

Enhanced basophil histamine release and neutrophil chemotactic activity predispose grain dust-induced airway obstruction

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Summary

Background The pathogenic mechanism of grain dust (GD)-induced occupational asthma (OA) remains unclear.

Objective To understand further the mechanism of GD-induced OA.

Methods Fifteen employees working in a same GD industry, complaining of work-related respiratory symptoms, were enrolled and were divided into two groups according to the GD-bronchoprovocation test (BPT) result: six positive responders were grouped as group III, nine negative responders as group II and five healthy controls as group I. Serum GD-specific immunoglobulin (Ig)E (sIgE), specific IgG (sIgG) and specific IgG4 (sIgG4) antibodies were detected by enzyme-linked immunosorbent assay. Basophil histamine release was measured by the autofluorometric method, and changes of serum neutrophil chemotactic activity were observed by the Boyden chamber method.

Results For clinical parameters such as degree of airway hyperresponsiveness to methacholine, duration of respiratory symptoms, exposure duration, and prevalences of serum sIgE, sIgG and sIgG4 antibodies, there were no significant differences between group II and III ($P > 0.05$, respectively). Serum neutrophil chemotactic activity increased significantly at 30 min and decreased at 240 min after the GD-BPT in group III subjects ($P < 0.05$, respectively), while no significant changes were noted in group II subjects ($P > 0.05$). Basophil histamine release induced by GD was significantly higher in group III than those of group I or group II ($P < 0.05$, respectively), while minimal release of anti-IgG4 antibodies was noted in all three groups.

Conclusions These results suggest that enhanced basophil histamine release and serum neutrophil chemotactic activity might contribute to the development of GD-induced occupational asthma.

Keywords: grain dust, occupational asthma, neutrophil chemotactic activity, basophil histamine release

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Introduction

Chronic inhalation of grain dust (GD) has been shown to cause acute and chronic airway injury characterized by bronchitis and airflow obstruction [1–4]. Longitudinal studies have shown an accelerated deterioration of pulmonary

function in these grain workers [5], and the severity of deterioration appears to be related to the concentration of airborne GD in the work environment [4,6].

There have been studies on the presence of serum specific immunoglobulin (Ig)E antibodies to GD in exposed workers, showing a prevalence of up to 40% of symptomatic workers [7]. Our previous study [8] on bronchial mucosa of GD-induced occupational asthma (OA) subjects using an immunohistochemical method showed that mast cell and

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neutrophil counts were significantly higher than those of allergic asthma and eosinophil and T-lymphocyte infiltrations. A few novel studies have shown that inhalation of GD in the healthy volunteers induced an airway inflammatory response characterized by neutrophil recruitment to lower respiratory tract [9,10]. *In vitro* studies indicated that extract of GD was capable of recruiting neutrophils or activating mast cells by several nonimmunologic mechanisms [9,11–13]. To our knowledge, there have been no in-vitro data for with neutrophil chemotactic activity and basophil histamine releasability in subjects with GD-induced asthma.

In this study, in order to deepen our understanding of the role of neutrophil and basophil in the pathogenesis of GD-induced asthma, we compared the changes of serum neutrophil chemotactic activity (NCA) during a GD-bronchoprovocation test (BPT) and basophil histamine releasability in subjects with GD-induced OA showing positive challenges to those working in the same workplace showing negative challenges.

Materials and methods

Subjects

Six asthmatic patients with significant bronchoconstriction on GD-BPT who had been working for the Dongbang feed

industry in Suwon, Korea (group III) participated in this study. Nine symptomatic employees from the same workplace, but showing negative results on GD-BPT (group II) and five unexposed healthy nonasthmatic subjects (group I) were enrolled as controls. The clinical characteristics of each group are described in Tables 1 and 2.

Sera

For serum NCA, sera from group II and III subjects was collected before, 30 min and 240 min after the bronchial challenge and stored at -20°C . All experiments were performed simultaneously.

