Sex-Related Differences in the Pattern of Coxsackievirus B-3-Induced Immune Spleen Cell Cytotoxicity Against Virus-Infected Myofibers

SALLY A. HUBER,* LILLIAN P. JOB, AND JACK F. WOODRUFF

Department of Pathology, The New York Hospital-Cornell University Medical College, New York, New York 10021

Spleen cells from adult BALB/c mice injected intraperitoneally with purified coxsackievirus B-3 were tested for cytotoxicity against ^{51}Cr-labeled syngeneic infected and uninfected myofibers. Both male and female immune cells were active against uninfected targets; this reactivity was evident by day 3 of infection and persisted throughout the first week. However, we observed marked sex-related differences in immune cell cytotoxicities against infected myofibers. Males exhibited a strong T-lymphocyte response 4 to 7 days after infection. In contrast, females exhibited a weak response, and only infrequently were the immune spleen cells of females significantly more reactive against infected myofibers than against uninfected myofibers. The demonstration of a stronger effector cell response against infected myocardial cells in male mice correlates with the observation that clinical adult coxsackievirus B myocarditis and pericarditis occur predominantly in males.

The group B coxsackieviruses are the most commonly identified causes of viral myocarditis and pericarditis in humans. The heart disease produced by these agents in adolescence and adulthood occurs predominantly in males (14, 15). The reason for this is not known.

Coxsackieviruses also replicate in mice and produce heart lesions that closely resemble those described in humans (9, 16, 20), thus providing an animal model in which the mechanism of viral myocarditis can be studied. Coxsackievirus heart disease in mice depends upon the presence of thymus-derived lymphocytes (T cells) in the heart, since (i) athymic (nude) mice or mice depleted of T cells by thymectomy, irradiation, and bone marrow reconstitution fail to develop significant myocarditis after infection, (ii) there is a marked reduction in animal mortality after coxsackievirus infection in T-lymphocyte-depleted animals, and (iii) cytotoxic T cells capable of lysing virus-infected fibroblasts are generated during infections with this virus (17, 18, 21).

Interestingly, male and female mice differ markedly in the capacity to generate virus-specific immune spleen cells. Thus, a strong virus-specific cytotoxic T-cell response has been detected in male animals but not in female animals (29), a finding which correlates with the predominance of clinical coxsackievirus heart disease in human males. However, the experiments with mice utilized fibroblasts as targets for detecting cytotoxicity. To investigate precisely the rela-

tionship of effector cells to myocardial injury, we have developed a system in which cultured myofibers are used to measure the immune cell activity induced during coxsackievirus infections (18). Such studies have confirmed that virus-specific cytotoxicity in males is mediated by T lymphocytes (5). The present work was undertaken to determine whether sex-related differences in reactivity could be found with this model. Our results demonstrate that male effector cells exert a stronger cytotoxic response than female immune cells against infected myocardial cells, whereas female effector cells are more active against uninfected targets. This finding supports the hypothesis that sex-related differences in immune reactivity during coxsackievirus infections influence the expression of heart disease.

MATERIALS AND METHODS

Animals. BALB/c mice were purchased originally from Cumberland Farms, Clinton, Tenn. Neonates and 6- to 8-week-old adults were obtained from colonies maintained at Cornell University Medical College.

Virus preparation and purification. A cardiotropic coxsackievirus B-3 (Nancy strain), which was grown originally in human epidermoid carcinoma of the larynx cells (H. Ep. No. 2), was passaged three times in HeLa cell monolayers (Gey strain; Flow Laboratories, McLean, Va.) and thereafter grown in HeLa cell suspensions (Mandel strain; courtesy of Richard Crowell, Hahnemann Medical College, Philadelphia, Pa.) by a previously described technique (3). The maintenance medium was Joklik modified minimal

essential medium (Flow Laboratories) containing double-strength essential amino acids and vitamins in Hanks basic salt solution, 5% fetal calf serum (FCS), and antibiotics (100 μg of streptomycin per ml and 100 U of penicillin per ml). HeLa cells were infected with 100 virus plaque-forming units per cell. After incubation at 37°C for 9 h, the cells were harvested, washed, and disrupted by a freeze-thaw technique. Virus purification was performed by using CsCl gradients according to the method of Oberg and Phillipson (11). The titers of purified virus ranged from 9×10^9 to 2×10^{10} plaque-forming units per ml; samples were stored at −70°C in phosphate-buffered saline without Ca^{2+} and Mg^{2+}. Purified virus was used in all experiments.

