Evaluation of the number of goblet cells in crypts of the colonic mucosa with and without fecal transit

Avaliação do número de células caliciformes nas criptas da mucosa colônica com e sem trânsito intestinal

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A B S T R A C T

Objective: To measure the thickness of the crypts and quantify the number of goblet cells of the colonic mucosa with and without intestinal transit, relating them to exclusion time. **Methods**: Sixty Wistar rats were divided into three groups of 20 animals each according to the time of the final operation for the removal of the colon, in six, 12 or 18 weeks. In each group 15 animals underwent colonic exclusion by left colon proximal colostomy and distal mucous fistula, and five underwent only laparotomy (control). The colons with and without fecal stream were removed, processed and submitted to histological sections stained with hematoxylin-eosin. The height of the colonic crypts and the number of goblet cells were measured by computerized morphometry. We used the Student t test and Kruskal-Wallis test for comparison and analysis of variance, using a significance level of 5% ($p < 0.05$). **Results**: The height of the crypts decreased in segments without fecal stream ($p = 0.0001$), reducing from six to 12 weeks of exclusion ($p = 0.0003$), stabilizing thereafter. The number of goblet cells in the crypts was smaller in segments without transit after 12 and 18 weeks ($p = 0.0001$), but increased as the time of exclusion progressed ($p = 0.04$) **Conclusion**: The exclusion of intestinal transit decreases the thickness of the colonic crypts and the number of goblet cells in the segments without transit. There is an increased number of goblet cells in the course of time exclusion.

Keywords: Colon. Colitis. Goblet cells. Image processing computer-assisted. Volatile fatty acids.

INTRODUCTION

The colonic epithelium is the most perfect functional barrier of the human body [1]. A single layer of cells juxtaposed with one another and attached to the basement membrane separating the interior of the intestinal lumen, with high bacterial concentration, from the sterile inner layers forming the intestinal wall [2]. This functional barrier is composed of multiple lines of defense, represented mainly by the layer of mucus covering the epithelial surface, the apical and basolateral membrane cell, the complex system of junctions and the basal membrane [1,3-5].

The mucus covering the intestinal epithelium, secreted by goblet cells present in the glands of entire digestive tract, form the first line of defense of the colonic mucosa.The goblet cells vary in number over the different segments of the large intestine, increasing as it progresses towards the more caudal segments, where they occupy virtually the entire length of the glands. Changes in goblet cell population, when comparing the different colon segments, are related to distinct physiological functions performed by each portion of the intestinal mucosa [6]. In the colon, the mucus layer, besides the lubricating function facilitating the progression of the fecal contents, provides protection against chemical attack caused by antigens, toxins and digestive enzymes existing within the intestinal lumen [3,4]. The mucus also possesses antibacterial properties, as it reduces the bacterial population in direct contact with the epithelial surface, hindering translocation to the internal environment [1,3,4,7].

Abnormalities in the secretion, composition and distribution pattern of mucins in the intestinal crypts have been demonstrated in several inflammatory diseases that affect the colon [6,8]. One example is the diversion colitis, a disease characterized by the development of chronic inflammation in the mucosa of segments without fecal

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transit [9,10]. In patients with diversion colitis, one of the most common complaints is the constant elimination of mucus from the excluded segment, suggesting that there is an increased production of mucus in these segments [9]. Model studies of diversion colitis showed changes occurring in the tissue content and in the expression pattern of the different subtypes of mucins in the colonic crypts when comparing segments with and without fecal transit [10-12]. However, it is unclear if these changes are related to changes in the population of goblet cells or increased production capacity of mucus by chronically inflamed epithelial cells. There is evidence of atrophy of intestinal crypts in excluded segments when comparing segments with and without intestinal transit, but controversy exists regarding the population of cells. Likewise, the influence of time of fecal exclusion in the number of goblet cells is not yet well established. It is believed that, over time, there is a proportional increase in the number of these cells in the excluded colon [10]. However, few studies have measured the population of goblet cells in colonic crypts comparing segments with and without fecal stream at different periods of exclusion, relating it to the thickness of the intestinal crypts [9,13,14]. The quantification of the number of goblet cells with a precise method of analysis could clarify whether there would be a proportional increase in the number of goblet cells, which could explain the symptoms of patients, despite the atrophy of crypts found in the segments without transit.

