Effects of the glucocorticoid betamethasone on the interaction of Candida albicans with human epithelial cells

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The glucocorticoid betamethasone (BM) is frequently employed in clinical practice because of its anti-inflammatory and immunosuppressive properties. In this study, we investigated the effect of BM (1 and 2 mM) on the ability of Candida albicans to adhere to, invade and damage oral, intestinal or vaginal epithelial cells, as well as to elicit cytokine and chemokine release. BM at 2 mM concentration stimulated adherence of C. albicans to vaginal cells and facilitated the invasion of intestinal and vaginal epithelia without influencing the growth rate of invading C. albicans hyphae at any type of epithelia and BM concentrations tested. In addition, BM at 2 mM concentration also augmented C. albicans-initiated cell damage of oral and intestinal cells. Furthermore, BM exposure decreased IL-6 cytokine and IL-8 chemokine release from oral and vaginal epithelial cells and also IL-6 release from intestinal epithelium after infection with C. albicans. These observations suggest that high-dose applications of BM may predispose patients to various epithelial C. albicans infections.

INTRODUCTION

The opportunistic human pathogenic fungus Candida albicans is part of the normal microbiota on mucosal surfaces (oral cavity, gastrointestinal tract and genitals) in humans (Odds, 1987; Naglik et al., 2011). On the other hand, C. albicans is also one of the most common causes of fungal infections including oral or vulvovaginal candidiasis (Calderone, 2001, 2002; Mayer et al., 2013). In addition, invasive candidiasis leads to bloodstream and deep infections, which are associated with high mortality (Calderone, 2002; Mavor et al., 2005). A number of risk factors predispose susceptible individuals to candidemia, for example, the use of broad-spectrum antibiotics or glucocorticoids, various immunosuppressive conditions, physiological conditions (e.g. very young or very old age) and chronic illnesses (e.g. diabetes and neutropenia as well as medical interventions like the use of intravenous catheters and surgery of the gastrointestinal tract) (Webb et al., 1998; Akpan & Morgan, 2002; Mayer et al., 2013; Koh, 2013).

An armoury of efficient virulence determinants enables C. albicans to occupy various anatomical niches in the human body (Sudbery et al., 2004; Sudbery, 2011; Polke et al., 2015). For example, the yeast-to-hypha transition, the expression of adhesins and invasins, the release of hydrolytic enzymes and the secretion of the Candidalysin toxin are essential factors in various stages of the infection of human epithelia by C. albicans including adhesion, invasion and damage (Sudbery et al., 2004; Naglik et al., 2011; Sudbery, 2011; Mayer et al., 2013; Moyes et al., 2016).

Glucocorticoids, e.g. betamethasone (BM), are frequently used therapeutic agents because of their anti-inflammatory properties.
METHODS

Fungal strain and culture media. *C. albicans* SC5314 wild-type reference strain was used in all experiments and the strain was grown as described before (Jakab et al., 2015). Briefly, the strain was maintained on YPD medium (1% yeast extract, 2% peptone, 2% dextrose and ±2% agar, pH 5.6). In standard assays, yeast pre-cultures were grown in 5 ml YPD broth at 30 °C, 3 Hz, for 18 h. Pre-cultures were diluted to an OD measurement of 0.1 (A600 nm) with fresh YPD broth ±1 or 2 mM BM (sodium phosphate; Sigma) and the cultures were further grown for 6 h at 30 °C and at 3 Hz shaking frequency. *C. albicans* cells were collected by centrifugation (5 min, 4000 g, 4 °C), washed three times with PBS and adjusted to 2 × 10^5 cells ml⁻¹ cell concentration in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) or Roswell Park Memorial Institute (RPMI; Gibco) medium prior to epithelial infections.

Epithelial cell lines and infection. Epithelial cell lines were cultured and infected with *C. albicans* (for measuring adhesion, invasion, filament length, cell damage and cytokine release) as described in previous publications (Giard et al., 1973; Dalle et al., 2010; Wächter et al., 2011 a, b; Wilson et al., 2013).

