INFECTION AND IMMUNITY, Nov. 1984, p. 448–452
0019-9567/84/110448-05$02.00/0

Vol. 46, No. 2

Effect of Thioglycolate on Phagocytic and Microbicidal Activities of Peritoneal Macrophages

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Received 4 June 1984/Accepted 24 July 1984

Brewer thioglycolate-elicited mouse peritoneal macrophages were as active as resident peritoneal macrophages in the phagocytosis of opsonized *Staphylococcus epidermidis* but were unable to kill ingested microorganisms. This decreased functional activity was restricted to Brewer thioglycolate-elicited macrophages, since peritoneal macrophages elicited with NIH thioglycolate, alone or supplemented with agar and methylene blue, were as active as resident peritoneal macrophages. No effect of agar on the functional activities of macrophages was observed. A defective intracellular killing by peritoneal macrophages due to Brewer thioglycolate was seen only after an intraperitoneal injection with thioglycolate, not after in vitro incubation of resident macrophages with thioglycolate. The results of this study show that, depending on the kind of thioglycolate used, the functional characteristics of elicited macrophages may alter. However, none of the forms of thioglycolate investigated induced the recruitment of activated macrophages.

In studies with peritoneal exudate macrophages, thioglycolate (TG) is often used as an eliciting agent. This stimulus has the advantage of recruiting a large number of cells to the site of inflammation (2–4, 11) but does not increase the microbicidal activity of macrophages and therefore does not activate macrophages (2, 5). After an intraperitoneal injection of TG, the resistance of mice to infecting microorganisms is decreased (7), probably due to insufficient bactericidal activity of the elicited macrophages (1, 10, 11). The question of which mechanism underlies this insufficient defense mechanism has not yet been answered.

This paper reports a detailed investigation of the effect of TG on the phagocytosis and intracellular killing of microorganisms by peritoneal macrophages.

MATERIALS AND METHODS

TG. Brewer TG (Difco Laboratories, Detroit, Mich.), NIH TG broth (incomplete TG; Difco) with the same formula as Brewer TG except for the omission of agar and methylene blue, and NIH TG broth supplemented with 0.05% (wt/vol) agar and 0.02% (wt/vol) methylene blue (complete TG) were prepared by the directions of the manufacturer. Solutions were stored in the dark for at least 4 weeks before use. Unless otherwise stated, undiluted (i.e., 4% [wt/vol]) solutions were used. Agar (Special Agar [Noble]; Difco) was prepared as a stock solution of 0.5 mg/ml in phosphate-buffered saline (PBS).

Mice. For these studies, we used specific pathogen-free male Swiss mice (Central Institute for the Breeding of Laboratory Animals, TNO, Bilthoven, The Netherlands) weighing between 25 and 30 g.

Peritoneal cells. Resident peritoneal cells were harvested by lavage of the peritoneal cavity as described elsewhere (8, 9). Elicited macrophages were similarly obtained at various intervals after an intraperitoneal injection of 1.5 ml of the eliciting agent under investigation. In control experiments mice received an intraperitoneal injection of 1.5 ml of sterile PBS 4 days before harvesting.

Morphometry. Morphometric measurements were performed on Giemsa-stained cells on cover slips, as described in detail elsewhere (M. van Schadewijk-Nieuwstadt, R. van Furth, and C. Cornelisse, submitted for publication). In short, contours of the cells and nuclei were traced on the surface of a graphic tablet interfaced to a MOP-AM 03 minicomputer (Kontron Messgeräte GMBH, München, Federal Republic of Germany) by using a cursor with a light-emitting diode visible as a bright red spot in the microscopic image. Before measurements were started, the graphic tablet was calibrated in relation to the combined magnification of the objective and the ocular lenses and the drawing tube. The printed output provided the area of the cytoplasm and nuclei. Measurements were done on 50 cells from each cell suspension.

Serum. In phagocytosis and intracellular-killing experiments, newborn calf serum (NBCS; GIBCO, Grand Island, N.Y.) was used.

