Cytokine expression profile of sensitized human T lymphocytes following *in vitro* stimulation with amoxicillin

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ABSTRACT. Since the withdrawal of penicillin determinants from the market, in addition to the hazard of re-exposing the patient to the drug, skin testing for the diagnosis of penicillin allergy has become less accurate and less standardized. The assay currently used, the lymphocyte transformation test (LTT), lacks sufficient sensitivity, and requires the use of radioactive material. The objective of this study was to establish an accessible and reliable method for the safe diagnosis of penicillin allergy. Peripheral blood mononuclear cells (PBMC) were isolated from 18 patients who were allergic to penicillin and 12 control subjects using the Ficoll-Hypaque method. The isolated, sensitized cells were stimulated *in vitro* with amoxicillin (1 mg/mL). Stimulation with phytohemagglutinin A (PHA) was used as the positive control. Transcriptional expression of specific cytokines (IL-2, -4, -5 and -13, TGF-β, TNF-α and IFN-γ) was assessed by RT-PCR. IFN-γ expression was also evaluated by ELISPOT. Secreted levels of IL-2, -5 and IFN-γ were measured by ELISA. All of these assays were performed two or five days, post-stimulation. This study of the *in vitro* diagnosis of penicillin allergy by the measurement of cytokine concentration in the supernatants of sensitized lymphocytes cultures involved the largest number of patients to-date. The Δ values (difference in cytokine concentration in the supernatants before and after stimulation) were compared between cases and controls using different statistical tests (Student's t test and the Mann-Whitney rank test). Of the various tests performed in this study, measurement of secreted cytokines using ELISA was the most sensitive and specific (80% and 100% respectively). *In vitro* stimulation of human lymphocytes sensitized to amoxicillin is a safe and useful test for the diagnosis of penicillin allergy if the ELISA is used to measure cytokine expression. The advantages are that it can be performed by many laboratories since kits to determine cytokines are widely available, and it can be done without the need for particularly specialized equipment.

Keywords: penicillin allergy, sensitized lymphocytes, *in vitro* stimulation, cytokines

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In the light of their effectiveness and low toxicity, penicillins are among the most commonly prescribed antibiotics. However, during treatment with penicillin, the incidence of allergic reactions is estimated at about 1 to 10% in the general population, representing the most frequent cause of drug allergy [1, 2].

The skin is the usual target in immunological reactions to penicillin [3], presenting two types of cutaneous allergic reaction. The immediate reaction or type I hypersensitivity (HS), is IgE-mediated, and includes urticaria, angioedema or anaphylaxis. The delayed reaction or type IV hypersensitivity, is T cell-mediated and includes several manifestations such as maculopapular exanthema, pustular or vesicular-bullous exanthema [4, 5].

Pseudoallergy is a hypersensitivity reaction that is neither IgE- nor T cell-mediated. It is characterized by the same features as type I or occasionally type IV allergy, and is sometimes more frequent than true IgE-mediated reactions [6-8].

The usual diagnostic methods for evaluating allergic reactions to penicillin are skin testing and *in vitro* lymphocyte stimulation. Penicillin skin testing is the most reliable method for evaluating IgE-mediated penicillin allergy provided that the necessary reagents are available. The skin provocation tests (patch, prick and intradermal) are performed using penicillin reagents or a preparation containing a commercialized form of the drug (pills, injectable) [9, 10]. Penicillin reagents include the major determinants (penicilloyl-polylysine/PPL), and the minor determinant mixture/MDM (penicilloate/penilloate). Skin testing with penicillin reagents was widely used by allergists/immunologists until Allergopharma (Reinbek, Germany) and Hollister-Stier (Spokane,Washington) announced, in

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2004, their decision to stop production of PPL and MDM (Allergopen, PrePen, respectively). When performed with both major and minor determinants, the negative predictive value of penicillin skin testing for immediate reactions approaches 100%, whereas the positive predictive value is between 40% and 100% [11, 12].With aminopenicillin- and betalactam-associated maculopapular exanthema, delayed positive reactions to the intra-dermal test (IDT) have been reported [10, 13, 14].

However, the skin test may cause an unpredictable, severe allergic reaction [15] and the possibility of a false-positive reaction [16]. Recently, some European pharmaceutical companies have begun to produce penicillin reagents again, which are still being evaluated by clinical researchers [17].

Some studies have shown that skin testing has no optimal sensitivity and that *in vitro* stimulation might be required as a complementary diagnostic test [7, 18, 19].

Several methods are used for *in vitro* stimulation tests such as the lymphocyte transformation test (LTT), the study of cytokines produced by lymphocytes after drug stimulation, and the basophil activation tests [20-22], which are routinely performed in research laboratories specializing in allergology.

The LTT measures the proliferative response of T cells and peripheral blood mononuclear cells (PBMC) to the drug under *in vitro* conditions [20]. PBMC are obtained from a penicillin-sensitized patient and cultured in the presence of penicillin for six days. The proliferative response of the lymphocytes is measured by the incorporation of ^{3}H-thymidine during DNA synthesis.

However, the LTT has some disadvantages, such as the use of radioactivity (^{3}H-thymidine), which limits the application of the method to only certain research laboratories, it is a fairly lengthy procedure, it is not sufficiently sensitive (60-70%) [23], and it depends upon the conditions employed for the culture medium (serum used, etc.).

Because of the central role of T cells in the pathogenesis of delayed HS, and their requirement for immediate HS, the study of T cell cytokines is helpful for *in vitro* testing. The study of cytokines is centered around their production [24, 25]. In fact, different subsets of T lymphocytes produce distinct cytokine expression profiles. CD4 helper T lymphocytes (Th cells) produce lymphokines such as interleukins (IL) IL-2, -4, -5, -6, -10 and interferon gamma (IFN-γ), whereas the CD8 subpopulation produces granzyme B, perforin, IFN-γ and tumor necrosis factor-α (TNF-α), which induces target cell lysis and has an anti-viral activity [26]. Th cells are functionally heterogeneous, with distinct cytokine patterns. Th1 cells produce IL-2 and IFN-γ, while Th2 cells produce IL-4, IL-5, IL-6 and IL-10 [27].

