DIFFERENTIATION OF DENDRITIC CELLS IN CULTURES OF RAT BONE MARROW CELLS

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The dendritic cell (DC)[1] provides accessory activity required for responses of T lymphocytes initiated by mitogens (1–3) or antigens (4), acts as a potent stimulator of an MLR (5–7), and serves as a critical passenger cell, which elicits rejection of transplanted tissues (8). The DC belongs to a group of related or identical cells that includes interdigitating cells (9–11), veiled cells (12–14), and Langerhans cells (15–17); the relationship among these cells has not yet been clarified (18–20). With the exception of the nervous system, DC exist as a minor population in all tissues examined (6, 21–23), peritoneal exudates (6), lymph (14, 24, 25), and blood (26). As with other blood-borne cells, the DC has a bone marrow origin (23, 27–29).

Previous studies (1, 6) identified the DC as the principal accessory cell required for responses of T lymphocytes to treatment with periodate. Measurements on a number of tissues, combined with fractionation procedures and morphological examination, permitted the identification of DC in these tissues. Despite their origin in bone marrow, DC were not identified by these means in fresh preparations of bone marrow cells (BMC). Further studies, presented in this paper, indicate that DC develop in short-term cultures of BMC. Moreover, they arise from precursors having a number of properties that differ from DC.

Materials and Methods

Materials

Reagent grade sodium periodate was purchased from Matheson, Coleman, and Bell (Norwood, OH). The mAb, OX3 (anti–rat Ia) and OX7 (anti–rat Thy-1), were obtained from Accurate Chemical and Scientific Corp. (Westbury, NY); G489.17 was kindly provided by Dr. Carl Beyer (The Rockefeller University, New York), who has shown (personal communication) that it specifically immunoprecipitates rat Ia. Rabbit anti-mouse IgG, F(ab′)$_2$ fragments, was purchased from Cappell Laboratories (Malvern, PA).

Animals

Bone marrow cells. BMC were obtained by flushing the femurs and tibias with cold HBSS. Single-cell suspensions were prepared by resuspending the tissue in HBSS. Cells were filtered through sterile cotton, washed in HBSS, and counted in a hemocytometer or on a Coulter Counter (Model ZBI; Coulter Immunodiagnostics, Hialeah, FL). An average of 3×10^8 BMC/ rat was obtained.

Lymph node cells (LNC). The preparation of cells from cervical and mesenteric lymph nodes was previously described (30).

Cell Culture Conditions

BMC were cultured in 35-, 60-, or 100-mm Petri dishes (Falcon Labware, Oxnard, CA) at concentrations up to 5, 15, or 50×10^6 cells/dish, respectively. The serum-free medium consisted of 45% Ham's F-12 medium (Gibco Laboratories, Grand Island, NY), 45% Iscove's medium (Gibco) and 10% Click's additive; the medium contained a final concentration of 0.1% bovine plasma albumin (BPA) (Armour Fraction V; Reheis Chemicals, Phoenix, AZ). The cells were cultured in a humidified atmosphere of 7% CO_2 in air at 37°C.

Fractionation of BMC and LNC

BMC and LNC were fractionated in a discontinuous gradient of BPA. The light and dense BPA solutions were prepared as described previously (31). Cells were suspended to a maximum concentration of 10^8 cells/ml in 5 ml of dense (ρ = 1.085 g/ml) BPA, overlayed with 1 ml less dense (ρ = 1.048 g/ml) BPA, and centrifuged at 10,000 g for 30 min at 4°C. Low-density (LD) cells at the interface and high-density (HD) sedimented cells were collected separately and washed.

Opsonized Erythrocyte (EA)-rosetting

Formation and removal of EA-rosettes were performed according to our previous description (6).

Periodate Treatment and Assay for Accessory Activity

HD-LNC to be used in the assay for accessory activity were treated with freshly prepared 1.2 mM sodium periodate in PBS for 15 min at 0°C, centrifuged at 535 g for 5 min at 4°C, washed once in HBSS at room temperature, resuspended at a concentration of 6 × 10^6 cells/ml in RPMI 1640 (KC Biologicals, Lenexa, KS) containing 10% heat-inactivated horse serum (Gibco), and dispensed in 100 µl aliquots to wells of a Microtest III plate (3072; Falcon Labware). Cell suspensions to be tested for accessory activity were serially diluted in the same medium and added as 100 µl aliquots to triplicate cultures.