Microorganisms. *Staphylococcus epidermidis* was cultured overnight in nutrient broth no. 2 (Oxoid Ltd., London, England) at 37°C, harvested by centrifugation for 10 min at 1,500 × *g*, washed twice with PBS, and suspended in Hanks balanced salt solution (HBSS) containing 0.1% gelatin (gelatin-HBSS) to a concentration of about 10^7 cells per ml. When necessary, bacteria were opsonized by incubation with 10% NBCS for 30 min at 37°C under rotation, after which the excess serum was removed by two washes with gelatin-HBSS, and the bacteria were suspended in gelatin-HBSS to a concentration of 10^7 cells per ml.

Phagocytosis assay. Phagocytosis of microorganisms was measured as a decrease in the number of viable extracellular bacteria during incubation of macrophages and bacteria (bacteria-to-cell ratio, 1:1; concentration, 5×10^6 cells per ml) in the presence of 10% NBCS at 37°C under rotation (4 rpm) as described elsewhere (4). Phagocytosis was expressed as the percent decrease in the number of viable extracellular bacteria. Values for the phagocytosis of *S. epidermidis* corrected for the extracellular growth of these bacteria were calculated as described previously (6, 9).

Intracellular killing. Intracellular killing of *S. epidermidis* by macrophages was measured as a decrease in the number of viable intracellular bacteria during incubation of macrophages containing ingested bacteria after phagocytosis of opsonized bacteria for 20 min in the presence of 10% NBCS at 37°C at 4 rpm, as described elsewhere (4).

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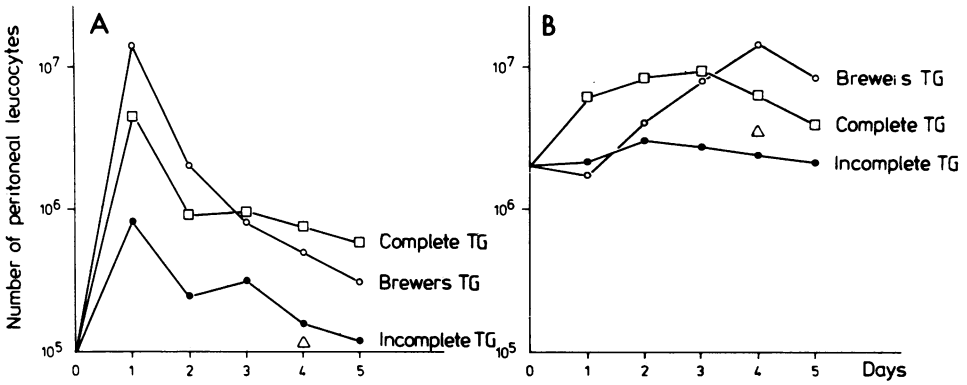

FIG. 1. (A) Course of the number of peritoneal granulocytes after an intraperitoneal injection of 1.5 ml of undiluted (i.e., 4% [wt/vol]) thioglycolate of various kinds: Brewer TG (○), complete TG (□), and incomplete TG (●). The number of granulocytes 4 days after an intraperitoneal injection of 1.5 ml of 0.05% agar (△) is also given. (B) Course of the number of peritoneal macrophages after an intraperitoneal injection of 1.5 ml of thioglycolate of various kinds (for symbols, see above).

Statistics. All values represent the mean and standard deviation of at least three experiments. Statistical analysis was performed by Student's *t* test for unpaired observations.

RESULTS

Course of the number of leukocytes in the peritoneal cavity after injection of TG. Intraperitoneal injection of various kinds of TG led to changes in the number and composition of leukocytes in the peritoneal cavity. With all three kinds of TG, a maximum number of granulocytes was observed 1 day after injection, followed by a return to normal values (Fig. 1A); the number of peritoneal lymphocytes remained almost unchanged during 5 consecutive days (data not shown).

Injection of Brewer TG increased the number of peritoneal macrophages from ca. 2×10^6 on day 0 to ca. 1.5×10^7 on day 4 (Fig. 1B). Injection of complete TG led to almost the same increase in the number of peritoneal macrophages as did the injection of Brewer TG. Injection of incomplete TG had no effect on the number of peritoneal macrophages. Intraperitoneal injection of 1.5 ml of 0.05% agar resulted in a slightly inflammatory response reflected by doubling of the number of peritoneal macrophages at day 4 after injection (Fig. 1B). Injection with PBS had no effect on the number of peritoneal macrophages (data not shown).