T cells are crucial for immediate HS because they release IL-4/IL-13, which are switch factors for IgE synthesis by B cells [28]. In drug-induced maculopapular exanthema, immunohistochemical studies have shown that the cell infiltration of the affected skin is composed of predominantly CD4 subpopulation cells and to a lesser degree of CD8 cells. Interestingly, a greater number of CD8 than CD4 cells are activated [29]. Up to 20% of the infiltrating T cells express granzyme B and perforin, causing keratinocyte damage. A high production of IFN-γ causes an upregulation of major histocompatibility complex class II (MHC) on keratinocytes enabling drug presentation to CD4 cells [30]. In addition, drug specific T cells orchestrate the inflammatory skin reaction through the release of various cytokines (IL-5,-6,-8, TNF-α, IFN-γ, eotaxine, etc.) contributing to the generation of tissue and blood eosinophilia, evidence for many types of drug-induced allergic reactions [31].

The study of cytokine expression and secretion can be evaluated by several methods such as reverse transcription polymerase chain reaction (RT-PCR), flow cytometric analysis, and enzyme linked immunosorbent assay (ELISA). RT-PCR is used to study a cytokine's transcriptional levels [32] while flow cytometric analysis are used to study intracellular cytokines [33]. Finally, ELISA, the most clinically relevant method, is used to measure the amount of secreted cytokines in cell culture supernatants [33, 34]. These *in vitro* tests represent important tools for diagnosis; however, they are used more as research methods than for routine procedures. Therefore, new or simply improved *in vitro* tests are necessary.

The aim of our study was to assess a new approach for *in vitro* testing using methods that could be applied in many laboratories and extended to other drugs. Based on the results of previous data [23], we studied the cytokines IL-2, -5 and IFN-γ in the supernatants of sensitized cell cultures using the ELISA method. With regard to cytokine gene expression, RT- PCR was used to study IL-2, -4, -5 and -13, TGF-β, TNF-α and IFN-γ. ELISPOT was also used for evaluating IFN-γ expression.

DONORS AND METHODS

Patients

A total of 30 Lebanese subjects, recruited from our outpatient clinic (18 patients and 12 controls) were selected for this study. Patient and control group characteristics are summarized in *tables 2* and 3. Only 15 patients were included for analysis because three samples were discarded for technical reasons (difficulty in obtaining an adequate volume of blood, low number of PBMN, contamination of the culture medium by microbes). The study was conducted between November 2005 and February 2007. A questionnaire, which included a full history related to the allergic reaction (dose, duration of treatment, chronology of the appearance of the symptoms, and clinical manifestation) was used. The withdrawal of the major determinants for skin testing from the market in 2004-2005, and amoxicillin being the most frequent reported antibiotic to induce allergic reactions [35, 36] led us to include patients with a known history of penicillin allergy and who developed a cutaneous allergic reaction after re-exposure to amoxicillin during the study period. It is worth noting that in Lebanon, antibiotics can be purchased without a doctor's prescription, which explains why patients may inadvertently take penicillin repeated, believing it to be a safe drug. After a detailed history and description of the clinical manifestations, in addition to a thorough examination of the skin, the following cutaneous symptoms/signs are reported as allergic reactions: pruritis with maculopapular exanthema (delayed-type hypersensitivity), urticaria or angioedema (immediate-type 1).

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Patients who developed allergy who had taken other, newly introduced drugs during the two to four weeks prior to the study were excluded. Delayed positive results for the ID test with Allergopen performed between 2002 and 2004 were available for patients 2, 3, 7, 9 and 10 (Batch number: Ch.-B.:30004462 PPL/30004589MDM). Delayed reactions occurring after 24 h or later, were considered positive when there was an infiltrated, erythematous reaction [10]. Positive prick test were available for patients 4 and 13.

The morphological differential diagnosis should exclude other causes such as the classical infections exanthemas like measles, rubella and syphilis.

Only patients with a known history of penicillin allergy and who have a positive re- exposure to amoxicillin were included in the study.

The control group included patients who, in the last twelve months, had been exposed to amoxicillin with no clinical signs of a drug allergic reaction or patients with no previous intake of the drug. The present study was approved by the university's ethics committee (number of the approval: fm 115). Informed consent for all the diagnostic procedures was obtained from all patients and control subjects in accordance with the Declaration of Helsinki.

Sample collection, cell isolation and culture system

Twenty to 30 mL of peripheral blood from patients and controls were obtained by venipuncture, and collected in ethylenediaminetetraacetic acid-coated (EDTA) tubes. In order to obtain good cellular viability and functionality, we adhered to the recommendations and instructions proposed by Pichler and Tilch for blood sampling, medium culture, and storage time, etc. [21].

Sample collection was performed more than three months after the allergic reaction had occurred because during the acute phase, lymphocytes are activated and may still produce high levels of cytokines. Two to four hours after blood sampling, PBMC were isolated by Ficoll-Hypaque density gradient centrifugation. The cells were washed twice in RPMI 1640 and re-suspended in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Gibco). The cells were cultured at 37°C in a humidified, 5% CO_2 atmosphere, in tissue culture flasks.

Cell treatment

For cell stimulation, we used free amoxicillin intended for injection in hospitals, in attempt to achieve conditions mimicking the clinical setting. Pure amoxicillin was not used for cell stimulation because patients included in the study had had two episodes of allergy to the drug, which led to the conclusion that allergy to excipient is unlikely. Amoxicillin was derived from stock (vial of Amoxil® 500 mg for intravenous use, Glaxo Smith Kline, batch number: BN 207025), and then added to the suspension of cells at a final concentration of 1 mg/mL. Each vial of amoxicillin was dissolved in 2.5 mL of sterile water and used immediately. Cells were cultured with media alone or in the presence of amoxicillin for two and five days (two or six hours for some patients), and with/without phytohemagglutinin A 150 µg/mL (PHA; Gibco) for two and/or five days. Cultures stimulated with PHA were considered as positive controls. Tetanus toxoïd (TT) or tuberculin-purified protein derivative, (extract of *Mycobacterium tuberculosis*) (PPD) could not be used as a positive control for memory-cell proliferation because most of the adult Lebanese population have not been correctly vaccinated against tetanus nor vaccinated with bacille Calmette-Guérin (BCG). Cell counts were performed using the trypan blue assay. At least 2×10^6 cells were used for RNA extraction, 10^6 cells were used for each proliferation, cytoxicity and ELISOPT assays.