The epithelial cell line TR146, derived from a squamous carcinoma of buccal mucosa, was obtained from Cancer Research Technology, London. TR146 cells were grown (passes 4–20) in DMEM with 10% FBS (PAA), supplemented with 1 mM pyruvic acid and 2 mM L-glutamine. The C2BBe1 (clone of Caco-2) colon carcinoma cell line was obtained from the American Type Culture Collection. These cells were routinely cultured (passages 4–25) in DMEM supplemented with 10% FBS and 0.01 mg ml⁻¹ holotransferrin (Merck). The A-431 vaginal epithelial cell line (ACC 91; Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany) was grown (passes 4–20) in RPMI medium with 10% FBS, 1 mM pyruvic acid and 2 mM L-glutamine. None of the culture media contained antibiotics or antifungotics.

All cell types were maintained in a humidified incubator at 37 °C in 5% CO₂ atmosphere. For adhesion, invasion and filament length assays, 1 × 10^5 epithelial cells were seeded on glass coverslips, placed in 24-well culture plates and incubated 2–3 days prior to infection. For damage and cytokine release assays, 2 × 10^4 epithelial cells were seeded in each well of a 96-well plate and incubated for 1 (TR146 and A-431 cells) or 3 days (C2BBe1 cells) prior to infection. For infection, concentration-adjusted *C. albicans* cells (either BM pre-treated or not pre-treated) were co-incubated with epithelial cells for 1 h (adhesion assay), 3 h (filament length, invasion assay) and 24 h (damage and cytokine assay) in serum-free DMEM or RPMI medium ±1 or 2 mM BM at 37 °C and 5% CO₂ to a final concentration of 1 × 10^4 ml⁻¹.

Adherence and invasion of *C. albicans* cells to epithelial cells. In adhesion assays, epithelial and *C. albicans* cells were co-incubated for 1 h, non-adherent yeast cells were removed by rinsing with PBS and the epithelial cells were fixed with 4% paraformaldehyde. Adherent *C. albicans* cells were stained with calcofluor white (CFW; Sigma) and stained fungal cells were visualized with a fluorescence microscope (Leica DM5500B, Leica DFC360 FX). The number of adherent cells was determined by counting at least 50 high power fields of 200 × 200 µm size. Adhesion (%) was calculated using the following formula: [(average cell count in the field × area of the well in µm²)/(area of the field in µm² × inoculated fungal cells in each well)] × 100.

To characterize invasion and determine filament length, epithelial and *C. albicans* cells were co-incubated for 3 h, and cells were washed with PBS and fixed with 4% paraformaldehyde. Non-invading *C. albicans* cells were stained with the green-fluorescent Alexa Fluor 488 conjugate of succinylated concanavalin A (ConA; Invitrogen) for 45 min. The subsequent permeabilization with 0.5% Triton X-100 and staining with CFW, as described above in the adhesion assay, allowed for differentiation of invading (only stained by CFW) and non-invading (stained by both CFW and ConA) hyphae. The number of invading cells and filament length were determined using fluorescence microscopy and at least 50 invading fungal cells were counted on each coverslip. Invasion (%) was calculated using the following formula: [(number of invading hyphae stained by CFW but not by ConA)/(total number of hyphae stained by CFW)] × 100.

Cell damage and interleukin release of epithelial cells. After 24 h co-incubation of epithelial cells with *C. albicans* cells, epithelial cell damage was estimated by measuring the release of lactate dehydrogenase (LDH) using the LDH cytotoxicity detection kit (Roche Applied Science). Culture supernatants were incubated with cytotoxicity detection mix for 25 min and the LDH release was determined spectrophotometrically at A=490 nm. Values obtained from resting cells were regarded as ‘low cytotoxicity controls’ and subtracted from the obtained values. As full lysis controls, non-infected epithelial cell layers were treated with 0.1% Triton X-100 for 5 min, and the measured LDH values were attributed to 100% cell damage and were regarded as ‘high cytotoxicity controls’ in relative cytotoxicity calculations. The same supernatants that were prepared for the cytotoxicity assays were used to determine IL-6 and IL-8 release by ELISAs (eBioscience).