Preparation of grain dust extracts

GD was obtained from the subject's workplace. It was extracted with phosphate-buffered saline (PBS, pH 7.5; 1:5 w/v) at 4°C for 1 h, followed by centrifugation at 5000 r.p.m. The supernatants for BPT were used at a concentration of 1:10 (w/v). Some of the supernatants were dialysed (the cut-off molecular weight was 6000 Da) against 4 L of distilled water at 4°C for 48 h, and lyophilized for preparation of antigens used in enzyme-linked immunosorbent assay (ELISA) for detecting serum specific IgE and NCA.

Table 1. Clinical features of the study subjects

Patient	Sex/Age	Atopy	Exposure period (years)	Respiratory symptom duration (months)	Smoking	Peripheral eosinophilia (μL)
Group II						
Kos	M/35	P	12	0.5	S	300
KiY	M/44	A	13	0.5	N	300
LeH	M/46	P	7	0.5	S	200
ChB	M/26	P	6	2	S	600
KiC	M/28	P	12	3	S	1400
KoY	M/32	P	13	2	N	300
Jun	M/34	P	10	1	S	0
Jin	M/36	P	4	1	S	200
LeJ	M/42	P	13	1	S	1700
Group III						
PC	M/48	P	16	2	S	1000
JD	M/46	P	11	2	S	300
LB	M/40	P	7	1	S	100
IH	M/42	P	1	0.5	N	1800
YJ	M/36	P	3	1	S	100
IJ	M/26	P	6	1	S	300

Atopy was defined as a positive reaction to more than one common inhalant allergen on skin-prick test. M, Male; P, presence; A, Absence; S, smoker; N, Non-smoker.

Table 2. Results of specific antibodies and grain dust-bronchoprovocation (BPT) test

Patient	PC20 methacholine (mg/ml)	Specific IgE to grain dust	Specific IgG to grain dust	Specific IgG4 to grain dust	BPT response (percent of all of FEV ₁)
Group II					
Kos	>25	+	+	+	Negative
KiY	>25	+	+	+	Negative
LeH	10.3	–	+	+	Negative
ChB	2.2	–	+	+	Negative
KiC	2.3	+	+	+	Negative
KoY	>25	–	+	+	Negative
Jun	>25	–	+	+	Negative
Jin	>25	+	–	–	Negative
LeJ	18	–	+	+	Negative
Group III					
PC	24	–	+	+	Dual (35%, 25%)
JD	>25	+	+	+	Early (24%)
LB	>25	+	+	+	Early (21%)
IH	2.5	–	+	–	Early (34%)
YJ	>25	+	+	+	Early (26%)
IJ	25	–	+	+	Early (25%)

+, Positive; –, negative. Ig, immunoglobulin.

Protocol

All subjects who had complained of work-related symptoms while exposed to GD in the workplace were admitted to the Allergy Clinic at Ajou University Hospital and a standard protocol was followed in all cases before the inhalation challenges. A screening evaluation, including a complete history and physical examination, pulmonary function tests, including a methacholine bronchial challenge test, chest X-ray, and electrocardiogram were performed on admission. A peripheral blood sample was obtained for the eosinophil and leukocyte counts, and basophil histamine release test. Baseline forced expiratory manoeuvres were performed using a spirometry (MultiSPIRO SX/PC, MultiSPIRO, AZ, USA) with standard protocols.

Bronchoprovocation test with grain dust

Airway responsiveness to methacholine was tested as previously described with some modifications [14]. BPT were performed by the method previously described [7]. Normal saline was administered from a nebulizer 646 connected to a dosimeter (DeVilbiss Co., PA, USA) operated at a pressure of 20 psi. The subject was asked to breathe the aerosol five times to the vital capacity and was observed for 7 h. On a different day, GD extract was inhaled from 1:1000 (w/v) to 1:10 (w/v). In the pulmonary function test, forced vital capacity, the forced expiratory volume in 1 s (FEV₁), and

FEF_{25–75} were measured with a spirometer (MultiSPIRO SX/PC, MultiSPIRO, USA) before and 10 min after each inhalation. Subsequently, FEV₁ and FEF_{25–75} were measured every 10 min during the first 1 h. The pulmonary function tests were performed every hour for 7 h after the challenge. A positive reaction was defined when FEV₁ decreased more than 20% from the baseline value. In order to exclude nonspecific reaction, the same procedure was performed in two house dust mite-sensitive asthmatic subjects.