Proliferation assay

Proliferation studies were conducted using CellTiter 96 Non-Radioactive (Promega). This assay is an MTT-based method, which measures the ability of metabolically active cells to convert tetrazolium salt into a formazan product. The absorbance is recorded at 595 nm. Briefly, cells were counted and seeded at 10^4 densities in a 96-well-round bottomed plate. Cell proliferation was measured on days two and five. One hundred µl were seeded into each well (in triplicate). For background measurements, three wells containing medium alone were included. Fifteen µl of dye solution were added to all wells. Cells were then incubated for four hours at 37°C, 100% humidity, 5%CO_2. Two 100 µL aliquots were then removed from each well and transferred to a 96-well-plate. One hundred µL of stop solution were then added to all wells. Cells were then incubated for minimum of one hour at 37°C, 100% humidity, 5% CO_2. Absorbance was measured at 595nm.

Detection of cytokine secretion in culture supernatants by ELISA

Cell culture supernatants were harvested after six hours, two days and five days, centrifuged at 2 000 g for 15 minutes, filtered through 0.22 micron filters and stored frozen at -80°C. ELISA for human (Hu) IL-2, IL-5, and gamma interferon (IFN-γ) were performed in triplicate wells in accordance with the manufacturer's instructions (Chemicon International, CA, USA). Hu IL-5, IL-2 and IFN-γ titers were measured in supernatants (pg/mL) from untreated and amoxicillin-treated two ml cultures from patients and controls. Prior to ELISA, the supernatants were subjected to centrifugation (16,000 xg, 1 min) to pellet all cell debris. A solid phase, sandwich ELISA was used. Briefly, monoclonal antibodies specific for Hu IL-2, IL-5 or IFN-γ were coated onto the wells of the microtiter strips provided. Samples, including standards of known Hu IL-2, IL-5 or IFN-γ content, supernatants from patients and controls were pipetted into these wells followed by the addition of a second, biotinylated monoclonal antibody. During the first incubation, Hu IL-2, IL-5 or IFN-γ antigens bound simultaneously to the immobilized antibodies on one site, and to the solution-phase, biotinylated antibody on a second site. After removal of excess second antibody, streptavidin-peroxidase was added. This bound to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove the entire unbound enzyme, tetramethylbenzidine substrate solution was added, which was acted upon by the bound enzyme to produce color. The intensity of the colored product was measured using a microplate reader at 450 nm. This intensity was directly proportional to the

concentration of Hu IL-2, IL-5 or IFN-γ present in the supernatant.

RT-PCR

Total RNA was extracted using NucleoSpin® RNA II (Macherey-Nagel). Quantification was performed using either Nanodrop or a GeneQuant spectrophotometer. Total RNA was reverse-transcribed into first strand cDNA at 37°C for 30 min. RT-PCR was performed with the *AB gene* kit in accordance with manufacturer's instructions. Amplification was achieved under the following conditions: 95°C for three minutes as an initial denaturing step; followed by an amplification step for 30 cycles of denaturing at 94°C for 20 s, annealing at 55°C (IL-13, IL-10, TNF-γ), 50°C (IL-2, IL-4, IL-6, IFN-γ) and 58°C (TGF-β) for 30 s and extension at 72°C for 1 min; a final extension step at 72°C for five minutes was followed by a holding temperature of 4°C. PCR products were electrophoresed on 1.5% agarose gel and visualized with ethidium bromide staining. The resulting images were captured using an eight-bit CCD camera. Subsequent analysis was performed by measuring the mean gray levels of each band by ImageJ (NIH imaging software). Correction was performed by subtracting for local background and normalization against β-actin (used as an internal standard). In this study, oligonucleotide primers for nine cytokines were designed to amplify cDNA fragments, summarized in the following *table 1*.

ELISPOT assay

Ninty six-well PVDF membrane ELISPOT plates (Millipore) were coated with purified anti-IFN-γ antibody by incubating the plates overnight at 4°C with 100 µL/well of functional grade purified antibody. The plates were then rinsed twice with 200 µL/well of sterile ELISPOT coating buffer and blocked with 200 µL/well of complete RPMI-1640 to reduce non-specific binding of proteins, and incubated for one hour prior to addition of cells. The wells were seeded with 1 x 10^6 cells. The assay was performed in duplicate wells with and without antigen stimulation for each cell density in all experiments. The seeded plates were placed undisturbed in a 37°C, 5% CO_2 humidified incubator for 24-48 hrs. After incubation, the plates were washed three times with ELISPOT wash buffer (phosphate-buffered saline (PBS) containing 0.05% Tween 20). Biotinylated anti-IFN-γ antibody was added to plates at 100 µL/well and incubated for two h at 37°C. The plates were washed again with ELISPOT wash buffer, the wells being allowed to soak for 1 min for each wash. Avidin horseradish peroxidase reagent was added to plates at 100 µL/well and incubated at room temperature for 45 min. The plates were rinsed three times with ELISPOT wash buffer and then washed twice with 1x PBS. Spots were developed by the addition of 100 µL/well of freshly prepared, AEC substrate solution prepared in accordance with the manufacturer's instructions (Sigma). After spot development, the plates were emptied, washed three times with 200 µL/well distilled water and allowed to dry. Spots were observed under a dissecting microscope. No spots were observed in wells without antigen.

STATISTICAL ANALYSIS

The cytokine concentrations were compared between cases and controls at different post-stimulation times (two or six hours, days two and five) using Student's t test when the variables were normally distributed in both cases and controls; elsewhere we used the non-parametric Mann-Whitney rank test for the comparison. The analysis was performed on Stata v8 and SPSS v13. A p value less than 0.05 was considered to be statistically significant. We calculated the stimulation index (ratio of cytokine concentration before and after stimulation with amoxicillin), and the Δ value (difference in cytokine concentration before and after stimulation).

We then drew the receiver operating characteristic (ROC) curves for the variable Δ, which was significantly higher in cases than in controls. After that, we calculated the area under the curve (AUC), the best discriminative cut-off with the corresponding sensitivity and specificity, and finally the cut-off at which we had 100% sensitivity and corresponding specificity.