Statistical analyses. All adhesion and invasion samples were assessed in duplicate, while the experiments of damage assay and IL-6 and IL-8 measurements were performed in triplicates (technical replicates). All experiments were performed in at least three independent sets (biological replicates). Only mean±SD values of biological replicates were compared in statistical analyses and are presented. Statistical significance was calculated using two-way ANOVA and the Tukey’s test for multiple comparisons (GraphPad Prism 7). A P value <0.05 was considered to be statistically significant.
RESULTS

Detailed summary statistics (mean±SD) of all measured phenotypes and all detailed statistical tests can be found in Table S1 (available in the online Supplementary Material).

Selection of BM concentrations

Although BM did not influence the growth of C. albicans in YPD broth at up to 4 mM concentrations (Jakab et al., 2015), preliminary cytotoxicity assays indicated that all tested epithelial cell lines were sensitive to 4 mM BM exposures (cell damage was >45 % for oral, >20 % for intestinal and >20 % for vaginal epithelial cell lines, after 24 h incubation). Therefore, we focused our investigations on treatments with lower (1 and 2 mM) BM concentrations. After 24 h, cell damage was negligible for both concentrations (<4 %) with the exception of 2 mM BM exposed TR146 cells, where higher, but statistically not significant, cell damage was recorded (15.5±12.6 %) (Fig. 1).

Effects of BM pre-treatments and treatments on C. albicans adhesion and invasion

When monitoring C. albicans cell adhesion, approximately 4–7 % of the initially inoculated C. albicans cells adhered to oral and intestinal cells after 1 h co-incubation, whereas about 10–16 % attached to vaginal epithelial cells. Importantly, C. albicans cells bound significantly more to the vaginal A-431 cells in the presence of BM at all pre-treatment (0, 1 and 2 mM BM) conditions (Figs 2 and 3 – Adhesion). This was not the case for the intestinal C2BBe1 or the oral TR146 cell lines, where no effect was observed due to pre-treatment or treatment with BM.

Hyphal length on any of the tested epithelial cells was not influenced after 3 h co-incubation by either BM pre-treatments or treatments of C. albicans cells. The filament lengths varied within the narrow range of 45.9±9.0 µm (non-pre-treated and 2 mM BM treated C. albicans cells on intestinal cells) and 61.1±11 µm (2 mM BM pre-treated and 2 mM BM treated C. albicans cells on oral cells) (Figs 2 and 3 – Hyphal length).

Considering epithelial cell invasion, typically 46–68 % of C. albicans hyphae invaded into oral and vaginal cells after 3 h co-incubation, while the ratio of invading hyphae was 12–23 % lower for the C2BBe1 intestinal epithelial cell line (Fig. 2 – Invasion). Both 1 and 2 mM BM treatments had a significant stimulatory effect on the invasion of C. albicans hyphae into intestinal and vaginal cell lines and BM pre-treatments had an effect on all tested cell lines, more accentuated for intestinal and vaginal cell lines (Fig. 3 – Invasion).

Combined effects of C. albicans infections and BM treatments on epithelial cell damage and interleukin release

Invading C. albicans cells caused considerable epithelial cell damage, ranging from approximately 20 % for untreated
Fig. 2. Effects of BM treatments on the interaction between *C. albicans* SC5314 (non pre-treated) and oral (TR146), intestinal (C2BBe1) and vaginal (A-431) epithelial cell lines. Bars indicate significant differences calculated by the Tukey’s test for multiple comparisons (*P*<0.05; **P*<0.001; ***P*<0.0001). Details on summary statistics are reported in Table S1.
Fig. 3. Effects of BM pre-treatments and treatments on all measured phenotypes describing the interaction between C. albicans SC5314 and oral (TR146), intestinal (C2BBe1) and vaginal (A-431) epithelial cells. Fungal cultures were pre-cultured in the absence or presence of 1 or 2 mM BM (BM pre-treatment). Infection was performed in the absence or presence of 1 or 2 mM BM (BM treatment). Two-way ANOVA outcomes (with $P$ values) are reported where significant (effects of pre-treatment and treatment). Details on summary statistics are reported in Table S1.
Fig. 3. (cont.)

