Factors Affecting the Lactate Dehydrogenase Isoenzyme Pattern of Cultured Kidney-Cortex Cells

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1. The lactate dehydrogenase isoenzyme pattern of cultured calf kidney-cortex cells was correlated to growth phase, changes in oxygen supply, mean generation time and changes in nutritional supply. 2. During culture of free cells and intact explants the lactate dehydrogenase isoenzyme pattern changed towards a dominance of isoenzymes containing the M subunit. 3. Of the shift in monomer proportion, 58% occurred during the lag phase and 42% during the initial part of the exponential growth phase. During the stationary phase the shift in monomer proportion reversed slightly. It was possible to relate the observed shift in monomer proportion to the glycolytic rate. 4. Factors that depressed glycolysis decreased the shift in monomer proportion. Oxygen was found to limit the decrease in the Hsubunit/Msubunit ratio caused by anaerobic culture *in vitro*. 5. The results obtained support the view that the altered lactate dehydrogenase isoenzyme pattern of urine in renal ischaemia may be explained by anaerobic changes in the lactate dehydrogenase isoenzyme pattern of cortical tubule cells.

Two types of LDH* monomers (H and M) exist, leading to the intracellular presence of five active tetrameric isoenzymes (Clausen & Hustrulid, 1968).

The LDH pattern depends on the tissue and cell type (Wilkinson, 1965). In a certain tissue population the LDH pattern may change as a function of developmental factors (Clausen & Hustrulid, 1969), but it can also be altered by malignant transformation (Gerhardt, Clausen, Christensen & Riishede, 1967) and by change in functional state of the tissue (Philip & Vesell, 1962). To elucidate some of the factors altering the LDH isoenzyme pattern, kidney-cortex tubule cells were cultivated under different environmental conditions. The oxygen supply, rate of glycolysis and cell division were all found to influence the LDH isoenzyme pattern.

MATERIALS AND METHODS

Animal material. Foetal calves of the Danish Red Landrace breed were obtained, just before delivery, from The Central Slaughterhouse of Copenhagen.

Chemicals. Chemicals were of the highest obtainable purity and, unless otherwise specified, were from British Drug Houses Ltd., Poole, Dorset.

Cell cultures. The kidneys of foetal calves were removed aseptically immediately after death, decapsulated, and the

outer cortex was minced with scissors. Cell suspensions were obtained by incubating the minced cortex for 15 min. at 37° in 0.25% (w/v) trypsin (Difco, code no. 0152) in Hanks balanced solution (BSS) (Paul, 1965, p. 83). The liberated cells were isolated by centrifugation at 300g for 10 min., resuspended in BSS and sieved to remove cell clumps. Finally, the number of cells was counted in a haemocytometer. About 7×10^5 cells were cultivated in glass Petri dishes, which allowed gas equilibrium with the atmosphere of the incubator (see below). After 24 hr. in culture half of the cells were attached to the dish and the medium was renewed to remove debris and dead cells. As a routine media were changed every second day.

The standard medium contained BSS enriched with 10% (v/v) calf serum, 0.2% (w/v) lactalbumin hydrolysate, 0.7% (w/v) Bacto Yeast Extract (Difco no. 0127-02), 2.05 mm-glutamine, 5.56 mm-glucose, 0.24 mm-penicillin and 0.86 mm-streptomycin; NaHCO_3 was added to maintain the pH at 7.20.

Tissue cultures. Tissue cultures of calf kidney cortex were performed in plastic Petri dishes (10 mm. $\times$ 60 mm.). Kidney cortex was sliced into pieces (1 mm. $\times$ 1 mm. $\times$ 0.5 mm.). About 50 explants were planted per dish. The medium (pH 7.20) contained: 40% (v/v) calf serum, 58% (v/v) Eagle's medium (Paul, 1965, pp. 95-99), antibiotics (as above) and 119 mm- NaHCO_3 .

Gas mixtures. The cultures were incubated at $36 \pm 0.5^\circ$ in a water-saturated atmosphere of $\text{O}_2 + \text{CO}_2 + \text{N}_2$ (25:5:70), or in some experiments in $\text{O}_2 + \text{CO}_2 + \text{N}_2$ (40:5:55) or $\text{O}_2 + \text{CO}_2 + \text{N}_2$ (25:1:74) or $\text{O}_2 + \text{CO}_2 + \text{N}_2$ (5:5:90). The composition of the different gas mixtures was controlled by the Haldane (1912) method.