Incorporation of [³H]thymidine was used to assess lymphocyte proliferation. Cells were labeled during the final 4 h of a 45–50-h incubation with 5 µl/well of RPMI 1640 containing [³H]thymidine (6.7 Ci/mmol; Research Products International, Mount Prospect, IL) and unlabeled thymidine. Final concentration of thymidine in each well was 10^{-5} M at 300 mCi/mmol, sp act (30). At the end of the labeling period, the cells were collected on glass fiber filter paper with a Titertek semiautomatic cell harvester (Flow Laboratories, Rockville, MD), and the radioactivity associated with the washed and dried filters was determined by scintillation counting. Values are means ±SD of triplicate cultures. Given the linear dose-response, a unit of accessory activity was defined as the number of cells producing a response 2,000 cpm above that of the control.

Panning

We used an indirect panning method according to the instructions of Dr. Carl Beyer, The Rockefeller University, who modified the original procedure of Wysocki and Sato (32). Depending on the number of cells to be panned, Falcon tissue culture dishes (3001, 3002, or 3003; Falcon Labware) were incubated overnight at 4°C with 1.5, 3, or 8 ml, respectively, of rabbit anti–mouse IgG, F(ab')₂ fragments, 1 mg/ml in PBS. The antibody solution was then removed, stored at 4°C for future use, and the antibody-coated dishes were washed three times with PBS.

Cells to be panned were incubated with the desired antibody (OX3 or G489.17) for 1 h. In the case of BMC, the maximum number panned on the three sizes of dish was 8, 20, or 50×10^6, respectively. After incubation, the dishes were gently rocked and swirled, the nonadherent cells were removed, the process was repeated three times after addition of PBS, and the rinsings were combined. The cells were then centrifuged (375 g, 10 min),

washed once in HBSS, and counted. In some experiments, the panned cells were subjected to a second panning on another antibody-coated petri dish.

Immunoperoxidase Staining

Slides were coated with poly-L-lysine (100 μg/ml in PBS) for 1 h at 4°C, drained, and air-dried. 20 μl of a cell suspension (2 × 10^6 cells/ml in HBSS) was placed on the slide, and the cells allowed to adhere during a 20-min incubation at 4°C in a humidified atmosphere. Slides were dipped in 0.1% gelatin in PBS, fixed for 30 s in an ace-tone/formaldehyde, phosphate-buffered fixative, rinsed well in water, and air-dried. After treating the slides with 3% H_2O_2 in 0.1 M Tris buffer, pH 7.4, for 5 min at room temperature to inactivate any endogenous peroxidase activity, and rinsing well with buffer, 10% horse serum in PBS was added, and the slides were incubated for 20 min at room temperature. The horse serum in PBS was aspirated, and then, without washing, 20 μl of mAb, titrated to establish a low concentration that was still effective for staining, was added in a 0.1% BSA solution and the slides were incubated 30 min at room temperature. After a rinse with Tris buffer diluted 1:10 in PBS (Tris-PBS), 20 μl of affinity-purified, biotinylated horse anti–mouse IgG, diluted 1:200 in 0.1% BPA in PBS, was added, the slides were incubated for 30 min at room temperature, and rinsed with Tris-PBS. 20 μl of a mixture of avidin DH and biotinylated horseradish peroxidase was added, prepared according to the instructions for Vectastain (Vector Laboratories, Burlingame, CA) and allowed to stand 30 min before use; the incubation was carried out for 1 h at room temperature, and the slides were rinsed. Finally, 20 μl of a diaminobenzidine solution (6 mg/ml in 0.1 M Tris buffer, pH 7.4, containing 0.03% hydrogen peroxide and 0.01 M imidazole) were added, and the slides were incubated at room temperature for 10–20 min, rinsed, and mounted with coverslips. In all experiments, >1,000 cells were counted for each duplicate sample.