ELISA for specific IgE antibodies to grain dust extract

The presence of specific IgE antibodies to GD was determined by ELISA according to the method previously described [7]. Microtitre plates (Dynatech, VA, USA) were first coated with 50 µL/well of GD extract (5 µg/well) and were left at 4°C overnight. Each well was washed three times with 0.05% tween phosphate buffered saline (PBST), and the remaining binding sites were blocked by incubation with 350 µL of 3% bovine serum albumin (BSA)-PBST for 1 h at room temperature. The wells were then incubated for 2 h at room temperature with 50 µL of either the patients' sera (undiluted) or control sera from 27 patients who showed negative skin-prick test responses to common inhalant allergens as well as to GD. After washing three times with PBST, 50 µL of the 1:1000 (v/v) biotin-labelled

goat antihuman IgE antibody (Vector, Burlingame CA, USA) was added to the wells and incubated for 2 h at room temperature. The wells were then washed three times with PBST and incubated with 1:1000 (v/v) streptavidin-peroxidase (Sigma, St Louis MO, USA) for 30 min before another washing step which was followed by incubation with 100 μ L of 2 N sodium azide. The absorbance was read at 410 nm by an automated microplate reader. All assays were performed in triplicate. The cut-off absorbance of positive IgE binding was determined as 0.064, which was derived from the mean and two standard deviations of the absorbance value of the control subjects.

Measurement of neutrophil chemotactic activity

NCA was measured using Boyden chamber method with some modifications [15,16]. Neutrophils were obtained from heparinized whole blood of AB type normal volunteers by sedimentation in 6% dextran dextrose solution followed by centrifugation on Ficoll-Hypaque solution (Sigma; specific gravity of 1.077) and hypotonic lysis. Cells containing more than 95% neutrophils were suspended in Hank's balanced salt solution (HBSS) with 0.4% of BSA at the concentration of 1×10^6 cells/mL. Polycarbonate filter with 5 μ L pore (Millipore, CA, USA) was topped over chemotaxin followed by distribution of neutrophil suspension. The chamber was incubated for 90 min at 37 °C in a humidified incubator containing 5% CO₂. Thereafter, the filter was removed, fixed in 100% methanol, and subsequently stained with Diff Quick stain solution (Sigma). The number of neutrophils, which migrated through the filter, was determined microscopically by a 40 $\times$ objective. Five fields were counted per well and the experiments were conducted in quadruplicate. The results were expressed as the mean neutrophil counts migrated per field in the postchallenge sample to the mean number of neutrophils per field of the prechallenge sample. Opsonized serum was used in positive control and HBSS with BSA in negative control.

Basophil histamine release

The techniques involved in preparing peripheral blood leukocytes for performing histamine release, in staging the histamine release reaction, and in the fluorometric measurement of histamine have been described previously [17–19]. Briefly, 20 mL blood were obtained from the subjects and mixed with 2.5 mL of 6% dextran-3% dextrose-physiologic saline and 1 mL of ethylenediamine tetraacetic acid. The mixture was allowed to sediment at room temperature for 2 h before the leukocytes containing plasma were removed by centrifuged at 4 °C and 250 g for 8 min. The leukocyte pellet was washed twice with PIPES-A (25 mM piperazine-*N,N'*-bis-2 ethane sulphonic acid, 110 mM NaCl, 5 mM

