preincubation on the binding of spin-labeled 48/80 to mastocytoma cells. Mastocytoma cells were divided into aliquots (1 ml) and preincubated for 30 min in the following solutions. SL-48/80 (300 μg) was then added and the cells were spun down and resuspended in Locke's solution. The spectra were scanned immediately. A spectrum similar to the solid line spectrum (A) was seen with cells preincubated in 0.9% NaCl, morphine (1000 μg/ml), curare (500 μg/ml, and concanavalin A (500 μg/ml). The dotted line spectrum (B) was seen with cells pre-incubated in polymyxin B (300 μg/ml) and unlabeled 48/80 (300 μg/ml).

signal was lost due to the interaction of the nitroxide group with the paramagnetic nucleus of the iron. In contrast, when SL-48/80 was bound to mastocytoma cells, the E.S.R. signal of the spin label was not affected by the presence of ferricyanide. However, the sharper peaks, due to unbound SL-48/80, were broadened and disappeared from the spectrum (Fig. 4).

Spin-labeled 48/80 bound to mastocytoma cells can be displaced both by unlabeled 48/80 and polymxin B (Fig. 5). In contrast, morphine, curare, 48/80 monomer (*p*-methoxy-*N*-methyl phenethylamine), and concanavalin A could not displace bound SL-48/80 from its binding sites. The E.S.R. spectrum of spin-labeled 48/80 bound to cells that had been preincubated with unlabeled 48/80 showed evidence for a more uniform population of highly immobilized labels (Fig. 6). The spectra recorded from cells that had been preincubated in morphine, curare, 48/80 monomer, concanavalin A, and polymyxin B showed that none of these drugs except polymyxin B had any effect on the subsequent binding of SL-48/80. The effect of preincubation with polymyxin B on the E.S.R. spectrum of added SL-48/80 was similar to that seen after preincubation with unlabeled 48/80.

DISCUSSION

Compound 48/80 is an ideal molecule for spin labeling as it contains several secondary amine groups. The covalent attachment of a nitroxide moiety to one (or more) of these, therefore, does not greatly reduce either the charge density or the binding properties of the polymer. The elution profile, binding characteristics, and histamine-liberating activity of the labeled drug after partial purification were also similar to those of the unlabeled drug.

The E.S.R. spectrum of SL-48/80 bound to mast cells and to mastocytoma cells was highly complex and could not be interpreted in terms of a correlation time or an order parameter. The general characteristics of the spectrum, however, suggest that the nitroxide moiety was highly immobilized. The width of the low field hyperfine extremum and the absence of a well defined high field extremum were somewhat atypical when compared to the spectra of the more commonly used lipid spin labels. Temperature studies of highly immobilized SL-48/80 in an isotropic solution show that a spectrum similar to that seen with mastocytoma cells can be obtained. This spectrum was intermediate between a very highly immobilized spectrum containing well defined high and low field extrema (splitting = 70 G) and a weakly immobilized spectrum exhibiting Lorentzian line shapes. This would indicate that motional restriction was the major factor in this cell spectrum and that the anisotropic contribution was minimal. Since the anisotropic contribution to the character of the signal cannot be analyzed, however, any interpretation of this type of spectrum in terms of motion or order is speculative. Although the possibility of multiple binding sites is not excluded by the spectral shape of cell-bound SL-48/80, the generation of a similar spectrum in an isotropic medium also indicates the possibility of a single binding site.

It was difficult to study purified mast cells using SL-48/80 for several reasons. Strong immobilization of the probe, binding site heterogeneity, and the possibility of magnetic interactions all contributed to the very broad nature of the spectrum. This made it very difficult to resolve the E.S.R. spectrum of purified mast cells at drug concentrations close to the range of normal pharmacological activity. The second problem with purified mast cells was the high concentration of intracellular binding sites for 48/80 located within the mast cell granules. When SL-48/80 concentrations that display a good signal (> 100 mg/ml) were used, there was extensive cell penetration and binding to granular sites (unpublished results). Signals coming from SL-48/80 bound to the mast cell granules, therefore, interfere with those from a putative membrane binding site. When low concentrations of SL-48/80 were bound to purified mast cells, the E.S.R. signal was similar to that seen with the mastocytoma cells; it was, however, very weak and decayed rapidly. Pure murine mastocytoma cells could be obtained in high quantities, and since they have no granules, they were useful in many experiments to study the basic properties of 48/80 binding to cell membranes.