Since the highest number of peritoneal macrophages (increase of 650%) was reached 4 days after injection of undiluted Brewer TG, the effect of injecting various concentrations of Brewer TG on the number of macrophages at day 4 was investigated. The results revealed that a 2% solution of Brewer TG increased the number of peritoneal macrophages to 8×10^6 at day 4, i.e., an increase of 300%, whereas injection of a 1% solution resulted in an increase of only 100%.

Effect of Brewer TG on the morphometric parameters of peritoneal macrophages. To investigate whether Brewer TG affects the morphology of macrophages, the surface areas of

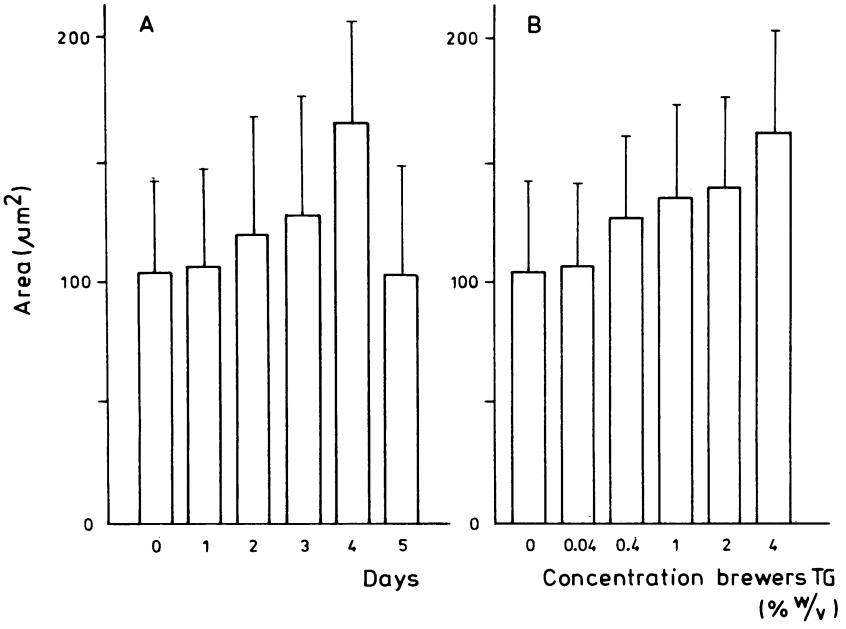

FIG. 2. (A) Changes in the surface area of peritoneal macrophages after intraperitoneal injection of 1.5 ml of undiluted Brewer TG. (B) Surface area of macrophages 4 days after intraperitoneal injection of various concentrations of Brewer TG.

the cytoplasm and of the nucleus were determined in cytocentrifuge preparations of Brewer TG-elicited peritoneal macrophages. The surface area of elicited macrophages increased up to a maximum at day 4; on day 5, the surface area had returned to a normal value (Fig. 2A). No differences in the nuclear surface areas of the cells were observed (data not shown). Comparison of the effect of various concentrations of Brewer TG on the cytoplasmic area of macrophages 4 days after injection showed a dose-related increase in the surface area of these cells (Fig. 2B).

Phagocytosis and intracellular killing of *S. epidermidis* by peritoneal macrophages elicited by TG. Since a maximal number of macrophages was obtained 4 days after injection with Brewer TG and the number of granulocytes was less than 4% of the number of macrophages at day 4, functional assays were performed with TG-elicited macrophages harvested 4 days after injection. Incubation of 5×10^6 peritoneal macrophages obtained from mice injected with PBS, Brewer TG, complete TG, incomplete TG, or agar together with 5×10^6 *S. epidermidis* cells in the presence of 10% NBCS at 37°C led in all cases to an identical decrease in the number of viable extracellular bacteria (Fig. 3). These results indicate that injection with TG or agar does not affect the phagocytic capacity of peritoneal macrophages.

Intracellular killing of *S. epidermidis* determined after incubation of 5×10^6 PBS-elicited macrophages per ml containing bacteria (after 20 min of ingestion of *S. epidermidis* at a bacteria-to-macrophage ratio of 1:1), in the presence of 10% NBCS, amounted to 82.8% at 60 min and 94.9% at 120 min (Fig. 4). The killing indices of macrophages elicited with complete TG, incomplete TG, or agar were similar to those for PBS-elicited macrophages (Fig. 4). However, macrophages elicited with Brewer TG showed an almost complete absence of intracellular killing (Fig. 4). Comparison of the effect of various concentrations of Brewer TG on the intracellular killing by peritoneal macrophages showed a dose-related impairment of the intracellular killing of *S. epidermidis* by Brewer TG-elicited macrophages (Fig. 5).