**Damage**

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**IL-6**

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**IL-8**

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BM pre-treatment of *C. albicans* cultures
infected oral TR146 cells to 36–37 % recorded for intestinal C2BBe1 and vaginal A-431 cells (Fig. 2 – Damage). Damage increased after treatment of oral and intestinal epithelia with 2 mM BM (Fig. 3 – Damage). BM pre-treatments of C. albicans cultures did not have a significant overall effect on epithelial cell damage, although a trend was observed indicating that fungal pre-treatment could induce higher damage, especially on TR146 oral epithelial cells.

Exposure of epithelial cells to BM during infection with C. albicans typically caused decreased production of IL-6 and IL-8 stimulated by the pathogen, except for IL-8 release from intestinal cells (Fig. 2 – IL-6 and IL-8) and similar trends were observed for uninfected epithelial cells treated with BM (Fig. 1 – IL-6 and IL-8). To be noted, IL-6 levels released by intestinal C2BBe1 and vaginal A-431 cells were very low and at the limit of detection. Therefore, statistical analysis performed on these samples should be considered cautiously. Furthermore, it is noteworthy that BM pre-treatments of C. albicans cultures had a significant inhibitory effect on the release of IL-6 from oral cells and of IL-8 from vaginal cells. In general, damage results were mirrored by the levels of IL-6 and IL-8 release (Fig. 3 – Damage, IL-6 and IL-8).

Interestingly, the C. albicans-elicited increases in cytokine production were less altered in BM-exposed intestinal epithelial cells (Fig. 3 – IL-6 and IL-8), which may be attributed to the overall low IL-6 and IL-8 release by this cell line. Nevertheless, the suppressive effect of BM treatments on IL-6 levels was also documented in intestinal cells under most conditions.

**DISCUSSION**

In this study, the physiological effects of the glucocorticoid BM employed at high (1–2 mM), pharmacologically relevant concentrations were tested in oral, intestinal and vaginal epithelial cell cultures infected by C. albicans. Relying on the data gained in this set of experiments, we can aim at answering the following important questions: (i) Are high-concentration BM treatments cytotoxic to various epithelia? (ii) Does BM increase the virulence of C. albicans cells present at the sites of infections? (iii) Are these BM-elicited effects on fungal physiology and virulence long-lasting or temporary?

**The cytotoxicity of high-concentration BM on epithelial cells may narrow the therapeutic window for this glucocorticoid**

The BM concentrations used in this study (1–2 mM) were comparable to those used topically in various forms (drops, ointments and lotions) in a wide spectrum of dermatological, gynaecological and ophthalmological applications (Gyetvai et al., 2007; Jakab et al., 2015). Importantly, BM employed at a higher (4 mM) concentration was toxic for all epithelial cell layers tested, especially for the buccal carcinoma epithelial cell line TR146 where extensive cell damage >45 % was measured. These observations suggest that limits of glucocorticoid concentrations should be applied in such drug formulae.

**BM treatments increase the virulence of C. albicans infecting epithelial cell cultures**

Glucocorticoids with anti-inflammatory properties enhance the adherence and colonization rate of C. albicans to the oral mucosa and increase the frequency of C. albicans translocation from the gastrointestinal tract to the bloodstream (Myerowitz, 1981; Bykov, 1989; Maraki et al., 1999). In addition, these compounds, e.g. methylprednisolone and BM, directly stimulate hypha formation and phospholipase secretion by this human pathogen, interfere positively or negatively with the action of antifungal drugs and make yeast cells more vulnerable to oxidants like menadione (Gyetvai et al., 2007; Jakab et al., 2015). Since the mechanisms of these physiological changes on the fungus are still largely unknown, this study aimed at dissecting the role that BM pre-treatment (of C. albicans cultures) and treatment (during infection) might play in influencing the adhesion and invasion potential of C. albicans to and into various (oral, intestinal and vaginal) epithelial cell types, as well as the consequent cell damage and cytokine release (Figs 2 and 3).