Variation in medium composition. The glucose content of the standard medium (5.56 mm) was in some experiments

* Abbreviations: LDH, lactate dehydrogenase (EC 1.1.1.27); H₄, LDH-1 (the anodic migrating LDH isoenzyme); H₃M, LDH-2; H₂M₂, LDH-3; HM₃, LDH-4; M₄, LDH-5 (the cathodic migrating isoenzyme).

replaced by 0.56mM- or 0.28mM-glucose, containing 0.84mM-sodium acetate, or 0.56mM-sodium lactate or 0.56mM-sodium pyruvate. In some experiments these replacements were supplemented by the addition of 0.1mM-NAD⁺ and 0.1mM-NADP⁺.

Hanks salt solution was in one experiment replaced by medium 858 (Paul, 1965, p. 91), and in another experiment extract of calf kidney cortex was added to a concentration of 50% (v/v). This extract was prepared by placing minced kidney cortex in the barrel of a sterile 20ml. syringe and squeezing the contents into a centrifuge tube. The minced tissue was centrifuged at 20000g for 20min. at +4°. The supernatant was sterilized by two successive filtrations (filters from Jena Glaswerk, Mainz, Germany) through no. 4g (9–15 μ m. pore size) and no. 5g (1.7–3.0 μ m. pore size) respectively.

Subcultures. Subcultures were prepared by mechanical separation of full-grown monolayers.

Cultivation on Millipore filters. To minimize the thickness of medium covering the cells, suspensions of kidney-cortex cells were planted on sterilized Millipore filters placed on a Teflon ring at the surface of the standard culture medium.

Harvesting and enzyme extraction. Before harvesting, medium was drawn off the cells and the monolayer was washed twice in BSS at 4°. The cells were harvested in 10ml. of cold BSS with a Teflon-coated glass rod, and then centrifuged at 600g for 10min. at 4° and resuspended in 1ml. of 0.16M-NaCl at 4°. The cell suspension was homogenized in an all-glass homogenizer (Kontes Glass Co.; size B) at 0°. The homogenate was centrifuged at 18000g for 20min. at 4°.

Cell counting and estimation of growth rate. To 50 μ l. of the cell suspension was added 50 μ l. of 0.1M-citric acid. After incubation for 1hr. at 37° the tubes were shaken vigorously and 100 μ l. of Crystal Violet solution (1mg./ml. of 0.1M-citric acid) was added. The stained nuclei were counted in a haemocytometer. The mean generation time in the exponential growth phase was calculated (Paul, 1965, p. 49).

Determination of LDH isoenzymes. Microelectrophoresis on agar-gel slides was performed as described by Wieme (1959) at pH8.4 in 50mM-sodium barbitone buffer containing 1% Difco Special Noble Agar. The separated isoenzymes were made visible by formazan formation (Van der Helm, 1962). The relative proportions of the different isoenzyme bands were measured as described by Clausen & Øvliisen (1965) by scanning the slides at 546nm. in a Vitatron photometer equipped with a scanning device, an automatic recorder and an integrator. Previous systematic investigations have revealed that Beer's law is valid as long as the integrator can be used (Clausen & Øvliisen, 1965). From the results of the scanning, the H subunit/M subunit ratio was determined. This ratio was estimated on the basis of the tetrameric composition of the LDH.

Assay of LDH activity. This was performed on 100 μ l. samples of suitable dilution (Clausen & Øvliisen, 1965). All results of total LDH determinations are mean values of double determinations.

The LDH unit is defined as the amount oxidizing 1 μ mole of NADH/min. at pH7.5 at 25° with a reaction mixture containing 1.0mM-sodium pyruvate and 0.33mM-NADH in 50mM-sodium phosphate buffer (Clausen & Øvliisen, 1965). The LDH specific activity is expressed as units/mg. of protein in the sample.

Determination of protein content. Protein was determined by the Lowry method as modified by Lous, Plum & Schou (1956). Tyrosine was used as standard and the protein content was calculated on the basis of the tyrosine content of bovine serum albumin.