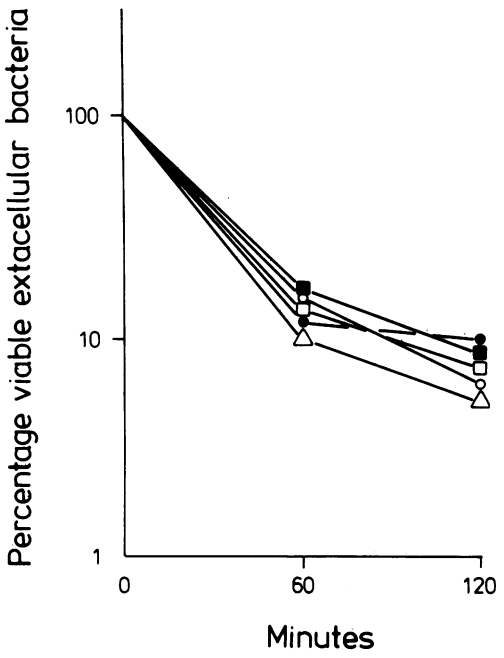

FIG. 3. Phagocytosis of *S. epidermidis* by macrophages elicited 4 days after an intraperitoneal injection of 1.5 ml of agar (○) or undiluted TG of the following kinds: Brewer TG (△), complete TG (□), incomplete TG (■). In control experiments macrophages were harvested 4 days after injection of 1.5 ml of PBS.

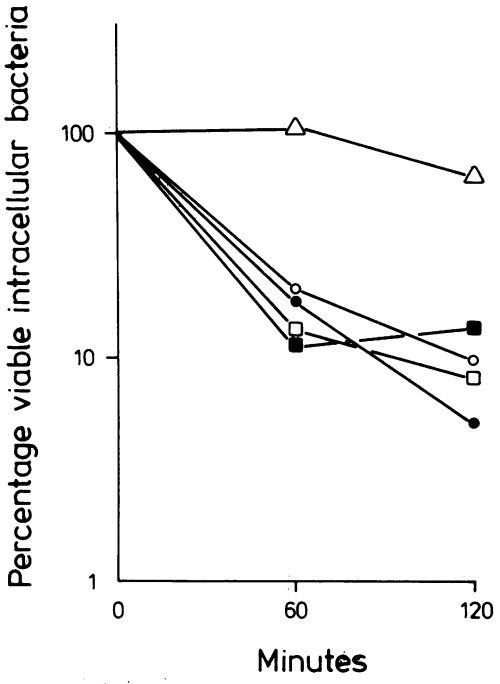

FIG. 4. Intracellular killing of *S. epidermidis* by macrophages elicited 4 days after an intraperitoneal injection of 1.5 ml of agar (○) or undiluted TG of the following kinds: Brewer TG (△), complete TG (□), and incomplete TG (■). In control experiments macrophages were harvested 4 days after injection of 1.5 ml of PBS.

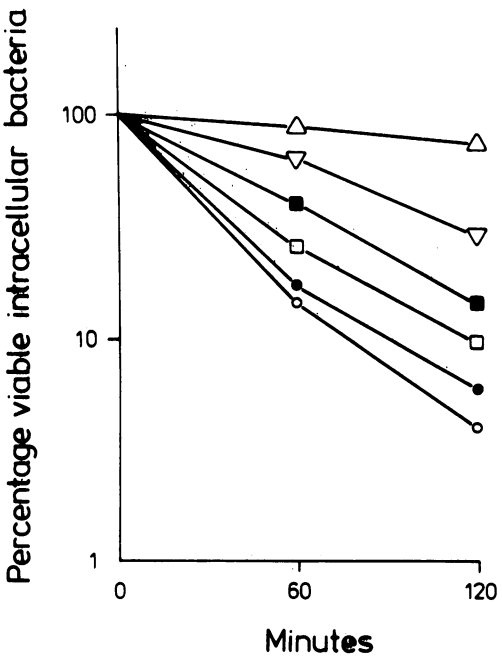

FIG. 5. Intracellular killing of *S. epidermidis* by peritoneal macrophages elicited 4 days after an intraperitoneal injection of the following concentrations (%) of Brewer TG: △, 4.0; ▽, 2.0; ■, 1.0; □, 0.4; ●, 0; ○, 0.04.