Results

Accessory Activity in Freshly Isolated BMC.   Freshly isolated BMC had low accessory activity (<0.001 mU/cell) when tested using periodate-treated lympho-cytes as responders. After fractionation of BMC on a discontinuous gradient of BPA, some accessory activity (0.003–0.007 mU/cell) was found in the minor fraction of LD-BMC (4.9 ± 1.6% of total fractionated BMC, $n = 87$). The LD fraction contains nearly all of the accessory activity present in a number of fractionated rat tissues (6). In comparison, the accessory activity measured on LD-BMC was 20–40-fold lower than that found for LD-LNC (0.132 ± 0.066 mU/cell; 4.9 ± 1.9% of total fractionated LNC, $n = 60$). HD-BMC had unde-tectable accessory activity.

Appearance of DC and Accessory Activity for BMC Cultured for 5 d.   Although not seen in fresh preparations of BMC, DC were present after BMC had been cultured for 5 d. They were identified by their characteristic morphology and tendency to form clumps. Accessory activity increased to 0.03–0.07 mU/cell, while recovery of viable BMC was 5–15% of the BMC originally plated.

If BMC were fractionated after 5 d of culture, both accessory activity and DC were recovered almost entirely in the LD fraction (Table I). The small number of DC in the HD fraction had a lower accessory activity per DC, but mixing experiments failed to reveal either suppression or enhancement. Accessory activity did not increase in HD-BMC cultured up to 12 d.

The LD fraction, containing ~20% DC, was subfractionated after EA-roset-ting. Rosetted cells had negligible accessory activity. The LD fraction freed of rosetted cells retained both DC and accessory activity. Moreover, the accessory

TABLE I

Bone Marrow Cells Fractionated after 5 d in Culture

Cells	Dendritic cells		Accessory activity		Accessory activity per dendritic cell (mU/cell)
	× 10⁻⁶	%	U	%	
BMC	1.58	—	1,424	—	0.9
LD-BMC	1.28	87.1	1,256	95.6	1.21
HD-BMC	0.19	12.9	58	4.4	0.3

3.70 × 10⁸ BMC were cultured for 5 d. 2.1 × 10⁷ viable BMC were recovered, and a portion were fractionated into LD-BMC and HD-BMC; 73% of the total cells recovered were present in LD-BMC, and 27% in HD-BMC. The number of DC and accessory activity are based on unfractionated, 5-d cultured BMC. Overall recoveries of DC and accessory activity in the fractions were 93 and 92%, respectively.

TABLE II

Bone Marrow Cells Fractionated before Being Cultured for 5 d

Fraction	Accessory activity of cells recovered after culture		LD cells recovered after fractionation of cultured cells				Accessory activity per DC (mU/cell)
	U	%	Accessory activity		Dendritic cells		
			U	%	× 10⁻⁶	%	
BMC	2,000	—	1,795	—	0.84	—	2.1
LD-BMC	1,470	92	1,779	91	0.56	69	3.2
HD-BMC	120	8	177	9	0.25	31	0.7

BMC were fractionated in a discontinuous gradient of BPA, and LD-BMC, HD-BMC, and unfractionated BMC were then cultured for 5 d. The recovery of viable cells, based on the cells initially cultured, was 11.3% for LD-BMC, 18.8% for HD-BMC, and 18.8% for unfractionated BMC. Recovery of accessory activity is based on that found for unfractionated BMC cultured for 5 d.
Each group was then refractionated, and the accessory activity and number of DC present in the LD fraction for each was determined. Based on the cells initially cultured for each group, the recovery of viable cells in the LD fraction was 4.6% for LD-BMC, 0.2% for HD-BMC, and 2.2% for unfractionated BMC.

activity per bone marrow–derived DC was similar to that found for DC purified from lymph nodes and assayed at the same time. Taken together, the results suggest that cultures of BMC give rise to DC that do not differ morphologically or functionally from those isolated from other tissues.

Density of BMC Giving Rise to DC. We wanted to determine whether the BMC that give rise to LD-DC initially have a low or high density. Freshly isolated BMC were therefore fractionated into HD- and LD-BMC, cultured for 5 d, and assayed for accessory activity. Each group was then refractionated on a discontinuous gradient of BPA, and the DC among the cells recovered in the LD fraction were counted and assayed for accessory activity. The results, presented in Table II, show that most of the accessory activity originated from the minor population of cells in the LD-BMC fraction. Regardless of origin, however, accessory activity measured after 5 d was almost totally recovered in the LD fraction after refractionation. Although the HD-BMC fraction contributed 30% of the morphologically identifiable DC that, upon refractionation, showed a low density characteristic of DC, accessory activity per DC was only 25% of that found for