Infection of mice. Each animal was infected by intraperitoneal inoculation of 0.5 ml of phosphate-buffered saline containing 2×10^5 plaque-forming units of purified coxsackievirus B-3. Spleens were removed 4 to 7 days after infection.

Spleen cell suspensions. Spleens from three to five mice were pooled and teased in cold basal medium Eagle (BME) containing 5% FCS, 100 μg of streptomycin per ml, and 100 U of penicillin per ml. The cells were washed twice ($300 \times g$ for 10 min), suspended in a solution containing 9 volumes of 0.83% NH_4Cl and 1 volume of 0.17 M tris(hydroxymethyl)aminomethane (pH 7.2) in order to lyse erythrocytes, and then washed with medium. Adherent cells were removed by incubating spleen cells in plastic petri dishes (60 by 15 mm; Falcon Plastics, Oxnard, Calif.) at a concentration of 20×10^6 cells per ml for 1 h at 37°C. The nonadherent cells were harvested, washed once, and suspended in BME containing FCS at a concentration of 10^7 viable nucleated cells per ml; these cells were kept on ice until they were used in cytotoxicity assays. Cell viability was at least 90%, as assayed by trypan blue dye exclusion.

Preparation, culture, and characterization of myofibers. Hearts were removed aseptically from BALB/c mice within 48 h of birth. Single-cell suspensions of myofibers were prepared by using a modification of the procedure of Bollon et al. (2). Briefly, hearts were minced and then subjected to stepwise enzymatic digestion with 0.25% pancreatin (GIBCO Laboratories, Grand Island, N.Y.). The isolated cells were washed with BME containing penicillin, streptomycin, 5% FCS, 10% horse serum, 0.2 mg of crystalline insulin, and 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (complete BME) per 100 ml of medium and then depleted of endothelial cells and fibroblasts by two sequential 1-h adsorptions in 25-cm² plastic flasks (Falcon Plastics) at 37°C (7; B. Landau, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, S124, p. 233). The nonadherent myofibers were recovered, washed once, resuspended in complete BME, and then dispensed into 6-mm tissue culture wells (Linbro Chemical Co., Hamden, Conn.). After 48 h (the time needed for these cells to become firmly adherent to plastic), the myofibers were used in the cytotoxicity assay.

In a separate experiment 2×10^6 myocardial cells in complete BME were seeded onto cover slips (9 by 22 mm) and maintained in Leighton tubes (16 by 100 mm; T.C. Wheaton Co., Millville, N.J.) for examina-

tion by light microscopy. At 48 h the cultures contained individual cells and cell clusters which contracted rhythmically (50 to 80 beats per min). In addition, 90% or more of the cells were identified as muscle fibers after staining with phosphotungstic acid-hematoxylin; sarcomeres were observed under light microscopy.

Cytotoxicity assay. The technique used was similar to that described in previous reports (17, 18). Briefly, 7×10^4 neonatal myofibers in 0.2 ml of complete BME were dispensed into 6-mm plastic tissue culture wells and incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 48 h the cells were infected with 100 plaque-forming units of virus per cell, a dose known to result in infection of 20% of the myofibers by 6 h and 75% by 16 h (5). After 1 h, unabsorbed virus was removed by washing the monolayers. Target cells not exposed to virus were processed in an identical fashion. Myofibers were then labeled by adding 5 μCi of ^{51}Cr to each well ($Na_2{}^{51}CrO_4$; Amersham/Searle, Arlington Heights, Ill.). After 45 to 60 min at 37°C, the monolayers were washed three times and overlaid with 0.2 ml of immune or nonimmune spleen cells in BME containing FCS or with just BME containing FCS. The cultures were incubated at 37°C for 18 h at a ratio of spleen cells to target cells of 100:1, unless otherwise noted; all assays were performed in triplicate. One representative experiment is reported from a minimum of three replicate experiments done for each table or figure. The ^{51}Cr levels in the supernatants and in the cells were determined as described previously (15); radioactivity was measured by using an Intertechnique CG 4000 gamma counter. ^{51}Cr release was calculated by using the following expression: [(counts per minute in supernatant)/(counts per minute in supernatant + counts per minute in cells)] $\times$ 100.