RESULTS

Table 2 summarizes the clinical characteristics and biological correlations for each patient. The mean age was 46.9 years (18-68), and the gender ratio F/M was 4 (12/3). Five patients had type I reaction allergic symptoms. The rest had maculopapular exanthema suggesting a type IV reaction. One patient (P11), had mixed symptoms of type I and IV reactions. Analysis of cytokine patterns did not show any significant differences between these groups.

Table 1
Primer information

Cytokine	Sense primer (5'- 3')	Anti-sense primer (5'- 3')	Product size
IL-2	ATGTACAGGATGCAACTCCTGTCTT	GTTAGTGTTGAGATGATGCTTTGAC	450bp
IL-4	GTCTCACCTCCCAACTGCTTC	CTTGTGCCTGTGGAACTGCTG	250bp
IL-5	CTTGGCACTGCTTTCTACT	TATCCACTCGGTGTTCATT	295bp
IL- 6	CCTTCTCCACAAGCGCCTTC	GGCAAGTCTCCTCATTGAATC	352 bp
IL- 10	CTTCGAGATCTCCGAGATGCCTTC	ATTCTTCACCTGCTCCACGGCCTT	312 bp
IL-13	CCACGGTCATTGCTCTCACTTGCC	CCTTGTGCGGGCAGAATCCGCTCA	318bp
TGF-β	GCTGTGGCTACTGGTGCTGACGCC	GAGCCTCAGCAGACGCAGCTCTGC	435bp
TNF-α	CAGAGGGAAGAGTTCCCCAG	CCTTGGTCTGGTAGGAGACG	324bp
IFN-γ	AATGCAGGTCATTCAGATG	TTGGACATTCAAGTCAGTT	273bp

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Table 2
Clinical characteristics of patients, and cytokine values in the supernatants before (B) and after (A) stimulation with amoxicillin

Patient	Age (Yrs)	Gender	Reaction interval (from exposure)	Reaction type	IL- 2 Day2 B/A	IL- 5 Day2 B/A	IFN- γ Day2 B/A	IL- 2 Day5 B/A	IL- 5 Day5 B/A	IFN- γ Day5 B/A
P1	43	F	4-6 H	AP/ AO	60/100 (50/100)■	10/30	50/50 (30/50)■	60/95	20/45	30/52
P2	65	M	24-48 H	MP	90/140 (90/450)■	15/20	13/18 (8/ 160)■	110/120	20/30	30/60
P3	67	F	72 H	MP	45/70 (40/60)■	15/20	130/350 (90/115)■	135/200	25/35	60/120
P4	45	F	6-12H	AO/ UR	35/80 (30/50)■	25/70 (15/20)■	50/125 (30/40)■	40/100	50/250	70/140
P5	24	F	48 H	MP	33/35	10/25	23/33	33/40	20/40	250/280
P6	55	F	24- 48H	MP	8/40	8/20	120/210	8/300	13/25	230/280
P7	58	F	24 H	MP	33/40	15/20	33/500	40/30	25/40	210/600
P8	67	F	48- 72H	MP	100/350• (120/140)□	20/30• (20/35)□	570/600• (380/600)□	-	-	-
P9	52	F	72 H	MP	50/150	10/30	190/650	90/100	50/60	60/110
P10	68	F	48 H	MP	90/150	90/120	370/1000	100/155	90/120	93/250
P11	26	M	72 H	MP/AO	95/200	30/40	46/400	90/110	60/80	300/400
P12	34	F	48 H	MP	40/70	20/40	200/400	90/110	40/70	60/100
P13	58	M	<6 H	AP	110/200	20/30	220/300	120/150*	50/100*	320/400*
P14	24	F	48- 72H	MP	150/200	60/80	700/750	230/250*	70/90*	500/800*
P15	18	F	24 H	MP	50/80	30/45	300/400	70/85	60/70	400/500

P: patient; Yrs: years; F: female; M: male; H: hours; MP: maculopapular.AP: anaphylaxis; AO: angioedema ; UR: urticaria; •: Day 3; *: Day 7; ■: 6 hours; □: 2 hours.
NB: Cytokine levels were high after PHA stimulation, which suggests good cell viability. Results are not shown. Concentrations varied between 50 pg/mL and 2 800 pg/ mL.

Table 3
Clinical characteristics of controls and cytokine values in the supernatants before (B) and after (A) stimulation by amoxicillin, or after stimulation by PHA

Control	Age (Yrs)	Gender	Interval exposure/reaction	IL- 2 Day 2 B/A- [PHA]	IL- 5 Day 2 B/A- [PHA]	IFN- γ Day 2 B/A-[PHA]	IL- 2 Day 5 B/A-[PHA]	IL- 5 Day 5 B/A-[PHA]	IFN- γ Day 5 B/A-[PHA]
C1	17	M	4 m	50/70-[600] (40/45)*	15/20 (10/15)*	80/90-[400] (30/40)*	-/- -[112]	11/0-[112]	-/100-[470]
C2	74	F	2 yrs	- (100/110)*	- (15/20)*	- (50/50)*	-	-	-
C3	24	M	6 m	25/20	2/5	200/210	35/- [900]	10/0-[130]	0/57-[705]
C4	25	F	0	30/23	10/20	100/110	40/25-[700]	30/50-[500]	120/125-[500]
C5	30	F	18 m	25/18-[600]	100/110-[400]	100/110-[700]	30/25	120/125	120/130
C6	53	F	12 m	8/-	10/-	100/-	10/- [500]	15/- [50]	100/-[500]
C7	27	F	21 m	90/100	3/10	65/70	125/130	30/50	100/125
C8	41	M	9 m	90/100	30/32-[250]	80/85-[750]	100/110	35/40	95/100
C9	39	F	16 m	85/90	60/70-[500]	90/92-[800]	92/100	65/70	85/95
C10	56	F	11 m	-/70	-/30	-/60	-	-	-
C11	47	F	0	-/85	-/40	-/80	-	-	-
C12	40	F	3 m	60/80-[1500]	40/50-[400]	400/550-[1700]	-/100	-/80	-/400

C: control; M: male; F: female; m: months; Yrs: years; *: 6 hours. Concentration in pg/mL.