C. albicans cells adhere to epithelial cell layers, undergo a yeast-to-hypha morphological transition and invade host cells, eventually causing cell damage (Naglik et al., 2011; Moyes et al., 2015, 2016; Polke et al., 2015). BM added at 1 or 2 mM concentrations typically stimulated the adhesion of C. albicans cells to the tested epithelial cells, although this was only statistically significant on vaginal epithelial cells (Figs 2 and 3 – Adhesion). BM also promoted the invasion of the fungus into these cells. This effect was observed for all cell types when considering the effect of pre-treatments and only for intestinal and vaginal cells, when considering the effect of treatment (Figs 2 and 3 – Invasion). Importantly, the observed (trends for) increased invasion rates were not mirrored by an increase in hyphal length due to BM exposures. Therefore, BM is directly responsible for increased invasiveness of the fungus and not indirectly through unspecific effects on hyphal length.

In our experiments, the invasion of TR146 oral and A-431 vaginal epithelial cells by C. albicans was more efficient and occurred more frequently and faster than invasion into C2BBe1 intestinal cells (44–68 vs 12–23 %; Table S1) after 3 h incubation. The explanation for this difference is probably attributable to the fact that oral epithelial cells can be invaded by either active penetration or induced endocytosis whereas the invasion of intestinal epithelium occurs only via active penetration (Dalle et al., 2010).

As shown in Figs 2 and 3 (Damage), 2 mM concentrations of BM especially augmented the C. albicans-initiated cell damage observed for oral (TR146) and intestinal (C2BBe1) epithelial cell lines, but no such harmful effect was observed for A-431 vaginal epithelial cell cultures. The invasion of
various types of epithelia activates epithelial cell responses, which may include the expression and release of both cytokines (G-CSF, GM-CSF, IL-1α, IL-1β and IL-6) and chemokines (IL-8, RANTES and CCL20). These proteins recruit immune cells, e.g. macrophages, monocytes, dendritic cells, neutrophils and T-lymphocytes, to the site of infection (Ashman & Papadimitriou, 1995; Steele & Fidel, 2002; Villar et al., 2005; Naglik et al., 2011; Moyes et al., 2015; Polke et al., 2015; Höfs et al., 2016).

For example, C. albicans invasion of oral epithelial cells induces a Th1-type cytokine response with high levels of IFN-γ and TNF-α and also stimulates the production of the interleukins IL-1α, IL-1β, IL-6 and IL-8 (Schaller et al., 2004; Höfs et al., 2016). Moreover, IL-23, IL-1β, IL-6 and TGF-β release induces Th17 response with release of IL-17A, IL-17F, IL-22 and IL-26, which also governs the response to oral candidiasis (Mostefaoui et al., 2004; Villar et al., 2005; Jayatilake et al., 2007; Naglik et al., 2011; Moyes & Naglik, 2011; Höfs et al., 2016). These literature data are in good accordance with our observations with IL-6 and IL-8 production by TR146 oral epithelial cells shown in Figs 2 and 3.

Considering other epithelia, C. albicans enhanced the production of the chemokine IL-8, but not the cytokines IL-6, MCP-1, SCF and TNF-α, in Caco-2 and H4 intestinal epithelial cells (Seagusa et al., 2007; Murzyn et al., 2010; Falgier et al., 2011). We found significant increases in both IL-6 and IL-8 levels in C. albicans-exposed C2BBe1 intestinal cells (Fig. 1 vs Fig. 2), but the IL-6 production of the cell line was particularly low (Table S1). As shown in Table S1, both IL-6 and IL-8 production by TR146 oral and C2BBe1 intestinal epithelial cells differed by about two orders of magnitude irrespective of C. albicans infections or the actual BM pre-treatments/treatments. Nevertheless, the ratio of C. albicans invading intestinal cells was 12–23 % of total hyphal cells after 3 h incubation.

Local vaginal cell-mediated immunity of healthy women exposed to vulvovaginal candidiasis shows a Th1-type cytokine response (IL-2, IL-12 and IFN-γ) with significantly increased levels of TNF-α and IL-1α, whereas interleukins like IL-1α, IL-6, IL-8, IL-10 and IL-12 as well as IFN-γ and the monocyte chemotactic protein MCP-1 are produced in low concentrations (Steele & Fidel, 2002; Schaller et al., 2004; Höfs et al., 2016). In our studies, IL-6 levels measured in C. albicans-infected but not BM-treated A-431 vaginal epithelial cultures were very low (1.53±0.08 pg ml⁻¹) and the IL-8 levels (118.7±26.2 pg ml⁻¹) were also considerably one order of magnitude lower than those released by TR146 oral epithelial cells (1197±370 pg ml⁻¹) but were higher than those found in C. albicans-exposed C2BBe1 intestinal epithelial cell cultures (43.2±3.0 pg ml⁻¹; Table S1).