Cytotoxicity was expressed as the percentage of lysis, as calculated by the following expression: [(average percentage of ^{51}Cr released from test group) − (average percentage of ^{51}Cr released from medium group)]/[(average percentage released after freeze-thaw) − (average percentage released from medium group)].

The percentage of specific lysis represented the percentage of lysis by sensitized lymphocytes minus the percentage of lysis by nonimmune lymphocytes.

Anti-thy 1.2 serum treatment of spleen cells. Monoclonal anti-thy 1.2 serum (OLAC Ltd., Blackthorn, England) was titrated as described previously (21). The antiserum used in these experiments had a cytotoxicity titer against 1.25×10^6 BALB/c thymocytes of $1:5 \times 10^5$. Lysis of thy 1.2-positive cells was performed as described previously (18), using a 1:1,000 dilution of anti-thy 1.2 serum and a 1:5 dilution of guinea pig complement (GIBCO) which had been absorbed with BALB/c thymocytes. This treatment increased the percentage of immunoglobulin-containing spleen cells from 43 to 91%.

Adsorption of spleen cells to nylon- or glass-wool. A total of 3×10^7 spleen cells suspended in 3 ml of BME containing FCS were incubated with 0.1 g of nylon-wool (Fenwal Laboratories, Morton Grove, Ill.) or glass-wool for 30 min at 37°C. Nonadherent cells were recovered by washing the wool with 30 ml of

medium.

Trypsin treatment of spleen cells. A total of 3 × 10^7 spleen cells were suspended in 1 ml of Ca^{2+}-free, and Mg^{2+}-free phosphate-buffered saline containing 0.05 or 0.25% trypsin (GIBCO) and then incubated at 37°C for 15 min. The trypsin was neutralized by adding 1 ml of cold BME containing 5% FCS, and the cells were washed twice. This procedure was a modification of the procedure used by Kim et al. (8).

Statistical analysis. Student's t test was used to analyze the significance of the differences among the mean values for the various experimental groups.

RESULTS

Male and female immune spleen cells obtained 3 to 7 days after coxsackievirus B-3 infection were assayed for reactivity against ^{51}Cr-labeled infected and uninfected myofibers by using conditions previous shown to be optimal for detection of virus-specific cytotoxicity (5). Initially, male effector cells lysed both infected and uninfected targets, but by day 4 activity against infected myofibers was significantly greater than activity against uninfected cells. Typically, preferential lysis of infected targets peaked on day 5 and then declined (Fig. 1), although significant virus-specific lysis persisted through day 7 of the infection (data not shown).

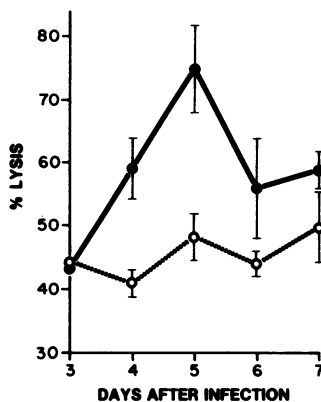Female cells were also cytotoxic, but the specificity of this reaction was markedly different from that observed when male immune cells were used. Early after infection, female effector cells manifested comparable levels of activity against infected and uninfected targets. However, between days 5 and 7 the pattern changed, and the activity against uninfected targets increased, whereas that against infected myofibers decreased. This pattern of cytotoxicity was observed in five of seven experiments (Fig. 2a). A somewhat different response was observed in two other experiments (Fig. 2b). In this case, some virus-specific activity was detected, but only on day 4; thereafter, effector cells lysed both infected and uninfected myocardial cells to comparable degrees. Thus, consistent virus-specific lysis by immune spleen cells was detected only in male animals.