Table 3 summarizes clinical characteristics for each control subject. The mean age was 39.4 years (17-74), and the gender ratio F/M was 3 (9/3). In the patient group, the type I or IV allergies are better correlated with the symptoms and cutaneous signs than with the interval between the exposure to amoxicillin and the occurrence of the reaction. Note that in IgE-mediated and pseudoallergic reactions, the symptoms occurred after an interval, which varied between few minutes to several hours. In T-cell-mediated reactions, the time delay is typically between a few hours to two days. For this reason, the "one hour" interval, as a cut-off to distinguish between immediate and non-immediate reactions, may not be accurate [7, 37].

Proliferation assay

Both cell assessment by MTT assay and RNA quantification were performed for controls and patients. Two million cells/2ml were incubated in the medium. A mild to moderate cell proliferation was observed after incubation with 1mg/ml amoxicilline *(figure 1)*. All cell cultures were also tested for viability and proliferation throughout the period of the experiment *(figure 2)*. Neither amoxicillin treatment nor activation by PHA resulted in any cytotoxicity: amoxicillin was not toxic to cells to up to 3 mg/ml over a six hour or two day incubation period. However, increased proliferation was seen on days two and five.

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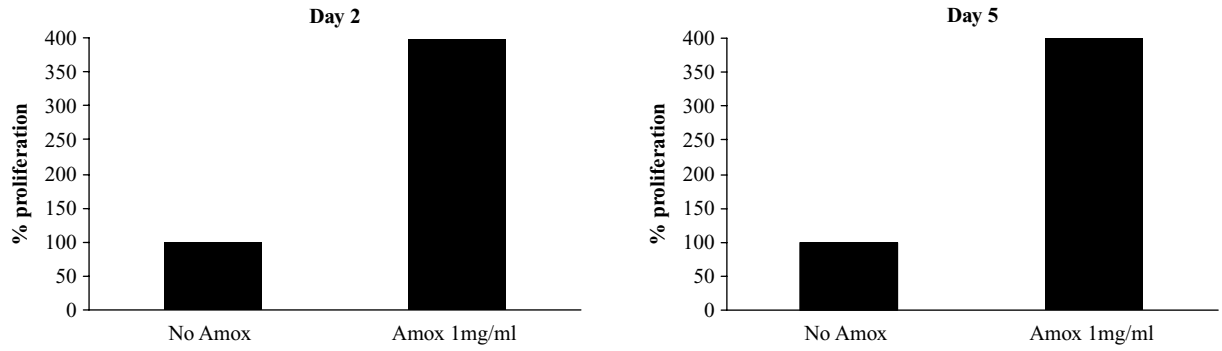

MTT results

P3	Absorbance at 595nm (day 2/day 5)		
Cell culture without amox	**Cell culture with amox**	**Medium alone**	**Medium with amox**
0.119/0.125	0.251/1.096	0.131/0.101	0.197/0.709
0.117/0.109	0.311/0.878	0.102/0.095	0.191/0.771

Cell count and RNA results

P3	Without amox day 2/day 5	With amox day 2/day 5
Cell count (2 mL)	176 x 10^4/182 x 10^4	210 x 10^4/208 x 10^4
RNA (20 µL)	9.2/30.6 ng/µL	14/13 ng/µL

Figure 1

Proliferation assay.
Non-radioactive MTT assay is used. The absorbance is recorded at 595 nm. Cells were counted and seeded at 10^4 density. Cell proliferation was measured on days 2 and 5. Media were included for background measurements. Cell proliferation is observed after incubation with amoxicillin.

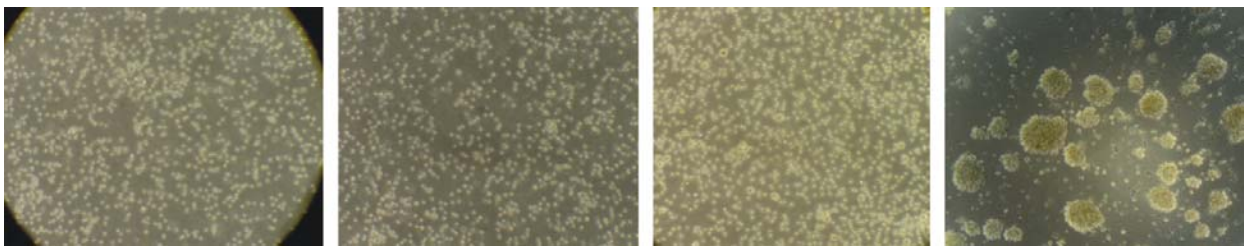

Figure 2

Effect of Amoxicillin and PHA on PBMC proliferation.
Microscopic evaluation clearly reveals an increase in cellular proliferation and activation due to treatment with amoxicillin or PHA. The effect of PHA appears to be maximal (clumping).

Although RT-PCR is valuable as a research tool for studying transcriptional expression of cytokines in cells, it seems not to be reliable enough for clinical assessment of amoxicillin hypersensitivity. In quantifying total RNA from various samples, we were faced with variable yield, sparse amounts in resting cells, and inaccurate densitometric analysis *(figure 3)*. The use of quantitative PCR may have alleviated some of these problems; however, it would have become an expensive, specialized and time-consuming undertaking.

Measurement of cytokine content in the culture supernatants

Measurement of the cytokines released in the culture supernatants concerned IL-2, IL-5 and IFN-γ (*tables 2* and *3*). The time to peak concentration ranged from two hours, six hours, two days or five days. For IL-2 and IFN-γ, the time to peak concentration was observed mainly on day two rather than on day five. For IL-5, the peak time is the same on day 2 and 5. For patients 2, 3, 8, and 9, stimulation with amoxicillin was as strong as stimulation with PHA (positive control). A strong PHA-induced cytokine secretion indicates a good proliferative capacity of the isolated cell population and shows that the cells are viable. For patients 8, 10, 11, 13 and 14, levels of all cytokines in the supernatants, particularly IFN-γ, were high before stimulation, reaching as high as 700 pg/mL for IFN-γ. For the other cytokines, concentrations were as low as 8 pg/mL. For this reason, positive stimulation should not be based on a cut-off value. Other parameters should be considered for positive response such as stimulation index (SI), which is calculated as the ratio between the concentration of cyto-