Determination of DNA. DNA was determined on whole tissue or cell homogenates after extraction of RNA with 1.6M-trichloroacetic acid for 18hr. at 4°. The remnant containing DNA was dissolved directly in the 1M-3,5-diaminobenzoic acid reagent of Kissane & Robins (1958) and DNA was determined fluorimetrically (Kissane & Robins, 1958) (436nm. exciting, 520nm. emitting wavelength). The DNA standard used (Sigma type I; calf thymus, 7.4% P, i.e. 78.4% pure), was appropriately diluted with 1M-NaOH.

Determination of substrates in metabolic experiments. Lactate was determined during metabolic experiments (see below) by the method of Hohorst (1962). In routine studies the standard procedures and reagents of C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany) were used.

Glucose was determined by using glucose oxidase, peroxidase and o-dianisidine. In routine studies the standard Boehringer procedures and reagents were used.

Electron microscopy. Specimens were fixed in 1% OsO₄ in 0.15M-sodium barbitone buffer, pH7.40, dehydrated and embedded in Vestopal (Martin Jäger, Vésenaz/Genève, Switzerland). Examinations were performed at magnifications of $\times 13000$ –75000 in a Philips 100B electron microscope.

RESULTS

Growth of cells. After a lag phase of 2½ days the explanted kidney-cortex cells began to grow steadily. In control cultures the mean generation time in the exponential phase was about 29hr. and monolayers were developed within 7 days. Fig. 1 shows a typical growth curve illustrated by the DNA content per culture.

As indicated in Table 1, however, some of the factors that influenced the final LDH isoenzyme pattern also affected the growth rate. The cells grew readily in 5% oxygen, but failed to grow in an oxygen concentration higher than 50% at 760mm. Hg. In the incubator a concentration of 40% oxygen decreased the growth rate by nearly half. Replacement of some of the glucose in the medium with acetate, lactate, pyruvate or kidney-cortex extract also affected the growth rate, but to a smaller extent (Table 1).

Morphological features. Most of the cells were of the epithelial type, arranged in groups of 5–15 cells surrounded by spindle-like cells of the fibroblastic type. Only the cells grown in acetate with 40% oxygen developed vacuoles. Electron-microscopic examination of cells after 7 days in culture, when compared with the cortex cells obtained from a cell suspension before culture, revealed a structure with a marked lack of differentiation in the cytoplasmic organelles. This involved an apparent decrease in the number and size of mitochondria, as well as

Fig. 1. Average concentration of glucose (○) and lactate (□) in the medium of kidney-cortex cells plotted against time of culture. Samples were taken twice a day. The growth characteristics of the cells are indicated by the increase in DNA per culture (∇). The concomitant shift in LDH isoenzyme pattern is also shown (Δ). After 120 hr. the medium was changed.

occurrence of mitochondria with packed folds of cristae in concentric or parallel arrays.

Metabolic properties. Depletion of glucose and accumulation of lactate in tissue culture media were examined to correlate the glycolytic rate of cultured cells with the shift in LDH isoenzyme pattern. In four cultures the changes of medium every second day were omitted until the medium was exhausted of glucose. This occurred after 5 days of culturing (Fig. 1). Samples were taken twice a day. In Fig. 1 the average concentrations of glucose and lactate in culture media are plotted against time (hr.) of culturing. During the first 2½ days the glucose concentration decreased by about 1 mm and during the next 2 days by about 4 mm. During the first 4 days this decrease in glucose was accompanied by an equivalent increase in the concentration of lactate. During the fifth day of culturing a slight decrease in lactate in the medium was often observed (Fig. 1).

LDH activity and isoenzyme pattern. During the 7 days of culture the total amount of LDH units in kidney-cortex cells increased by about 60 milliunits/μg. of DNA.