The apparent inability of female immune cells to consistently lyse virus-infected myofibers to a greater degree than uninfected targets could be due to the use of a ratio of spleen cells to target cells that was not optimal. Therefore, female immune cells obtained 3, 4, and 5 days after infection were incubated with infected and uninfected myofibers at ratios of spleen cells to target cells of 50:1, 100:1, 150:1, and 200:1, and at each ratio the percent ^{51}Cr release was deter-

FIG. 1. *Kinetics of in vivo generation of male cytotoxicity against coxsackievirus-infected (●) and uninfected (○) neonatal myofibers. Assays were performed by using a ratio of spleen cells to target cells of 100:1. Each point represents the mean ± the standard error of the mean for three wells. Values for spontaneous ^{51}Cr release from infected and uninfected myofibers were 28.6 and 30.0%, respectively. The difference between lysis of infected myofibers and lysis of uninfected myofibers by immune cells was significant on days 4 and 5 (P < 0.05). At each interval studied the lysis of both infected and uninfected myofibers caused by immune cells was significantly greater than the lysis caused by nonimmune cells (P < 0.05 to P < 0.01).*
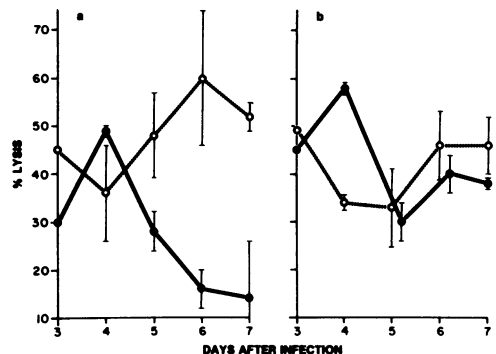

FIG. 2. *In vivo generation of female immune spleen cell activity against coxsackievirus-infected (●) and uninfected (○) neonatal myofibers. Assays were performed by using a ratio of spleen cells to target cells of 100:1. Each point represents the mean ± standard error of the mean for three wells. (a) and (b) represent separate experiments. (a) Values for spontaneous ^{51}Cr release from infected and uninfected targets were 38.4 and 38.0%, respectively. The immune cell activities against infected and uninfected targets were not significantly different on days 3 through 5, but thereafter produced significantly greater lysis of uninfected myofibers (P < 0.05). (b) Values for spontaneous ^{51}Cr release from infected and uninfected targets were 28.6 and 30.0%, respectively. Immune cells exhibited virus-specific cytotoxicity on day 4 (P 0.05) but at other times lysed both infected and uninfected myofibers to comparable degrees. In both (a) and (b) target cell lysis by nonimmune cells ranged from 0.0 to 3.0%.*

mined. At each ratio female cells exhibited comparable degrees of cytotoxicity against infected and uninfected myofibers; nevertheless, day 5 immune male cells caused significantly greater lysis of infected targets than of uninfected targets (data not shown). In another experiment, we tested the susceptibilities of infected myofibers to lysis by male and female immune cells obtained 4 to 6 days after infection. Table 1 shows that at all ratios of spleen cells to target cells used, male effector cells were more active ($P < 0.05$ to $P < 0.01$) than female cells.

The differences observed between male and female cytolytic responses indicated that more than one type of effector cell may have been involved. Male and female mice were infected for 3 to 6 days; the spleen cells were treated with anti-Thy 1.2 serum and complement before culture on uninfected or infected myofibers (Table 2). Cytolytic activity in day 6 male spleen cells was removed by anti-Thy 1.2 serum. No activity was lost from either day 3 or day 6 female spleen cells or from day 3 male spleen cells. In additional experiments to study the characteristics of the effector cells, spleen cells obtained from male and female mice 3 days postinfection were adsorbed to glass or nylon wool or treated with 0.05 or 0.25% trypsin and then assayed on uninfected myofibers (Table 3). There was little or no reduction in the cytolytic activity of either male or female spleen cells caused by adsorption to glass- or nylon-wool or by treatment with 0.05% trypsin. Activity was lost after treatment with 0.25% trypsin. This loss of activity did not result from toxicity of the enzymatic treatment to the effector cells since spleen cell viability after treatment was not decreased and since cytotoxicity could be regenerated after incubation of the spleen cells in serum-containing medium for 6 h at 37°C before assays were performed.