Therefore, the objectives of this study were to quantify the goblet cells and correlate this finding with the thickness of the colonic crypts when comparing segments with and without intestinal transit, also assessing the influence of time of exclusion in the population of these cells.

METHODS

We used 60 male Wistar rats, SPF, from the Multidisciplinary Center for Biological Research, State University of Campinas (UNICAMP-CEMIB), weighing between 300 and 320g and an average age of four months. Until the date of the operation, all animals were kept in individual cages in air-conditioned environment with controlled temperature and humidity and 12-hour light / dark cycles. The day before the surgical procedure they were fasted, except water, for 12 hours. The cages were identified by the number of animals, group and subgroup to which they belonged and the same data were tattooed on the tail of each rodent.

The animals were randomly divided into three experimental groups of 20 animals, according to the sacrifice being done in six, 12 and 18 weeks after surgery. In each group, 15 animals underwent colonic bypass through a left colon proximal colostomy and distal mucous fistula (subgroup study) and five, only laparotomy, without bowel bypass (control subgroup). On the day of surgery, the animals were weighed and anesthetized with xylazine + ketamine at a dose of 0.1 ml/100g administered intramuscularly into the left hind leg.

The abdominal cavity was opened by median incision, three inches long, the Peyer's patch being found, located at the antimesenterial edge of the rectalsigmoid transition. The distance between the Peyer's patch and the site chosen for the section of the left colon, four centimeters above the top of the patch, was measured with a caliper. After ligation of the marginal arcade vessels and section of the left colon, in all the experimental animals the proximal end was exteriorized as a colostomy in the left hypochondrium. The distal segment of the sectioned intestine was catheterized with a 12F polyvinyl tube and irrigated with 40 ml of 0.9% saline solution at 37° C until the effluent drained through the anus came out clean of feces. After the irrigation the catheter was removed and the distal colon exteriorized as a colostomy (distal mucous fistula) in the lower left side of the abdomen. The fixation of stomata to the skin was performed with the use of interrupted stitches of 4-0 monofilament absorbable sutures in the four cardinal points, and between them, always tied with three knots. The abdominal wall was closed in two planes: peritoneum and aponeurosis with running 4-0 polyglycolic acid sutures and the skin with 4-0 nylon interrupted ones.

When done, the animals were kept warm for 10 minutes and, after recovery from anesthesia, housed in their cages, being released to water and standard chow intake after recovering the waking state. Throughout the postoperative period they were isolated and kept in the same air-conditioned environment until the time of sacrifice. There was no additional care was taken in relation to wounds and stomata, nor were administered analgesics or antimicrobials. On the eve of the day set for the withdrawal to the colon, the animals were again weighed and fasted for 12 hours, except for water. They were then anesthetized for the procedure with the same technique described above and subjected to abdominal trichotomy and antisepsis. The abdominal wall was reopened through a wide midline incision. In animals from the experimental subgroup the colon with, and all the remaining segment without, fecal transit, including the anus, was removed. In the animals from the control subgroup the left colon was resected from the left flexure.

The segments of extirpated colon were opened at the antimesocolic edge in the longitudinal direction and arranged and secured on a plate of cork with the mucosal surface facing up. They were then fixed for 72 hours in a 10% buffered formaldehyde solution, dehydrated in increasing concentrations of ethanol and cleared in xylene. After this step they were embedded in paraffin blocks and subjected to 5ìm-thick longitudinal cuts for the preparation of histological slides, stained with hematoxylin-eosin (HE). The diagnosis of colitis was always established by the same pathologist with expertise in inflammatory

bowel disease, who did not know the origin of the material and the objectives of the study.