TABLE III

Immunospecific Staining for Cell Surface Markers on Unfractionated and Panned LD-BMC

Exp.	mAb used for staining	Percent positive cells in LD-BMF			
		Unfractionated	Sham panned	Ig-panned	Incubated with anti-Ia, then Ig-panned
I	OX3	3.4	2.8	4.2	−0.4
	G489	3.1	5.3	5.0	−0.1
	OX7	33.1	33.4	36.6	32.6
II	OX3	3.1	3.3	2.4	0.2
	G489	4.8	4.2	1.1	−0.4
	OX7	27.1	31.8	28.7	26.7
III	OX3	3.8	5.0	2.3	−1.1
	G489	5.6	6.4	1.8	0.1
	OX7	35.9	37.9	36.8	35.3
IV	OX3	2.2	4.4	3.5	0.5
	G489	4.5	4.8	4.8	0.1
	OX7	31.0	36.2	44.8	46.8

OX3 and G489 detect the same Ia-like molecule; OX7 detects Thy-1. Exps. I–III used immunoperoxidase; exp. IV used immunofluorescence. Background staining for each mAb in every group was determined, and has been subtracted; it averaged 7% for exps. I–III and 2% for exp. IV.

TABLE IV

Production of DC and Accessory Activity in Cultures of LD-BMC 5 d after Panning

Type of panning	DC per 10^7 LD-BMC ($\times 10^{-2}$)	Accessory activity per 10^7 LD-BMC (U)	Accessory activity per DC (mU/cell)
Blank dish	9.1 ± 5.0	1,386 ± 475	16.3 ± 5.7
Ig-coated dish	8.9 ± 5.1	1,510 ± 673	18.3 ± 4.0
Anti-Ia; Ig-coated dish	7.0 ± 2.8	1,636 ± 572	23.8 ± 5.4

The values given are the means ± SD for seven separate experiments. LD-BMF were panned as indicated. Cells recovered after panning, which were similar for the three types of panning, were cultured for 5 d, when the number of DC and accessory activity were determined.

DC produced by the LD-BMC. Because the majority of cells displaying accessory activity and DC morphology developed from LD precursors, the remainder of the studies focused on the LD fraction of BMC.

Expression of Ia on LD-BMC Giving Rise to DC. We wanted to determine whether DC arose from the 2–5% of LD-BMC that express Ia. To test this possibility, Ia⁺ cells were removed from LD-BMC by indirect panning on an IgG-coated dish after preincubation with OX3 (anti–rat Ia mAb). Controls included panning the same LD-BMC preparation on an IgG-coated dish and a blank dish. Immunoperoxidase staining or immunofluorescence was used to monitor the effectiveness of the panning procedure. As shown in Table III, Ia⁺ cells were removed by panning.

TABLE V

Accessory Activity Determined 5 d after Culture of Irradiated or
Unirradiated BMC

Viable cells tested ($\times 10^{-3}$)	[^{3}H]Thymidine incorporation (cpm)	
	Irradiated BMC	Unirradiated BMC
0	385 ± 105	385 ± 105
8	—	957 ± 106
15	384 ± 79	1,556 ± 504
30	619 ± 245	4,253 ± 747
60	378 ± 111	9,334 ± 1,253
120	713 ± 126	16,019 ± 444
240	497 ± 58	—

Bone marrow cells were irradiated with 1,000 rad before culture. Viable cells recovered after 5 d were 0.2% for irradiated and 12% for unirradiated BMC. Both groups were tested for accessory activity on 6 × 10^5 periodate-treated HD-LNC.

The nonadherent cells recovered after panning were then cultured for 5 d, when accessory activity was measured and DC were counted. Table IV records the results of seven separate experiments, including the four presented in Table III that were monitored by immunoperoxidase or immunofluorescence.

There were small differences among the three groups. LD-BMC panned for Ia$^+$ cells showed a slight decrease in the total number of DC produced, but total accessory activity was higher than in the controls due to a higher accessory activity per DC. We conclude that DC arise from LD-BMC that lack Ia.