DISCUSSION

Striking differences were observed in the abilities of male and female coxsackievirus-immune cells to lyse neonatal myofibers. Although male immune spleen cells obtained during week 1 of infection were capable of lysing uninfected targets, reactivity against infected myofibers was greater; virus-specific cytotoxicity was detected between day 4 and day 7 of disease. However, with females we most commonly found that immune spleen cells lysed infected and uninfected myofibers to comparable degrees on days 3 through 5, but thereafter reactivity against uninfected targets predominated. Occasionally, a significant degree of virus-specific female cytotoxicity was detected, but this occurred only on day 4. The poor response of female immune cells against infected targets was not changed by varying the concentration of effector cells.

These sex-related differences in coxsackievirus-immune cell activity parallel observations made in experiments in which skin fibroblasts were used as targets (19). In that study the weak virus-specific response of female cells was not improved by varying the in vitro assay conditions or the dose of virus administered to the animals. However, female mice were not immunologically deficient, since a vaccinia virus infection stimulated levels of virus-specific cytotoxicity comparable to the levels found in males. These results suggest that the altered cellular immune response in coxsackievirus-infected female mice is related to the type of virus and not to the strain of animals used.

Although the specific cause of the differences in the male and female patterns of cytotoxicity has not been determined, it is known that in infected female mice coxsackievirus is cleared more rapidly and higher titers of neutralizing antibody appear earlier (19). Such a situation could lead to a reduced population of the infected cells needed for efficient stimulation of cytotoxic cell precursors. Other factors which might contribute to the diminished virus-specific response include masking of antigenic determinants by virus-specific antibody, formation of immune complexes (10), and stimulation of suppressor cell activity (13).

TABLE 1. *Comparison of male and female immune spleen cell activities against virus-infected myofibers*

No. of days after infection	Sex of donor	% [51]Cr release at a spleen cell/target cell ratio of:			
		50:1	100:1	150:1	200:1
4	Male	32.5 ± 1.6[a]	51.8 ± 2.3	56.4 ± 1.8	57.8 ± 0.7
	Female	20.4 ± 0.6	29.6 ± 1.5	34.6 ± 1.8	44.7 ± 0.8
5	Male	34.1 ± 2.1	46.0 ± 2.7	47.7 ± 1.8	54.6 ± 1.1
	Female	21.6 ± 1.4	28.4 ± 1.1	24.2 ± 5.2	41.5 ± 2.9
6	Male	29.7 ± 1.1	50.5 ± 0.2	47.3 ± 2.2	60.1 ± 4.5
	Female	21.7 ± 1.3	29.5 ± 2.0	29.7 ± 3.8	35.6 ± 3.0

[a] Mean ± standard error of the mean for three wells per group. The average spontaneous ^{51}Cr release from infected myofibers was 19.5%.

TABLE 2. *Sensitivities of male and female effector cells to anti-Thy 1.2 serum and complement*

Spleen cells from:	Treatment	% Specific lysis[a]	
		Uninfected cells	Infected cells
Females	Day 3 untreated	45.3 ± 3.5	60.3 ± 8.5
	Day 3 anti-Thy 1.2 serum + complement	65.0 ± 2.9	61.0 ± 5.1
	Day 6 untreated	63.1 ± 3.0	55.2 ± 2.9
	Day 6 anti-Thy 1.2 serum + complement	73.0 ± 4.4	72.1 ± 6.3
Males	Day 3 untreated	32.8 ± 2.5	38.1 ± 3.5
	Day 3 anti-Thy 1.2 serum + complement	36.0 ± 2.6	25.3 ± 1.4
	Day 6 untreated	43.8 ± 2.9	71.9 ± 8.4
	Day 6 anti-Thy 1.2 serum + complement	18.9 ± 1.3	4.9 ± 0.9

[a] Mean ± standard error of the mean for four wells per group. Mean percent lysis values for infected and uninfected myofibers cultured with nonimmune female spleen cells were 7.5 and 5.4%, respectively, and the corresponding values with nonimmune male spleen cells were 3.1 and 5.5%, respectively. The average values for spontaneous ^{51}Cr release from infected and uninfected myofibers were 37.2 and 41.4%, respectively.