TABLE 1. Effect of various concentrations of brewer TG on the phagocytosis, intracellular killing, and viability of resident peritoneal macrophages

Concn (%) of Brewer TG	Phagocytic index (%) at 120 min		% Intracellular killing at 120 min (no. of expt; P_3[d])	% Viable macrophages[e] at 120 min (no. of expt.)
	Observed value (P_1[a])	Corrected value[b] (P_2[c])		
0	90 ± 6	93 ± 5	90 ± 6 (6)	94 (10)
0.04	93 ± 4 (NS)	96 ± 7 (NS)	N.D.	95 (3)
0.4	82 ± 12 (NS)	92 ± 6 (NS)	N.D.	92 (3)
1.0	73 ± 17 (<0.01)	94 ± 8 (NS)	88 ± 8 (3; NS)	93 (3)
2.0	59 ± 18 (<0.001)	90 ± 13 (NS)	87 ± 6 (3; NS)	91 (6)
4.0	20 ± 21 (<0.001)	63 ± 25 (<0.01)	81 ± 7 (3; NS)	70 (6)

[a] P_1, Significance compared with the observed phagocytic index in the absence of TG. NS, Not significant.
[b] The corrected value was calculated by the formula $F(t) = 1 - (N_t \times B_0/N_0 \times B_t)$ in which N_t is the observed number of viable bacteria at time t during incubation of macrophages and bacteria; N_0, this number at time $t = 0$; B_0 and B_t, numbers of bacteria during incubation of bacteria alone in the presence of TG, respectively.
[c] P_2, Significance compared with the corrected phagocytic index in the absence of TG. NS, Not significant.
[d] P_3, Significance compared with intracellular killing index in the absence of TG. ND, Not determined.
[e] Viability was assessed by trypan-blue exclusion.

Phagocytosis and intracellular killing of *S. epidermidis* by resident macrophages in the presence of TG. To find out whether the decreased intracellular killing by Brewer TG-elicited macrophages is due to residual TG on the surface of these cells during harvesting procedures, the effect of extracellular TG on macrophage functions was assessed. For this purpose, 5×10^6 resident macrophages per ml were incubated with 5×10^6 *S. epidermidis* cells per ml in the presence of 10% NBCS and various concentrations of Brewer TG. The results showed a concentration-dependent decrease in the ingestion of bacteria (Table 1). However, after correction of the phagocytosis index for the rapid growth of extracellular bacteria in the presence of TG, all indices were similar to those obtained in the absence of TG except that of 4% Brewer TG; with the latter concentration, the viability of the macrophages was also reduced (Table 1).

Incubation of 5×10^6 resident macrophages per ml containing ingested *S. epidermidis* in the presence of 10% NBCS and 2% Brewer TG showed that TG does not affect intracellular killing of previously ingested bacteria (Table 1).

To assess any effect of preincubation of macrophages with Brewer TG on functional capacities of these cells, 5×10^6 macrophages per ml suspended in HBSS containing 20% NBCS was incubated with 2% Brewer TG up to 24 h at 37°C at 4 rpm. The results showed a significant reduction in the number and viability of the macrophages after incubation for more than 3 h (Table 2). Investigation of the functional state of the viable cells revealed a marked impairment of ingestion and intracellular killing activity (Table 2). In control experiments in which the cells were incubated without TG, the initial number of macrophages was reduced by 20% after 24 h, and the surviving cells showed normal functional capacities (Table 2).

DISCUSSION

Brewer TG, a culture medium routinely used in bacteriology, is also used to elicit large numbers of peritoneal macrophages resembling activated macrophages in many respects (2, 5, 11). The present results show that 4 days after injection of at least 1% Brewer TG, the number of exudate macrophages is maximal, a finding compatible with earlier reports (3, 10). The time course of the number of macrophages was identical to that observed for the changes in the size of the macrophages. The size of TG-elicited cells on day 4 was almost double that of resident macrophages. On day 5, the number and size of the macrophages had returned to normal.