The measurements were always performed by computerized morphometry. The technician who performed the measurements was unaware of the origin of the specimen, as well as the experimental group to which the animal belonged. The measurements of the thickness of the colonic crypts and quantification of the number of goblet cells were always performed on a site where there were at least three contiguous and intact crypts in six random fields, a total of 18 crypts being studied in each segment. To measure the thickness of crypts and goblet cell count the selected image was focused by an ordinary optical microscope and captured by a video camera previously coupled to the microscope body. The captured image was digitized and transferred to a microcomputer and analyzed using NIS-Elements (Nikon Corporation, Japan). To measure the thickness of the crypts in the control group and in segments with and without transit of the subgroup experiment the program marke, the apical and basal limits of each crypt with a cross. After the measurement of 18 crypts, the program automatically provided the average of measured values with the standard error of each segment. The mean values were transferred to a data sheet. Immediately after measuring the thickness, the number of goblet cells was quantified in the same crypt and also transferred to the same sheet. Thus, the values for the two variables were always obtained in the same crypt. After the evaluation of the colon of all animals at the different times the final values found for the thickness of the crypts and goblet cell population within each group were also calculated and expressed as mean, with the corresponding standard deviation.

We used the Student t test to analyze the thickness of the intestinal crypts and number of goblet cells, comparing segments with and without fecal transit and the segments obtained from control animals. The test was applied separately for each period of exclusion established (six, 12 and 18 weeks). The Kruskal-Wallis test was used to evaluate the influence of time of exclusion for both variables. All data were analyzed by adopting a significance level of 5% in all tests. The significant values were marked with an asterisk.

This study was approved by the Ethics Committee of São Francisco University (2211/2007), and followed the guidelines of the Brazilian College of Animal Experimentation - (COBEA) and the Federal Law 11.794 of August 10th, 2008 (Sergio Arouca Law).

RESULTS

Figure 1A shows the colon wall with intestinal transit of an animal subjected to intestinal bypass for six weeks, while figure 1B, the colon wall devoid of fecal stream of the same animal. It was found that, after six weeks of exclusion, the thickness of crypts decreased in segments without fecal stream when compared to control animals and those segments with transit of animals from the experiment subgroup ($p = 0.0001$). By analyzing the variation of the thickness of the colonic crypts according to the time of exclusion, we found that, in the colon without transit, there was a reduced thickness between six and 12 weeks ($p = 0.0003$) and there was no change after that period ($p = 0.056$).It was found that, after 12 and 18 weeks of exclusion, the number of goblet cells was lower in segments without fecal stream when compared to control animals and those segments with transit of the experimental group ($p = 0.00001$).

It was found that, in control animals and in the segments with transit of the experimental animals, the number of goblet cells increased with time ($p = 0.001$). In segments without fecal transit we found that an increased number of goblet cells after 18 weeks of exclusion ($p = 0.04$).

DISCUSSION

Diversion colitis is an inflammatory disease that affects the lining of the colon or rectum devoid of intestinal transit [9]. The disease is a syndrome of nutritional deficiency caused by a deficiency of the regular supply of short-chain fatty acids, the main energy substrate for the proper trophism and metabolism of colonic epithelial cells [15-19]. The derivation of colonic transit deems mucosal cells unable to metabolize short chain fatty acids as an energy source.

Alternatively, then, they begin to use glutamine provided by the blood circulation [14]. However, systemic supply of glutamine appears to be unable to maintain adequate nutrition of the colonic epithelium, causing changes in the mechanisms of energy-producing cells of the intestinal mucosa [20]. These metabolic changes lead to increased production of oxygen free radicals by epithelial cells, molecules that have been blamed for the initial damage to the mucosa in experimental models of exclusion colitis, as well as in patients with ulcerative colitis [2,20]. The importance of a regular supply of short-chain fatty acids for cellular nutrition can best be gauged by the results of studies demonstrating that administration of substances that inhibit the metabolism of short chain fatty acids are capable of triggering the onset of colitis [15,21]. Its importance for proper cell nutrition is more evident with the results of studies showing that the restoration of intestinal transit, normalizing the supply of short-chain fatty acids, or the application of enemas containing solutions rich in them in the excluded segments, can reverse the inflammatory process [10,22,23]. They are capable of modulating the proliferation of goblet cells and, when instilled into the colon without fecal stream, stimulate production and disposal of mucini [24,25]. Amongst the short chain fatty acids, butyrate seems to be most effective in maintaining epithelial trophism and production