TABLE 3. *Characteristics of male and female effector cells cytolytic for uninfected myofibers*

Expt	Treatment	% Specific lysis[a]	
		Female spleen cells	Male spleen cells
1	None	49.3 ± 2.3	25.1 ± 1.9
	Adsorbed to glass-wool	38.6 ± 5.6	24.6 ± 2.4
	Adsorbed to nylon-wool	38.7 ± 3.3	30.6 ± 2.0
	Treated for 15 min at 37°C with 0.05% trypsin	50.6 ± 4.9	34.1 ± 1.5
	Treated for 15 min at 37°C with 0.25% trypsin	6.8 ± 0.5	0.8 ± 0.3
2[b]	None	50.4 ± 2.0	0 ± 0
	Treated for 15 min at 37°C with 0.25% trypsin and assayed at zero time	0 ± 0	0 ± 0
	Treated for 15 min at 37°C with 0.25% trypsin and assayed at 6 h	85.2 ± 22.0	17.2 ± 7.2

[a] Mean ± standard error of the mean for three wells per group. Mean percent lysis values for targets cultured with nonimmune male spleen cells were 3.6 and 0.8% for experiments 1 and 2, respectively, and for targets cultured with nonimmune female spleen cells the corresponding values were 5.4 and −18.5%, respectively. Immune spleen cells were obtained three days postinfection. The average values of spontaneous ^{51}Cr release from uninfected myofibers were 23.5% in experiment 1 and 45.5% in experiment 2.

[b] In experiment 2, spleen cells were treated with trypsin and then assayed for cytotoxicity immediately or were incubated at 37°C in medium containing 5% FCS for 6 h before assay.

The cytolytic responses in male and female mice can be divided into early (up to day 4) and late (after day 4) phases. Early effector cells in both males and females are equally lytic to infected and uninfected myofibers. These effectors are not sensitive to treatment with anti-Thy 1.2 serum or anti-immunoglobulin serum (data not shown). Also, they are not adherent to plastic or glass, which removes macrophages, or to nylon wool, which removes B lymphocytes (6). Finally, cytolytic activity is abolished by treatment with proteolytic enzymes, a procedure shown to inhibit natural killer cells but not K cells (8). Incubation of the trypsin-treated cells before they were used in the cell-mediated cytotoxicity assay allowed regeneration of cell surface receptors and restored cytolytic activity, indicating that the treatment employed was not toxic to the spleen cells. Therefore, these results suggest that early cytolytic activity is due to natural killer cells. In the late phase, the male response began to change. A characterization of effector cells 4 to 6 days after infection indicated that male mice produced virus-specific T lymphocytes, which are found infrequently in females. In addition, male effector cells cytolytic to uninfected myofibers were sensitive to anti-thy 1.2 serum, whereas the effector cells in female mice continued to have the characteristics of natural killer cells.

The inflammatory lesions may be the combined result of both virus-specific cytolytic T lymphocytes and autoimmune (natural killer) cell destruction of infected and uninfected myofibers in vivo. Nonetheless, natural killer cells alone would not be able to induce this destruction. Therefore, we assume that the initial reaction of specific T cells and infected myofibers results in the release of chemotactic factors, with subsequent accumulation of specific and nonspecific lymphoid cells in the heart. The evidence for this assumption is, first, that T-cell-deficient mice fail to develop significant myocarditis despite viral replication in the heart, and, second, that despite the appearance of natural killer cells within 48 to 72 h after infection, inflammatory lesions only appear after day 5, when cytolytic T cells are observed (5, 19).

The finding that coxsackievirus-specific effector cells are routinely elicited in males but infrequently detected in females may be related to the observation that in adolescents and adults clinical coxsackievirus B myocarditis and pericarditis occur predominantly in males (14, 15), with the exception of increased susceptibility in females during pregnancy (12). The stronger cytotoxic response in males also correlates with a higher mortality rate in BALB/c male mice during the first week of coxsackievirus B-3 dis-

ease. Thus, 16 of 26 male mice but only 3 of 27 female animals were dead by day 7 after infection ($P < 0.01$) (J. F. Woodruff, in H. Friedman, S. Spector, and J. Prier, ed., *Infection and Autoimmunity*, in press). A similar sex-related difference in mortality after a coxsackievirus B-1 infection of mice was also observed by Berkovich and Ressel (1). In addition, the present findings may relate to other manifestations of coxsackievirus disease. For example, infections of humans and mice in the islets of Langerhans may be associated with the development of diabetes mellitus (22, 23). This virus-induced pancreatic disease is known to occur predominantly in male animals (23).

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