KCL, 40 mM NaOH, and 0.03% human serum albumin adjusted to pH 7.4), and, prior to histamine release studies, it was resuspended in PIPES-ACMD (91 mM CaCl₂, 0.5 mM MgCl₂, and 100 mg% dextrose in PIPES) to a final concentration of approximately 5×10^6 cells/mL. Some 100 μ L of the GD extract dilutions (0.001, 0.01, 0.1 mg/mL), calcium inophore A23197 (1 μ M, 3 μ M, Sigma, USA) or antihuman rabbit IgG4 antibodies (1:100, 1:1000, 1:10000 v/v, Sigma, USA) were added to 200 μ L of leukocyte suspension and the suspensions were incubated for 15 min at 37 °C. In parallel, leukocyte suspensions were incubated with PIPES-ACMD alone to correct for spontaneous histamine release. Total cellular histamine concentration was measured by adding 0.1 mL of 55% trichloroacetic acid to leukocyte suspension. The tubes were centrifuged at 4 °C and 250 g for 15 min and the supernatants were collected and stored at –20 °C for subsequent assay of histamine. All the experiments were performed in duplicate. Histamine concentration was measured by the automated fluorometric method described (auto-fluorometer, UK). Histamine release was expressed as percentage of total cellular histamine, based to the following formula:

$$\begin{aligned} \text{Percent histamine release} &= 100 \\ &\times (\text{stimulated histamine release} \\ &\quad - \text{spontaneous histamine release} / \text{total cellular} \\ &\quad \text{histamine release} \\ &\quad - \text{spontaneous histamine release}) \end{aligned}$$

Statistics

To evaluate the statistical differences between the data, the Wilcoxon-signed rank tests and ANOVA, Mann-Whiney *U*-tests were applied using the SPSS version 7.0 (Chicago, USA): A *P*-value of 0.05 or less was regarded as significant.

Results

Serum-specific IgE, IgG and IgG4, and clinical parameters

Tables 1 and 2 summarize the clinical characteristics of the study subjects. Three subjects of group III had high sIgE antibody, while all of them had high sIgG antibody. There were no significant differences in the prevalences of serum specific Ig (sIg)E, sIgG and sIgG4 antibodies. No differences were noted in the duration of respiratory symptoms (*P* = 0.80), the degree of airway hyperresponsiveness to methacholine (*P* = 0.16) or the exposure duration between group II and III (*P* = 0.17).