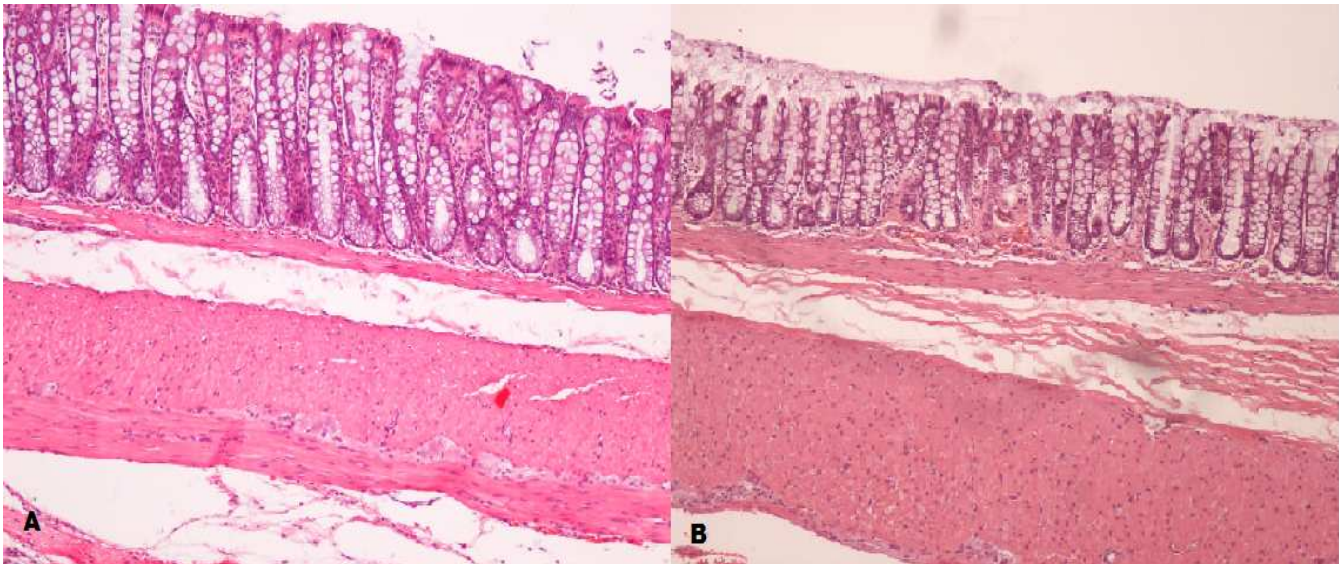

Figure 1 - *A) Photomicrograph of the colon wall segment provided with fecal transit six weeks after intestinal bypass; B) Photomicrograph of the colon wall segment devoid of fecal stream six weeks after intestinal bypass. (HE 200x).*

and release of mucus by the colon mucosa [26,27]. Studies have shown the importance of maintaining the provision of these acids for the expression of genes related to the formation of different subtypes of mucin synthesized by goblet cells [4,28].

Studies using experimental models of colitis exclusion describe a series of histological changes in the colon wall, similar to those occurring in humans, mainly represented by erosions or ulcerations in the intestinal mucosa, a significant reduction in the thickness of the intestinal crypts, inflammatory infiltrate, Focal nodular hyperplasia, submucosal vascular congestion, changes in the number of goblet cells and changes in the synthesis of mucins [10-15]. One of the most common complaints in patients with this type of colitis is the constant elimination of mucus from the excluded segment transit. The larger passage of mucus suggests that there may be due to the increased production of goblet cell population in these segments [10]. Yet, paradoxically, experimental and human studies have shown that, with the exclusion of colon fecal stream, there is significant atrophy of the mucous layer, mainly of intestinal crypts, because of the lack of regular supply of short-chain fatty acids. To explain these apparently contradictory findings, some studies suggest that despite the reduction in intestinal crypts by nutritional deficiency, the population of goblet cells does not change, even occurring a proportional increase when compared to the other cells constituent of colonic crypts [7,10]. However, no study has quantified the number of goblet cells compared with segments without fecal stream and linking it with the epithelial thickness and the time of fecal exclusion. The number of goblet cells indirectly reflects the ability of mucus secretion and activity of colonic glands, whose function is to produce and secrete mucins throughout the gastrointestinal tract [9]. Keli et al. [10] analyzed the population of goblet cells and measured the thickness of the colonic mucosa in segments without fecal stream, and found a reduction in thickness of the mucosa and maintenance of the population of goblet cells. They them suggested that, in absolute numbers, actually there was an increase in the population of goblet cells. For them it is possible that the proportional increase can be responsible for the increased production of mucus. However, they evaluated the population of goblet cells in a subjective manner, without describing any item in the quantification method that ensures greater result reliability [10].