Comparison of the inflammatory responses after injection of TG of various kinds and of agar showed that Brewer TG and complete TG both induced maximal recruitment of granulocytes by day 1 postinjection; Brewer TG gave the maximal number of macrophages by day 4 and complete TG gave the maximal number by day 3. Since on day 4 all suspensions of TG-elicited macrophages showed minimal contamination by granulocytes, which hamper assay of the functional activities of macrophages, further experiments were performed with cells obtained 4 days after TG injection.

Macrophages elicited with various kinds of TG or agar proved to be as active as resident macrophages with respect to phagocytosis of opsonized bacteria. Intracellular killing by TG-elicited macrophages was on the same level as that by elicited or resident macrophages, except that by Brewer TG-

TABLE 2. Effect of brewer TG on the number, viability, and functional activity of peritoneal macrophages[a]

Period (h) of incubation	No. ($\times 10^6$) of macrophages (P)	% Viability (P)	% Phagocytosis at 120 min (P)	% Intracellular killing at 120 min (no. of expt; P)
0	6.5 ± 0.5	99 ± 1	97 ± 2	90 ± 6 (6)
1	5.7 ± 0.8 (NS)	95 ± 4 (NS)	97 ± 2 (NS)	89 ± 6 (3; NS)
2	5.4 ± 0.8 (<0.1)	85 ± 10 (<0.01)	93 ± 4 (<0.1)	81 ± 7 (3; <0.1)
3	4.0 ± 1.7 (<0.1)	70 ± 10 (<0.001)	85 ± 6 (<0.01)	43 ± 14 (3; <0.001)
5	3.0 ± 1.0 (<0.01)	65 ± 8 (<0.001)	36 ± 10 (<0.001)	15 ± 10 (3; <0.001)
24	0.5 ± 0.4 (<0.001)	50 ± 11 (<0.001)	6 ± 5 (<0.001)	10 ± 8 (3; <0.001)
24[b]	5.6 ± 0.9 (NS)	87 ± 12 (<0.1)	91 ± 4 (<0.1)	85 ± 7 (6; NS)

[a] Macrophages (5×10^6/ml) were incubated with 20% NBCS and 2% Brewer TG at 37°C and 4 rpm; after various intervals, the cells were washed; the concentration was adjusted to 5×10^6; and viability, phagocytosis, and intracellular killing were determined. P, Significance compared with values at start of incubation; NS, significance ≥0.1.
[b] Incubation without thioglycolate.

elicited macrophages. The decreased ability of Brewer TG-elicited macrophages to kill ingested bacteria is in agreement with previous reports describing an increased mortality of mice infected with *Listeria monocytogenes* when Brewer TG was injected intraperitoneally (7) and describing a decreased in vitro bactericidal action of TG-elicited macrophages (11). However, because the latter studies were done with methods measuring ingestion and intracellular killing simultaneously, these studies did not indicate which phase of the phagocytic process was affected by TG. An increase in the number of viable bacteria instead of an expected decrease might be due to depressed ingestion, a higher growth rate of the extracellular bacteria, or reduced intracellular killing. The present studies using in vitro assays, in which the growth rate of bacteria without cells, the rate of phagocytosis, and the rate of intracellular killing were measured independently of each other, revealed that a functional defect of Brewer TG-elicited macrophages must be attributed to an impaired killing capacity, since the rate of phagocytosis by these cells equaled that of resident and PBS-elicited macrophages.

The decreased bactericidal activity induced by Brewer TG is in all probability due to the composition of this kind of TG, because incomplete TG (NIH TG), complete TG (NIH TG supplemented with agar and methylene blue), and agar elicit macrophages that kill ingested bacteria as efficiently as resident macrophages do. The observation that agar-elicited macrophages were as active as resident macrophages in the intracellular killing of bacteria disproves an earlier conclusion attributing the decreased functional activities of Brewer TG-elicited macrophages to the agar present in this kind of TG (4, 10, 11).

ACKNOWLEDGMENTS

This study was partially supported by the J. A. Cohen Institute for Radiopathology and Radiation Protection, Leiden, The Netherlands.

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