Although a high concentration of unlabeled 48/80 was required to displace SL-48/80 from mastocytoma cells, this requirement varied with the batch of unlabeled 48/80 (crude) used and the quantity of cells that were labeled. Since crude 48/80 is a mixture of polymers, the percentage of active constituents varies with each preparation, and therefore it is not possible to do competitive studies with any precision. Unlabeled 48/80 does, however, bind to sites in common with the spin-labeled analog. The chemical histamine liberators studied were unsuccessful in displacing SL-48/80 from its binding sites, with the exception of polymyxin B. These stimulators release histamine with different potencies and efficacies: polymyxin B > curare > morphine. Since morphine and curare, even at very high concentrations, could not displace SL-48/80, it must be concluded that these drugs have a low relative affinity for the 48/80 binding sites. The polypeptide antibiotic polymyxin B, however, easily displaced the SL-48/80 molecule, indicating that a polymeric nature is perhaps an important requirement for competition with 48/80. Concanavalin A stimulates histamine release in a manner similar to the anaphylactic response [16], and it may act by binding to carbohydrate residues of cell-bound immunoglobulins (IgE or IgG_2). This lectin was also unsuccessful in displacing compound 48/80, indicating that concanavalin A and SL-48/80 probably bind to two separate locations.

Results similar to the above were also obtained when mastocytoma cells were preincubated with the competitor drugs. Polymyxin B and unlabeled 48/80 were the only histamine liberators tested that changed the spectrum of SL-48/80 bound to 48/80 or polymyxin B pretreated cells. The high field extrema were shifted in a manner indicating that the SL-48/80 was more rigidly bound, and the general shape of the spectra was simplified. This was also shown by the appearance of well-defined low field extrema that were not present in the spectra of SL-48/80 bound to cells that had been preincubated in

Locke's solution. The SL-48/80 was therefore binding to sites that were more homogeneous and/or more highly immobilized. Although the mechanism for this phenomenon is unknown, there are at least two possible explanations. First, pretreatment with unlabeled 48/80 or polymyxin B blocks sites that loosely bind SL-48/80, leaving a more highly immobilized population that then binds the SL-48/80. Alternatively, treatment with 48/80 or polymyxin B may alter the conformation of the SL-48/80 binding site, making it bind the drug in a more highly immobilized manner.

Paramagnetic ferricyanide ions broaden the E.S.R. signal of nitroxides by intermolecular magnetic interactions. Since these negative ions are excluded from intact cells [17], they were useful in determining more precisely the location of the bound SL-48/80. The failure of ferricyanide to alter the E.S.R. spectrum of cell bound SL-48/80 suggests that the label was protected from the external aqueous medium. Previous studies, however, have shown that 48/80, bound covalently to Sepharose beads, can activate cells without penetrating their membranes [10]. Therefore, although 48/80 does not transverse the cell membrane to cause activation, it nevertheless penetrates to a point where it is not exposed to the external aqueous environment.

In summary, direct spin labeling of 48/80 has been successfully achieved with little change in the chemical properties, binding characteristics, or pharmacological activity of the polymers. The spectrum of cell-bound SL-48/80 differs from that of the spin-labeled stearic acids and, although the precise characteristics of probe motion and orientation are unclear, the label appears to be highly immobilized. Competition studies showed that 48/80 (crude) and polymyxin B could displace SL-48/80 from its binding sites, whereas curare, morphine, the 48/80 monomer, and concanavalin A had no effect. Finally, these sites are not in contact with the outside aqueous environment.

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