Surface Markers on Bone Marrow–derived DC. DC that developed in cultures of LD-BMC were characterized by immunoperoxidase staining and other methods. Every DC identified morphologically was found to be positive for the leukocyte common antigen (OX1), Thy-1 (OX7), and Ia (OX3, G489.17). Lymph node DC express the same antigens.

It was possible to remove Ia$^+$ DC by panning 5-d cultures of BMC. When periodate-treated lymphocytes were added to the adherent cells that remained after panning, the following responses were measured: 125 ± 32 cpm, blank dish; 267 ± 23 cpm, Ig-coated dish; 35,354 ± 1,667, anti-Ia preincubation, Ig-coated dish. Controls run at the same time with freshly prepared LD-LNC indicated that DC were specifically removed from these preparations by panning.

Effects of γ-irradiation. When BMC were given 1,000 rad of γ-irradiation before culture, no DC developed after 5 d, and there was no detectable accessory activity (Table V). This dose of irradiation did not affect the accessory activity of DC isolated from lymph nodes.

To determine more precisely when γ-irradiation affected DC development, a sufficient number of cultures was established so that each day a different culture of LD-BMC was irradiated and returned to the incubator. At the end of 5 d, DC were counted and accessory activity was determined. Table VI records the results of two experiments. Both the number of DC and accessory activity showed daily increases.

In the experiment presented in Table VI, it was possible that those cultures having longer intervals between the time of irradiation and assay suffered a

TABLE VI

Effect of γ-irradiation on Development of DC and Accessory Activity in Cultures of LD-BMC

Day irradiated	Exp. 1		Exp. 2	
	Accessory activity (U)	DC (× 10⁻³)	Accessory activity (U)	DC (× 10⁻³)
0	59	10	8	3
1	369	40	—	—
2	825	74	668	30
3	1,276	106	634	41
4	1,300	173	900	60
5	1,485	161	988	64

Six cultures of LD-BMC were established. Daily, starting on day 0, one was removed, irradiated, and returned to the incubator. After 5 d, accessory activity was determined and DC were counted. Units of accessory activity and number of DC are both given per 10^7 LD-BMC initially cultured.

TABLE VII

Number of DC in Cultures of LD-BMC and LNC Irradiated on Successive Days

Day irradiated	DC (× 10⁻³)	
	LD-BMC	LD-LNC
0	14	107
1	45	116
2	94	111
3	118	73
4	139	65
5	148	67

Six cultures of LD-BMC and LD-LNC were established. At daily intervals, one culture from each group was irradiated, the next day the cells were centrifuged in a discontinuous gradient of bovine plasma albumin, and the number of DC in the LD fraction was determined.

relatively greater loss of DC, thereby producing an apparent increase in the number of DC appearing daily. To investigate this possibility, separate cultures of LD-BMC, as well as LD-LNC, were irradiated at daily intervals. The next day, the cells were centrifuged in a discontinuous gradient of BPA, and the DC in the LD fraction were counted. The results of this experiment (Table VII) show that there are daily increases in the production of DC in cultures of LD-BMC. In contrast, no increase occurred for lymph node DC cultured under the same conditions; by the third day the number had decreased.

Discussion

Although DC originate from bone marrow (23, 27–29), they are not detectable in fresh preparations of BMC (33). In this paper, we have presented evidence indicating that DC arise when BMC are cultured in a serum-free medium. Moreover, our findings show that DC originate from a precursor that has a

number of properties that distinguish it from the DC. These findings confirm previous reports (34, 35), and provide new information on the properties of DC precursors.

Three criteria were used to identify the cells appearing in culture as DC: morphology, expression of Ia, and accessory activity for periodate-treated lymphocytes. During the later stages of culture, cells having characteristic DC morphology were observed either singly or aggregated in clumps similar to those seen in cultures of DC purified from other tissues (6). After fractionation of cultured BMC in a discontinuous gradient of BPA, cells recovered in the same LD fraction as lymph node DC were spread on poly-L-lysine–coated slides to facilitate identification (36). ~20% of the cells had DC features and stained strongly for Ia by the immunoperoxidase procedure.