Other authors evaluated the population of goblet cells in fecal exclusion of segments, comparing animals undergoing intestinal bypass for different periods of time, using histochemical methods for identification and subsequent morphometry to quantify the number of goblet cells rich in acidic and neutral mucins [13]. They found that there was no significant difference with respect to the total amount of goblet cells in comparing colon fragments collected at the time of intestinal bypass (control group) and from segments without fecal stream, regardless of the time of exclusion. Nevertheless, they found a reduction of goblet cells rich in acid mucins in the course of time of exclusion and, although they used computational analysis to quantify the goblet cells, the authors do not mention the use of any method of mechanical cleaning before performing the exclusion [13]. It has been shown that mechanical cleaning is an important step when using experimental models of colitis exclusion, because without it it is impossible to ensure that the excluded colon is completely free of fecal matter that would maintain a supply of short-chain fatty acids [29]. Research using routinely anterograde mechanical preparation of the colon found that both the content of acidic and neutral mucin is lower in the colon without transit, but there is an increase of total content as the time of exclusion passes [11]. Subsequently, the same

group, using histochemical techniques that allow identification of acidic mucin subtypes, found that in the colon excluded there is an increase in tissue acid-content mucins. However, only at the expense of sulfated mucins, since the ones rich in sialic acid almost disappeared [12]. These results suggest that the increased production of sulphated neutral and acidic mucins in the colon without transit can be related to the proportional increase in the number of goblet cells in the course of time exclusion [30].

In the present study, in order to be able to measure the thickness of the intestinal crypts and the number of goblet cells in an objective way, we chose the use of computerized morphometry due to familiarity with the method [11,12,15]. The computerized morphometry is an available and inexpensive methodology, which allows precise measurement of different histological features. Initially, it appeared interesting to measure the thickness of the colonic crypts, comparing segments with and without intestinal transit, in order to verify if the lack of supply of short-chain fatty acids could reduce the height of the colonic crypts. It was found that, in all animals the segments without fecal transit displayed a significant reduction in the height of the colonic crypts, regardless of the exclusion time considered. These findings confirm that the lack of supply of such acids to epithelial cells results in atrophy of the mucosal surface. It was further observed that the atrophy of colonic crypts in segments without transit occurred mainly during the first 12 weeks of exclusion and did not vary thereafter. These findings are similar to those found by other studies, suggesting that after the first 12 weeks of exclusion, considered a critical period, there might be a gradual adaptation of epithelial cells to the alternate supply of glutamine by the systemic circulation [14]. Nevertheless, this alternative supply is not able to maintain the trophism of intestinal crypts similar to what occurred with the colon with preserved transit because, independently of the time of exclusion, there was significant atrophy of the crypts in segments without short chain fatty acids.

In order to verify whether the number of goblet cells was altered in the segments without fecal stream, we also used the computerized morphometry to quantify it. Only two studies used computer analysis to determine the number of goblet cells relating to the exclusion time [13,14]. In the first, the different methodology employed does not allow comparison with results found in this study [13]. In the second, which found no alterations in cell number over the course of time, the authors did not established a relation between the total number of goblet cells found and the thickness of the crypts, making it impossible to assess the occurrence of a proportional increase [10,14].

Mechanical preparation with anterograde saline was used in all animals of the subgroup experiment, similar to what was previously proposed [29]. Only one study had evaluated the thickness of the intestinal crypts and the number of goblet cells in a model of diversion colitis which used systematic mechanical preparation [14]. The importance of the anterograde preparation was confirmed by noticing that, even after the fasting time proposed, in all animals subjected to the derivation of intestinal transit there was output on average of two (1-5) pellets of feces through the anus during the course of cleaning. Therefore, it was confirmed that without mechanical preparation it is impossible to ensure complete cleaning of the colon, which will certainly affect results.

When comparing the number of goblet cells between the control and experimental groups (segments with and without intestinal transit) we found a decrease in the segments without fecal stream, regardless of time of exclusion considered. Conversely, in the colon with the provision of short-chain fatty acids preserved there was an increase in the number of goblet cells after 12 and 18 weeks. These findings confirm that a steady supply of short-chain fatty acids is important to maintain the number of goblet cells throughout the experiment.