Typically, C. albicans infections resulted in an increase in interleukin production, whereas BM treatments (and in some cases also BM pre-treatments) resulted in a decrease in interleukin production (Fig. 3). Although the analysis of epithelial cell signalling responses to BM exposures was beyond the scope of this study, dual-specificity phosphatase 1, a key regulator of innate immunity (Abraham & Clark, 2006), may mediate the anti-inflammatory effects of BM in epithelial cells as well. More recently, dual-specificity phosphatase 1 has been shown to regulate negatively pro-allergic responses in airway epithelial cells (Golebski et al., 2015).

Importantly, our observations together with (i) previous data about the suppressive effects of BM on innate epithelial antimicrobial defence including the inhibition of human β-defensin 2 production (Meyer et al., 2004) and (ii) the positive physiological effects of glucocorticoids on C. albicans cells, e.g. hypha formation and the production of virulence factors including extracellular aspartic protease and phospholipase activities, which is stimulated by methylprednisolone and BM (Gyetvai et al., 2007; Jakab et al., 2015), warn us that the topical application of these anti-inflammatory steroids at high, ~1–2 mM concentrations is likely to increase the susceptibility of the patients to various epithelial Candida infections in the oral cavity, the gut and in the vagina.

**Pre-treatment of C. albicans cells with BM affects virulence to a lesser extent**

Interestingly, BM pre-treatments of the fungal cells had only small effects on epithelial cell damage and cytokine release (Fig. 3 – Damage, IL-6 and IL-8), almost no effect on adhesion and some minor effects on invasion without significantly affecting hyphal length (detailed multiple comparisons can be found in Table S1). In good accordance with these observations, Gyetvai et al. (2007) did not find any significant differences in colony-forming C. albicans cell counts in peritoneal washings of mice, which were intraepithecally infected with either 4 mM methylprednisolone pre-treated or not pre-treated C. albicans cells. These data clearly indicate that the versatile physiological effects of glucocorticoids on C. albicans are only temporary and fungal cells recover quickly after exposure to glucocorticoids is terminated.

Considering that C. albicans cells can adopt the GUT (gastrointestinal induced transition) morphotype in the intestine (Pande et al., 2013), further studies are required to estimate the effects of glucocorticoids, either pre-treatments or treatments, on the virulence of intestine-inhabiting C. albicans cells (Farkas et al., 2006; Kudo et al., 2010).

**Biomedical significance and perspectives**

The medical application of glucocorticoids has been recognized as a risk factor for the onset of candidiasis for a long time and these compounds have also been associated with candidemia (Lionakis & Kontoyiannis, 2003; Perlooth et al., 2007; Yapar, 2014). Glucocorticoids have been coupled to candidiasis diagnosed in patients with acute renal failure, cancer, transplantation and systemic lupus erythematosus (Pryor et al., 1996; Patterson, 1999; Hovi et al., 2000; Bodey, 2002; Sung et al., 2001; Rovin et al., 2005). Botas et al.

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(1995) demonstrated that hydrocortisone treatments increased the risk of disseminated *Candida* infections in preterm babies. In addition, organ transplant recipients (e.g. liver, bone marrow, lung and heart) were also more susceptible to invasive *Candida* infections after receiving exogenous glucocorticoid therapies (Patterson, 1999; Hovi et al., 2000; Bodey, 2002). Since *C. albicans* colonizes the mucosal surfaces of 30–70% of healthy individuals, the absence or suppression of immune recognition and response can lead to fungal infections (Moyes & Naglik, 2011; Sardi et al., 2013; Yapar, 2014; Moyes et al., 2015). Therefore, a deeper understanding of epithelium–*C. albicans*–glucocorticoid trilateral interactions seems to be of primary importance to prevent or alleviate nosocomial fungal infections (Pelroth et al., 2007; Karkowska-Kuleta et al., 2009; Moyes & Naglik, 2011; Sardi et al., 2013; Yapar, 2014).

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