Previous work (1, 6) has shown that rat DC function as the predominant, if not exclusive, accessory cell required for periodate-induced lymphocyte transformation. Cells from many tissues, including macrophages, either lacked accessory activity or inhibited responses. Although the percentage of DC in any given preparation of BMC rarely exceeded 30%, the evidence suggests that the accessory activity measured can be attributed to DC. When bone marrow–derived DC and more highly purified lymph node DC were assayed at the same time, similar accessory activities per DC were obtained. Removal of bone marrow macrophages from preparations by adherence or EA-rosetting did not result in a decrease of accessory activity. Temporally, accessory activity and DC developed concomitantly in cultures of LD-BMC, and in general, accessory activity correlated with the content of DC. Taken together, the evidence indicates that DC are produced in bone marrow cultures.

It is clear, however, that DC do not originate from mature DC present in fresh preparations of BMC. They are derived from precursors that differ in at least two respects. First, the precursor is a dividing cell. Irradiation administered before culture abolished the production of DC (Table V). With time, daily irradiation arrested the number of DC until a plateau was reached on the fourth or fifth day (Tables VI and VII). Preliminary studies with autoradiography show that Ia$^+$ bone marrow–derived DC are labeled in culture with [^{3}H]thymidine. Conversely, suicidal amounts of [^{3}H]thymidine in culture abolished the production of DC. In contrast, mature DC do not divide. Second, the cell giving rise to a DC lacks Ia, or at least expresses so little Ia that it cannot be selected by panning or detected by immunofluorescence and immunoperoxidase. The bone marrow precursor of the rat Ia$^+$ B cell also lacks Ia (37).

The precursor and the mature DC have in common a low density. Taking advantage of this property led to a 20-fold enrichment in DC precursors, since nearly all were recovered in an LD fraction that contains only 5% of the fractionated BMC. Castagnola et al. (38) found that rat CFUs were recovered almost entirely in an LD fraction after fractionation of BMC in a gradient of BPA.

Not all DC identified morphologically arose from LD precursors. HD-BMC accounted for as much as 30% of the DC produced, but the accessory activity per DC was only one-fifth that of the DC arising from LD precursors (Table II). The lower accessory activity was not due to inadequacies of either the assay

system or the culture period, which, if prolonged, did not result in an increased accessory activity. Good recoveries for both accessory activity and DC in the fractions relative to the unfractionated cells suggest further that no component essential for possible development of the HD-DC was lacking. It is possible that we are dealing with more than one population of dendritic cells, especially in light of the demonstration by Pugh et al. (24) and Fossum (25) of the heterogeneity of the nonlymphoid Ia$^+$ cells present in the thoracic duct lymph of lymphadenectomized rats. The relationship between the dendritic cells that develop from LD and HD precursors remains to be determined.

BMC were cultured in serum-free medium. Although the medium contained 0.1% BPA, which improved production of DC 3–4-fold over medium lacking BPA, it could be replaced by ovalbumin. Factors, if involved in DC differentiation, would have to be produced by BMC in culture. In this regard, it is of interest that Klinkert (35) has reported an increase in the production of DC when rat BMC are cultured in medium supplemented with a supernatant from Con A–treated spleen cells. The substance(s) responsible for the activity in the supernatant was found to have a molecular mass of 40 kD. It is not known what effect factors and other cells have on DC differentiation, as has been reported for the differentiation of many other bone marrow–derived cell types (39).

Summary

Although dendritic cells (DC) originate from bone marrow, they were not observed in fresh preparations of bone marrow cells (BMC). Likewise, accessory activity was barely measurable in a sensitive assay for this potent function of DC. However, both DC and accessory activity developed when BMC were cultured for 5 d. Based on fractionation before culture, nearly all of the accessory activity could be attributed to only 5% of the total BMC recovered in a low-density (LD) fraction.

The LD-DC precursors differed from mature DC in a number of important respects. Removal of Ia$^+$ cells from the LD fraction by panning did not decrease the production of DC when the nonadherent cells were cultured. Thus, the cell from which the DC is derived does not express or minimally expresses Ia antigens, in contrast to the strongly Ia$^+$ DC that is produced in bone marrow cultures. Irradiation of LD cells before culture prevented the development of DC. When irradiation was delayed by daily intervals, progressive increases in the number of DC resulted, up to the fifth day. These findings, together with preliminary autoradiographic data, indicate that cell division has occurred, in contrast to the DC, which does not divide. We conclude that bone marrow–derived DC arise in culture from the division of LD, Ia$^-$ precursors.

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