Trypsin-treated kidney-cortex cells in suspension initially exhibited the LDH isoenzyme pattern of kidney cortex (Fig. 2 and Table 1). However, in the first 2½ days in culture (the lag phase) the H subunit/M subunit ratio decreased from about 3.0 to 1.5. This shift in isoenzyme pattern, occurring before any appreciable cell division, was also found in the cells floating in the medium (Table 1). During the exponential growth phase the H subunit/M subunit ratio decreased even further, to approx. 0.6 at the

Table 1. Lactate dehydrogenase isoenzyme monomer proportion of cultured kidney-cortex cells correlated to growth phase, changes in oxygen supply, changes in nutritional supply and mean generation time

Culture conditions	H subunit/ M subunit ratio	Time when monolayer developed (days)
Standard conditions: 5.56 mm-glucose + O ₂ + CO ₂ + N ₂ (25:5:70)		
Cells attached	day 1	2.8
Cells attached	day 2½	1.5
Cells attached	day 3	0.9
Cells attached	day 5	0.6
Cells attached	day 7	0.6
Cells attached	day 12	0.7
Cells in suspension	day 0	2.7
Cells remaining in suspension	day 2	2.0
Cells remaining in suspension	day 3	1.1
	H subunit/ M subunit ratio†	
40% O ₂	1.2	10
Cells on a floating Millipore filter	0.9	9
1% CO ₂	0.7	8
5% O ₂	0.5	7
0.84 mm-Sodium acetate + 0.28 mm-glucose*	1.0	9
0.56 mm-Sodium lactate + 0.28 mm-glucose*	0.8	8
0.56 mm-Glucose*	0.6	7
0.56 mm-Sodium pyruvate + 0.28 mm-glucose*	0.5	8
Kidney-cortex cell strain (5.56 mm-glucose*)	0.9	15
Explants of kidney cortex in tissue culture*	0.6	
Extract of kidney cortex (50%, v/v)*	0.8	8
Medium 858 in place of Hanks BSS*	0.8	7
Medium changed every 24 hr.*	0.5	6

* Gas phase as in the standard conditions, i.e. O₂ + CO₂ + N₂ (25:5:70).

† At the time when the monolayer developed.

end of this phase (Fig. 2 and Table 1). The formation of a cell monolayer during the fifth to seventh day of culture was not followed by any further changes in the H subunit/M subunit ratio. During the stationary phase, however, the proportion increased slightly to about 0.7 (Table 1). Variations in the H subunit/M subunit ratio of kidney-cortex explants grown as tissue cultures were similar to those described in the cell cultures. The ultimate monomer proportion was 0.6 (Fig. 3).

During the period of cultivation, the cultures finally developed into a homogeneous monolayer.

Fig. 2. LDH isoenzyme shift of cultivated kidney-cortex cells. The numbers 0–5 indicate days in culture. At day 0 the trypsin-treated kidney cells exhibit the pattern of kidney cortex. At day 5 the M_4 and HM_3 isoenzymes predominate.

At this time the H subunit/M subunit ratio acquired values depending on environmental factors, i.e. oxygen supply, glycolysis, growth ratio and the addition of various components.

Oxygen supply. Cells cultured in 40% oxygen (Fig. 4) or in 20% oxygen on a floating Millipore filter had an H subunit/M subunit ratio twice that of cells cultured in 20% oxygen beneath a culture medium of 1 mm. thickness (Table 1 and Fig. 3).

Glycolytic rate. In control cultures grown in 5.56 mM-glucose the decrease in H subunit/M subunit ratio during the exponential growth phase was accompanied by an increase in glucose consumption (Fig. 1). Cells cultured in 0.84 mM-sodium acetate plus 0.28 mM-glucose had a final H subunit/M subunit ratio twice that of the controls (Table 1 and Fig. 5). Replacement of glucose by lactate had a similar but less-pronounced effect, whereas

Fig. 3. LDH isoenzyme pattern of kidney-cortex cells cultured in 20% O_2 on a floating Millipore filter (M) and the pattern of an explant culture of kidney cortex after 7 days *in vitro* (e).

Fig. 4. LDH isoenzyme pattern of kidney-cortex cells grown for 10 days in 40% O_2 (40) and in 20% O_2 (20). s, Isoenzyme pattern of a kidney-cortex cell strain during the twenty-first passage.

replacement by pyruvate lowered the proportion further (Table 1 and Fig. 5). A tenfold decrease of the glucose concentration in the medium slightly elevated the final H subunit/M subunit ratio (Table 1).