Likewise, the weight gain of animals can also be considered as a factor related to the increased thickness of the crypts and hence the number of goblet cells. In the segments without intestinal transit the number of goblet cells did not change during the first 12 weeks from the time of exclusion, although there was a transient decrease of the height of the colonic crypts. After 18 weeks, although there was no variation in height of the crypts in excluded segments, we found increased numbers of goblet cells. Since there is also a reduction of the crypts thickness in the same period, it suggests that the number of goblet cells has increased, as proposed above [10].

Several factors may explain the increased proportion of goblet cells in the segments without fecal stream with the passage of time. It is possible that the exclusion of feces reduces the need for absorption by the colonic gland, causing the cells from the proliferative zone to prioritize the differentiation to goblet cell over the ones with absorptive function. It is also possible that, with the worsening of the inflammatory process in the segments without intestinal transit, there is greater need for mucus-producing cells, in order to strengthen the first line of defense of the colonic mucosa against oxidative stress. Perhaps the greatest amount of mucus found in segments without fecal transit may result from the increased formation of goblet cells by the proliferative zone and from the highest rate of apoptosis of mature goblet cells in specialized portions of the crypts. Mucus has, as one of its main functions, the lubrication of the stool for easy transit within the colon. In the distal colon, as such feces have greater consistency, this feature becomes more important, being the main reason for the higher concentration of goblet cells in that region. It is possible that the absence of fecal stream arising from intestinal exclusion causes the mucus produced and secreted in larger quantities by the segment excluded to accumulate inside the colon. As the patient does not eliminate the daily amount of mucus due to lack of transit, the accumulated mucus, associated with bleeding caused

by epithelial erosions and ulcerations, might be the explanation for the symptoms of patients with diversion colitis who complain of periodic elimination of mucus and blood by the segments without transit.

The results of this study reinforce the importance of the fecal stream for the proper development of colonic crypts. The exclusion of intestinal transit reduces the thickness of the crypts and decreases the population of goblet cells in intestinal crypts. However, as the exclusion time passes, despite the reduction in colonic crypts, there is a proportional increase in the number of goblet cells. These histological findings are consistent with the symptoms reported by patients with diversion colitis.In conclusion, there is a reduction in the number of goblet cells in colon segments without intestinal transit. The proportional number of goblet cells in relation to the thickness of the crypts increases in colon segments after 18 weeks of fecal exclusion.

R E S U M O

Objetivo: *Medir a espessura das criptas e quantificar o número de células caliciformes comparando a mucosa cólica com e sem trânsito intestinal, relacionando-as ao tempo de exclusão.* ***Métodos:*** *Sessenta ratos Wistar, foram distribuídos em três grupos com 20 animais segundo a operação final para a retirada dos cólons, realizadas em seis, 12 ou 18 semanas. Em cada grupo, 15 animais foram submetidos à derivação do trânsito por colostomia proximal no cólon esquerdo e fístula mucosa distal e cinco apenas à laparotomia (controle). Os cólons com e sem trânsito fecal foram removidos, processados, submetidos a cortes histológicos corados pela hematoxilina-eosina. A altura das criptas colônicas e o número de células caliciformes foram mensurados por morfometria computadorizada. Foram utilizados os testes t de* Student *e Kruskal-Wallis para comparação e análise de variância, estabelecendo-se nível de significância de 5% (p<0,05).* ***Resultados:*** *A altura das criptas diminui nos segmentos sem trânsito fecal (p=0,0001), reduzindo entre seis e 12 semanas de exclusão (p=0,0003), estabilizando-se após este período. O número de células caliciformes nas criptas é menor nos segmentos sem trânsito após 12 e 18 semanas (p=0,0001), porém aumenta com o decorrer do tempo de exclusão (p=0,04)* ***Conclusão:*** *A exclusão do trânsito intestinal diminui a espessura das criptas colônicas e o número de células caliciformes nos segmentos sem trânsito. Existe aumento do número de células caliciformes com o decorrer do tempo de exclusão.*

Descritores: *Colo. Colite. Células caliciformes. Processamento de imagem assistida por computador. Ácidos graxos voláteis.*

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