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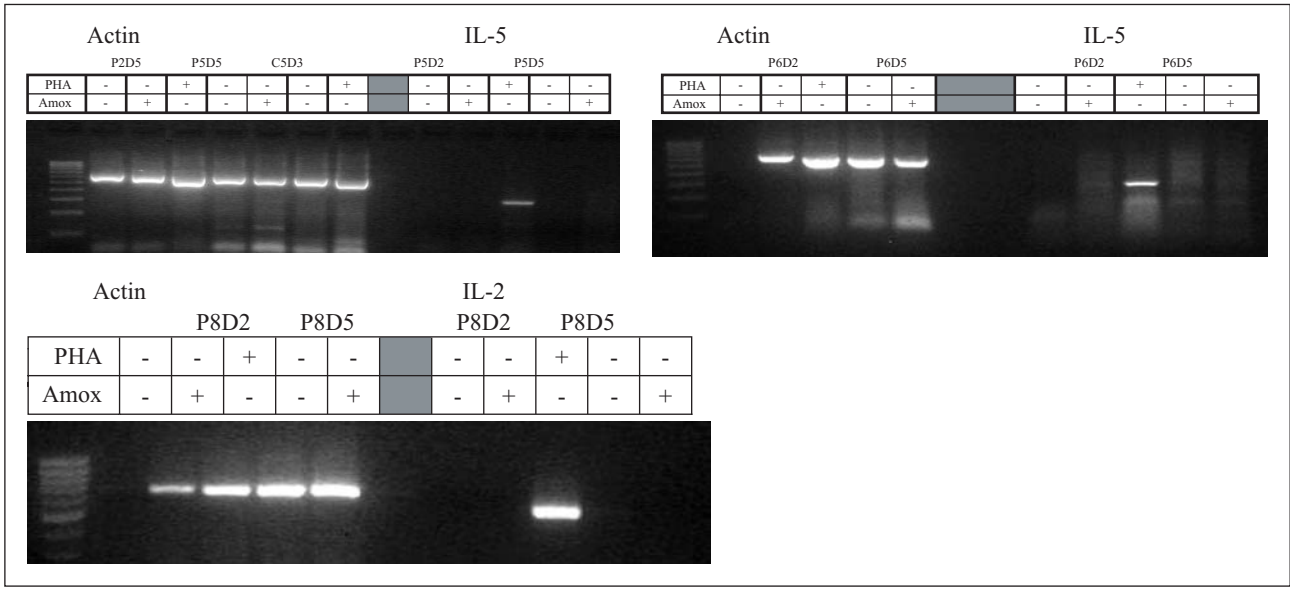

Figure 3

Cytokine mRNA expression by RT-PCR.
Samples showing IL-5 and -2 expression profiles in response to stimulation with amoxicillin and PHA at different time points.
D: day; P: patient; actin is used as an internal standard. The stimulation is evident only with PHA (same results as those with actin).

kines before and after stimulation, or Δ, which is the value of the difference in concentrations variation before and after stimulation.

Statistical results

The sensitivity of amoxicillin- induced cytokine secretion was determined with regard to the patient group. As mentioned in Donors and Methods, a patient was included only if he had a well-documented history of allergic cutaneous reactions to penicillin. Specificity was determined based on results from the control group. The sensitivity and the specificity of the test depends on the value chosen for these parameters. As an example, a positive SI can be 1.5, 2.0 or more; the difference in concentration, Δ, after stimulation can be 30, 40, or 50, more or less. If the ratio 3 is chosen for the SI, only three patients out of 15 will have a positive result for IL-2 and IL-5, and only four patients will have positive results for IFN-γ. The sensitivity will be only 20% or 26%. For the ratio ≥ 1.5, twelve patients will have a positive result for IL-2 and IFN-γ, and eleven patients a positive result for for IL-5. The sensitivity is then 80% or 73.3% respectively.
Furthermore, the Δ value is both sensitive and specific. The calculated sensitivity and specificity depend on the type of cytokine (IL or IFN) and the timing of dosage *(figure 4)*.
It is best to choose a value with an optimal sensitivity and specificity. Based on the ROC curves, the cut-off for Δ values giving the best sensitivity and specificity are the following: IL-5 on day five (cut-off ≥ 10, sensitivity 100%, specificity 62.5%, AUC 0.75), IFN-γ on days two and five (cut-off ≥ 140, sensitivity 78.57%, specificity 90%, AUC 0.84).
IL- 2 on day two (cut-off ≥ 25, sensitivity 86.67%, specificity 100%, AUC 0.925), IL-2 on day five (cut-off ≥ 10, sensitivity 85.71%, specificity 80%, AUC 0.9). For IL-5 on day two, the discrimination is not statistically significant (the AUC is not statistically different from 0.5).

Because a false negative result may be more dangerous for the patient than a false positive, it is preferable to choose a test that is 100% sensitive with the best achievable specificity.
Here are the cut-off points at which we have 100% sensitivity and the corresponding specificity: IL-5 on day five (cut-off ≥ 10, specificity 62.50%), IFN-γ on day five (cut-off ≥ 22, specificity 60.00%), IL-2 on day two (cut-off ≥ 2, specificity 37.50%).

IFN-γ expression using ELISPOT

The ELISPOT used to detect IFN-γ expression in response to stimulation with amoxicillin was weakly positive. Microscopic analysis of the wells showed a strong response to (non-physiological) PHA, however, amoxicillin-treated cells displayed weak signals (three-five spots) *(figure 5)*.