Growth rate. During the exponential growth phase the H subunit/M subunit ratio of control cultures decreased 50% (from 1.5 to 0.6) (Fig. 1). A slight increase in the proportion, however, was

Fig. 5. LDH pattern of kidney-cortex cells cultivated for 9 days in 0.84 mm-sodium acetate + 0.28 mm-glucose (a), for 8 days in 0.56 mm-sodium lactate + 0.28 mm-glucose (l), and for 8 days in 0.56 mm-sodium pyruvate + 0.28 mm-glucose (p).

found during the stationary phase, i.e. during the seventh to the twelfth days of culture (Table 1). A kidney-cortex cell strain that during the twenty-first passage possessed a mean generation time nearly twice as long as that of the controls exhibited a final monomeric proportion (0.9) 50% higher than the control cultures (Fig. 4 and Table 1).

Effects of various additives. The addition of a kidney-cortex extract resulted in a slight increase of the ultimate H subunit/M subunit ratio from 0.6 to 0.8 (Table 1). The addition of NAD⁺ or NADP⁺ failed to affect the ratio.

Other changes in environmental conditions. Replacement of Hanks salt solution by medium 858 resulted in a slight elevation of the final H subunit/M subunit ratio (Table 1). Culture in 1% carbon dioxide had the same effect, whereas medium change every day instead of every other day failed to elevate the final H subunit/M subunit ratio (Table 1).

Combined factors. On the basis of the results described above, an attempt was made to combine

the factors possessing the most pronounced ability to restore the H subunit/M subunit ratio of the kidney cells before culture. A cell suspension was cultured in a basic medium but contained 0.84 mm-acetate + 0.28 mm-glucose at 40% oxygen. After 10 days a monolayer was developed. The cells were vacuolated. The H subunit/M subunit ratio was 1.2.

DISCUSSION

The reported decrease in H subunit/M subunit ratio during culture of disaggregated calf kidney-cortex cells and explants agrees with results obtained from trypsin-treated cell suspensions (Philip & Vesell, 1962; Cahn, 1964; Goodfriend, Sokol & Kaplan, 1966) or explants (Philip & Vesell, 1962) of chick tissues, from human (Nitowsky & Soderman, 1964) and monkey (Goodfriend *et al.* 1966) cell strains, and from cultured lymphocytes (Bloom, Tsuchioka & Wajma, 1967; Rabinowitz, Lubrano, Wilhite & Dietz, 1967).

The observed shift in monomer proportion may be caused by selection of cells, a deranged metabolism of damaged cells, an adaptation to environmental conditions of culture *in vitro*, cell division or lack of organizers.

A possible shift in H subunit/M subunit ratio as a result of selection of certain cells cannot be excluded. The shift in monomer composition was, however, also found in the cells remaining in suspension, i.e. cells that did not give rise to monolayers and survive. The shift in monomer composition of the attached cells continued during the exponential growth phase, whereas cell morphology did not change.

Metabolites leak into the medium from newly inoculated cells. This may cause oxygen uptake to fall to low values (Danes, Broadfoot & Paul, 1963). This deranged metabolism may explain the shift in H subunit/M subunit ratio observed during the lag phase. The reported changes in monomer proportion of trypsin-treated cell suspensions could, however, be mimicked by intact explants of the same tissue. This indicates that enzyme changes are not simply due to the dissociation and trypsin procedures. Other unfavourable environmental conditions during culture, such as lack of oxygen, may offer an explanation.

In tissue culture normally the cells are kept beneath a culture medium at a depth of 0.5–1 mm. However, the oxygen diffusion coefficient, defined by Thews (1960) as Krogh's diffusion coefficient, of a 0.7% protein solution is 6 μ l. of oxygen/mm./min. at 0.2 atm. Since 10⁷ cells in primary cell culture will use approx. 6 μ l. of oxygen/min. (Danes *et al.* 1963) the oxygen partial pressure experienced by cells cultured at a depth of 1 mm. can be close to zero. Cultured cells, however, have variable needs

for oxygen and may hardly use oxidative pathways at all (Paul, 1965, p. 44; Horecker, 1965). Many cells grow satisfactorily irrespective of whether oxidative or glycolytic pathways are predominant (Leslie & Jarnell, 1960). The content of cytochrome oxidase and the formation of mitochondria in cultured cells depends on oxygen partial pressure (Leslie & Jarnell, 1960; Linnae, 1965; Hakami & Pious, 1967). The described decrease in H subunit/M subunit ratio may reflect the adaptation of cultured cells to a diminished supply of oxygen. A previous study has suggested that a correlation might exist between the amount of H monomers available and the mitochondrial content (Güttler & Clausen, 1967). Cahn (1964) has shown, however, that the decrease in the ratio of H monomers to M monomers is due mainly to an increased synthesis of M monomers. The electrophoretic technique employed in this investigation does not allow a selective evaluation of the synthetic rate of a single monomer.