DISCUSSION

The use of amoxicillin as the sole reagent for lymphocyte stimulation is a good choice for several reasons. Firstly, the use of several reagents requires a large volume of blood. Secondly, amoxicillin is the best inducer of allergic reactions and the most widely used antibiotic among the beta-lactams [35, 36, 38]. Lastly, the stimulation of PBMC by amoxicillin may be stronger than stimulation by other reagents such as PnG, PnV, carbenicillin, or flucloxacillin [19]. Heparinized tubes are usually used for blood collection. Some authors used EDTA-stored blood for RT-PCR studies, while heparinized blood was used only for immunofluorescence staining [39]. EDTA coated tubes for blood sampling were used equally by others, with good results [32]. Moreover, it has been shown that EDTA blood samples kept at 4°C maintain an acceptable viability for at least 24 h, which cannot be said for heparinized blood [40, 41]. The recommendations and instructions proposed by Pichler and Tilch for the proliferation test and blood sam-

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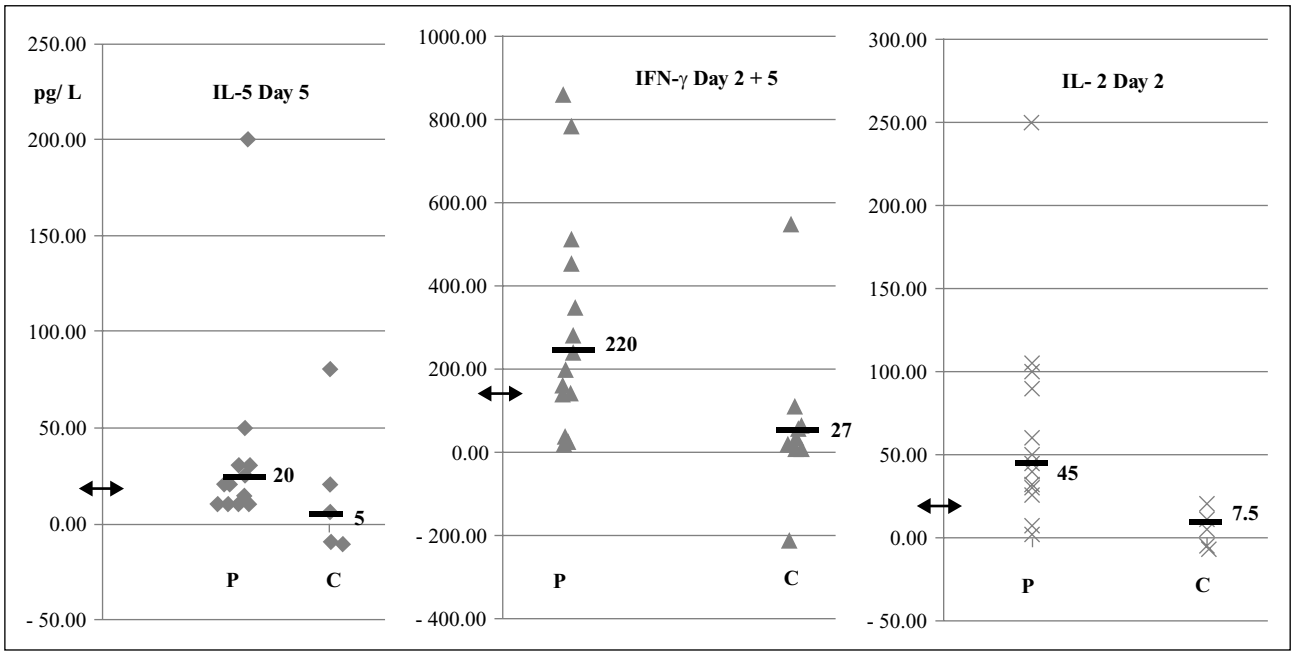

Figure 4

Comparison between patients and controls of the Δ variation in IL-5, IFN-γ and IL-2 concentrations before and after stimulation with amoxicillin. —: median value. ↔: cut-off of the best sensitivity and specificity obtained by ROC curves (↔: IL-2 and 5 = 10, IFN-γ = 140). The difference is statistically significant for IL-2 and IFN-γ (p = 0.000 and 0.005 respectively).

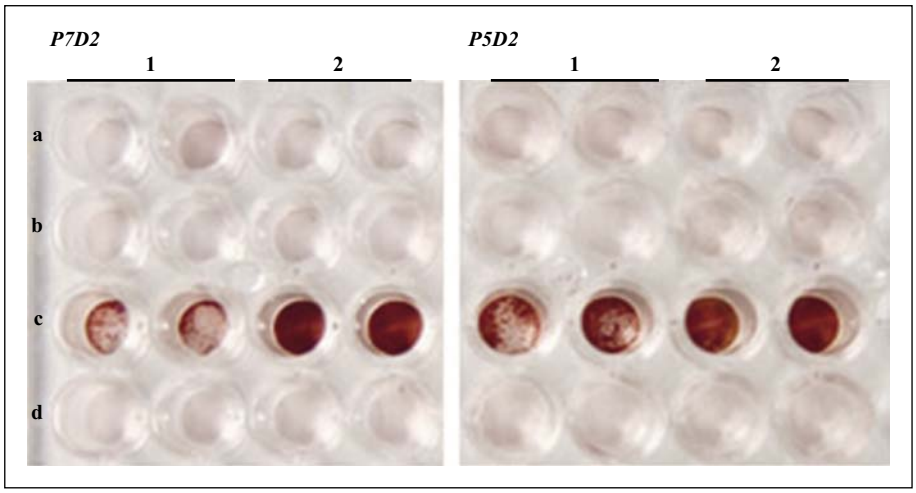

Figure 5

ELISPOT results for two patients (P 7 and 5) on Day two (D2).
a: control; b: with amoxicillin (1 mg/mL); c: with PHA; d: medium.
Two conditions are employed. 1:1 million cells/mL; 2 million cells/mL. Weakly positive results obtained from cells treated with amoxicillin. Strongly positive results only with PHA.

pling in EDTA tubes may be used [21]. For the first two patients and one control subject, heparin-containing tubes were used for blood collection. However, for the reasons cited above, EDTA tubes were subsequently used for blood sampling for all the patients and the control group and the blood sampling was repeated for the patients and control for whom heparin-containing tubes had been used. Cell counts were similar in both heparin and EDTA groups.

In order to achieve cell stimulation under conditions similar to the medical reality, we used an commonly available drug, *i.e.* amoxicillin intended for injection in hospitals. The concentration of 1mg/ml of amoxicillin was chosen because it has been proven to be optimal for the stimulation of PBMC in LTT in many studies [19, 32, 42, 43]. However, stimulation by 2 and 3 mg/mL is interesting to do in order to see the "dose-response" effect. In a number of studies, authors did not use pure substances for stimulation. Drugs were dissolved in PBS and diluted to the intended concentration [19]. The use of pure substance is strongly recommended for drug stimulation; however, the injectable form can be used as an alternative [21].