Further, Clausen & Hustrulid (1969) have shown that a decrease in H subunit/M subunit ratio during maturation of muscles is related to the function of striated muscles. Environmental conditions other than the oxygen supply can therefore modify the H subunit/M subunit ratio. The present investigation shows that changes in the glycolytic activity may induce changes in the ratio of H monomers to M monomers. This is consistent with the demonstration by Clausen & Øvlisen (1965) of a difference in the extent of product inhibition by L-lactate on pyruvate reduction by the two tetramers H₄ and M₄. The rate of glycolysis in cultured cells is directly related to the composition of the medium (Munyon & Merchant, 1959). A high rate of glycolysis can be maintained by renewing the medium daily to maintain a high glucose concentration (Munyon & Merchant, 1959; Wilson, Jackson & Brues, 1942). This procedure results in a decrease in H subunit/M subunit ratio, whereas a tenfold decrease in the glucose concentration in the medium limited this decrease. The observed 50% inhibition of the shift in the H subunit/M subunit ratio on replacing glucose with acetate suggests a further relationship between the glycolytic rate and changes in H subunit/M subunit ratio.

A relationship has been found between glycolysis and cell division (Kitos & Waymouth, 1964; Cristofalo & Kritchevsky, 1965; Westfall, Peppers & Earle, 1955). During different phases of growth, changes occur in the pattern of carbohydrate metabolism of monolayer cultures (Paul, 1965, p. 51). The glycolytic rate is high during the lag phase and the earlier part of the exponential growth phase (Paul, 1965, p. 51). This correlates with the present finding that 50% of the observed decrease in H subunit/M subunit ratio occurred during the

Table 2. *Lactate dehydrogenase isoenzyme monomer proportion of renal tissues correlated to the partial pressure of oxygen*

Tissue	H subunit/ M subunit ratio	pO ₂ (mm. Hg)
Renal cortex	2.9*	92–100†
Renal medullary zone	1.8*	38–68†
Cortex cells in culture:		
O ₂ at 250 mm. Hg.	1.2	100‡
O ₂ at 150 mm. Hg.	0.5	0‡
Papilla renalis	0.6*	18–22†

* Güttler & Clausen (1965).

† Leonhardt & Landes (1963).

‡ Calculated according to Thews (1960).

lag phase and the other 50% during the early part of the exponential phase. The fact that the combination of factors found were still unable to maintain the original isoenzyme pattern supports the view of Koskimies (1967) that differentiation also determines the H subunit/M subunit ratio.

Urinary LDH may originate in the kidneys (Plummer, 1965; Güttler & Clausen, 1965). As the cortical tubular cells show preponderances of H₄ and H₃M, as in urine, urinary LDH may be derived mainly from these cells (Mattenheimer, 1968; Raab, 1968). Urine and blood from the affected kidney in unilateral renal ischaemia show, however, an M-type pattern of LDH (Güttler & Clausen, 1965; Ringoir, 1967; Nielsen, Kemp & Laursen, 1968). As the activity of HM₃ and M₄ is low in total kidney extracts and only found in small amounts in the medulla and in the papilla tissue (Mattenheimer, 1968; Güttler & Clausen, 1965; Nielsen *et al.* 1968), the change in the urinary LDH isoenzyme pattern after hypoxia may be caused by a release of LDH of altered isoenzyme pattern from renal tissue.

If the oxygen supply to the cortex in renal ischaemia decreases to values normally characteristic of the inner medullary zone (Table 2), this could lead to a H subunit/M subunit ratio of the medullary type. The present study supports this hypothesis since it demonstrates that the oxygen partial pressure is one of the environmental factors changing the LDH isoenzyme pattern of cortical tubular cells into the medullary type. Previous studies have shown that this isoenzyme pattern actually is found in the urine from the affected kidney in unilateral renal ischaemia (Güttler & Clausen, 1965).

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