In our study, the use of pure amoxicillin was not mandatory because all patients had had two episodes of allergic reaction to the drug, which led to the conclusion that allergy to excipient was unlikely. Regarding the method we used, it is worthwhile mentioning that the amoxicillin used was free (unbound to human serum albumin [HAS]). In primary cultures containing polyclonal PBMC, free amoxicillin

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can activate $CD4^+$ and $CD8^+$ cells, which are both involved in drug allergy [34]. Similar results show that the use of free penicillin G can stimulate specific T-cell clones of the CD4 or CD8 type, whereas BPO-HAS (benzylpenicilloyl conjugated to HAS) stimulates proliferation of $CD4^+$ T clones only [43].

Free amoxicillin induces Th1- and Th2-like responses, as demonstrated by enhanced expression of IFN-γ and IL-4 mRNA. Conjugated amoxicillin is associated only with Th1 responses [32].

For many years, human serum albumin (HAS) has been used as a carrier for *in vitro* testing, but with limited results for diagnosis. Efforts to characterize hapten carrier conjugates have been unable to provide information concerning the true situation [43, 44].

The levels of cytokines released in the supernatants of cell culture after penicillin stimulation as assessed by ELISA, are not commonly reported and if they are, the sample size is small.

In the study by Sachs *et al*. [34], only 3/10 patients were studied for the IL-5, -10 and IFN-γ. In the study by Cederbrant *et al*. [45], involving 39 patients allergic to bacampicillin, the cytokine titres for IFN-γ, and IL-4 and -5 were measured in the supernatants of only six patients and 3/10 controls. Among these six patients, only two had allergic contact dermatitis. In the study by Brugnolo *et al*., cytokine production (IFN-γ and IL-4) was assessed in the supernatants of stimulated penG-specific T cell clones (TCC) derived from two subjects among 29 with a history of allergy to beta-lactams [42]. In Yawalkar *et al*.'s study, IL-5, -4, IFN-γ and TNF-α were measured for only two patients [46].

Similar to the studies by Sachs and of Cederbrant [34, 45], our study used polyclonal PBMC for drug stimulation *in vitro*, whereas in other studies, monoclonal TCC were investigated [42, 46].

In patients 1, 5 and 15 cytokine secretion was weak or not sufficiently high following amoxicillin stimulation. This can be explained by pseudoallergic reactions or by the fact that a very low proportion of drug-specific T- cells are activated as a result of drug allergy. In the case of cutaneous hypersensitivity reactions to sulfamethoxazole, patients have sulfonamide-specific, peripheral T-lymphocytes at a frequency of 1: 172 000 [47]. In contrast, lymphocytes of patients 2, 3, 8 and 9 were equally stimulated *in vitro* by amoxicillin or PHA. It was shown that the frequency of drug-reactive T-cells can be higher than the frequency of T-cells able to recognize a tetanus toxoid in the same subjects and that 1:250 to 1:10 000 of T-cells in the peripheral blood of drug-allergic patients are reactive to the relevant drug [48]. Moreover, the use of bulk PHA stimulation assays may have limitations related to the existing cellular makeup of the PBMC population [49, 50], or to a significant alteration in the proportion of T cells producing type 1 cytokines [51].

In our series, analysis of cytokine patterns did not show any significant differences between patients with type I or type IV reactions. This does not correlate with previous data that have shown that delayed HS reactions are Th1 type immune responses [32, 39] or a Th2-skewed profile in any type of allergy to beta- lactams [42]. A Th2-biased cytokine profile, with preferential expression of IL-5 in type IV b allergy, has also been observed [52].

However, it has been reported that PenG-stimulated PBMC show a heterogeneous cytokine pattern, as in our results, with clones secreting large amounts of IFN-γ [53].

The study of cytokines using the RT-PCR method has been applied by some authors to stimulated PBMC with the purpose of studying allergy to penicillin [32, 34, 39]. In the study by Gaspard *et al*. [32] involving 19 patients, both IL-4 and IFN-γ were tested by RT-PCR. The disadvantages of using RT-PCR for the study of penicillin allergy include the use of free and conjugated forms for different reagents, the synthesis of beta-lactam-serum conjugates and controlling the efficiency of conjugation, and the requirement of a large volume of blood, with the result that RT-PCR becomes a cumbersome method that requires a long time to carry out.

The ELISPOT method, despite being used by other researchers, does not yield reliably quantitative results unless a dedicated detector is employed.

This *in vitro* test is seldom used for the diagnosis of penicillin allergy. A study carried out by a French group was encouraging because 6/7 patients allergic to amoxicillin had a positive ELISPOT IFN-γ test (> 50 spots/1.10^6 cells) [54].

With Elispot, the frequency of T-memory cells present in PBMC is calculated by detecting the number of spots in the wells. The value used corresponds to the number of spots/1.10^6 cells [55-57].

When freshly isolated T-cells from PBMC are challenged with amoxicillin and subjected to IFN-γ ELISPOT assays (of 48 hours duration), only the memory cells that differentiated *in vivo* should produce the cytokine. Moreover, because the duration of such assays (24/48 hours) is too brief for *in vitro* proliferation and/or differentiation to occur, the number of spots detected should reflect both the frequency of memory cells present in the cell isolate and the commitment to produce IFN-γ imprinted on them *in vivo*.

To date, our study contains the greatest number of patients and control subjects compared to other published studies that have used the ELISA method for the diagnosis of penicillin allergy. The biologically effective cytokines are those released from cells. The use of ELISA for the quantification of specific cytokine release by stimulated cells is a good indicator for hypersensitivity. In addition to the fact that ELISA kits are readily available commercially, it can be used in many laboratories with basic cell culture facilities without the need for sophisticated and complicated equipment. The use of PCR and ELISPOT requires only transcriptional or the stored cytokine content of cells and may require special equipment.

Based on the results and supported by extensive statistical analysis of our data, we conclude that the quantification of IL-2 on day two and IFN-γ or IL-5 on day five, represents a specific and sensitive method for the assessment of penicillin allergy. The Δ (the variation before and after stimulation by amoxicillin) is a useful value for this assessment. It is essential to undertake larger studies that include other cytokines and immunological markers such as eotaxin, granzyme B, perforin, etc., to further refine cut-off sensitivities and specificities for this standard protocol. Finally, pseudoallergy, which is as frequent as or even more frequent than type I hypersensitivity, cannot be diagnosed by these methods because its mechanism is quite different.

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