Comparison of serum neutrophil chemotactic activity

Figure 1 shows the changes of NCA in the subjects of group

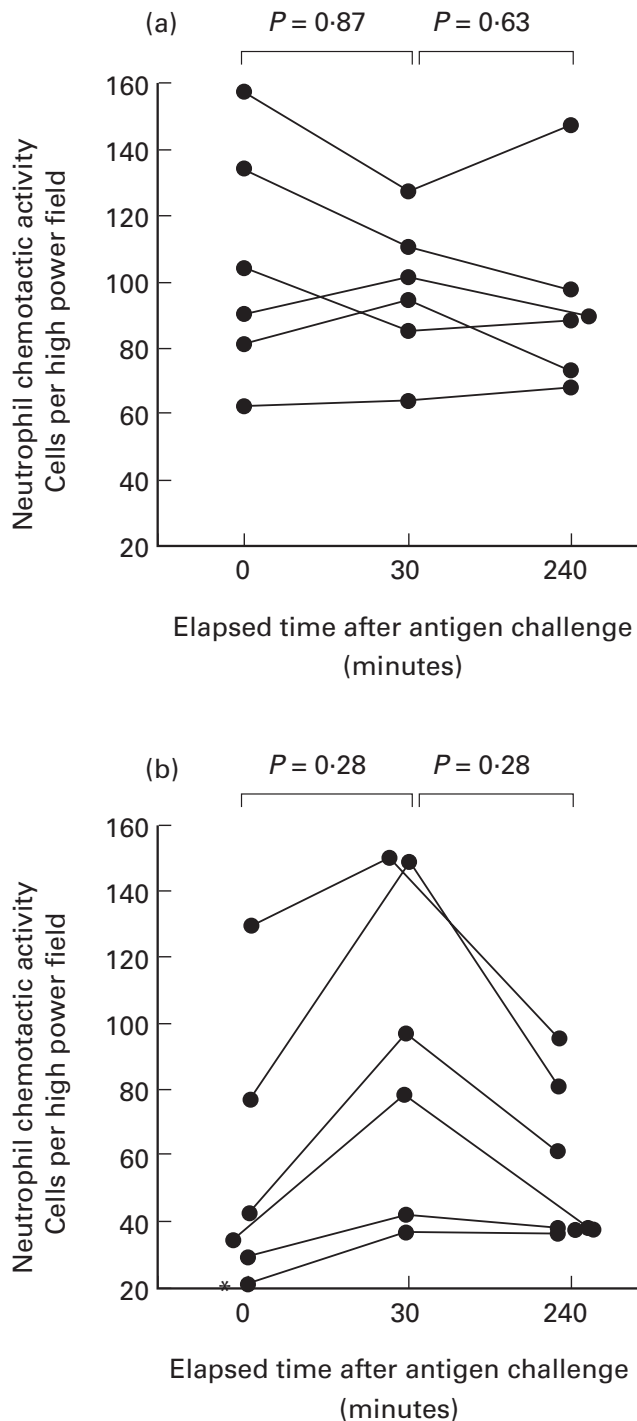


Fig. 1. Comparison of serum neutrophil chemotactic activity (NCA) during grain dust bronchoprovocation test between (a) group II and (b) group III. Significant changes were noted by Wilcoxon-Signed rank test in group III subjects.

II and group III. In the group III subjects, NCA peaked at 30 min and decreased significantly at 240 min during GD-BPT ($P = 0.028$, respectively). In contrast, no significant differences were noted among group II subjects ($P > 0.05$). Five subjects showed early asthmatic responses, and a patient with dual asthmatic response (indicated by an asterisk in Fig. 2) showed a persistent enhanced NCA for up to 240 min.

Basophil histamine release by grain dust

Figure 2 shows comparison of basophil histamine release in three individual groups with serial additions of GD antigen ranging from 0.001 to 0.1 mg/mL. Release of histamine by both GD and anti-IgE antibodies increased in a dose-dependent manner, while release by anti-IgG4 antibodies was minimal (data was not shown). In a comparison of the three groups, significantly greater amounts of histamine release with additions of 0.01 and 0.1 mg/mL of GD were noted in group III subjects ($P = 0.01$, $P = 0.01$, respectively). There was no significant difference in histamine release whether the subjects have serum specific IgE or not ($P > 0.05$, data not shown).

Discussion

This study demonstrates that difference patterns were noted in changes of the serum neutrophil chemotactic activity during the GD bronchial challenge test and the degree of GD induced basophil histamine release between exposed subjects with or without positive challenges. As the study subjects had worked in the same workplace and their exposure intensities to GD were similar, ranging from 0.4 to 1.0 mg/m³ which were measured by individual personal sampler, they were classified according to the GD-BPT results, and their clinical and laboratory parameters were compared in order to identify the determinant factors for the BPT results. There were no significant differences in clinical parameters with respect to the degree of airway hyper-responsiveness to methacholine, duration of respiratory symptoms, exposure duration and prevalences of specific antibodies. However, significant changes of serum neutrophil chemotactic activity after the bronchoprovocation test and basophil histamine releasability induced by GD were noted in group III subjects, while no significant changes were noted in group II subjects.

Several mechanisms may be involved in pathogenesis of GD-induced OA. Our previous study demonstrated a presence of serum specific IgE antibodies that was significantly inhibited by serial addition of GD antigens on ELISA inhibition test. Furthermore, IgE-binding components to GD were noted in 50% of the subjects with GD induced asthma, which suggests that the IgE-mediated reaction

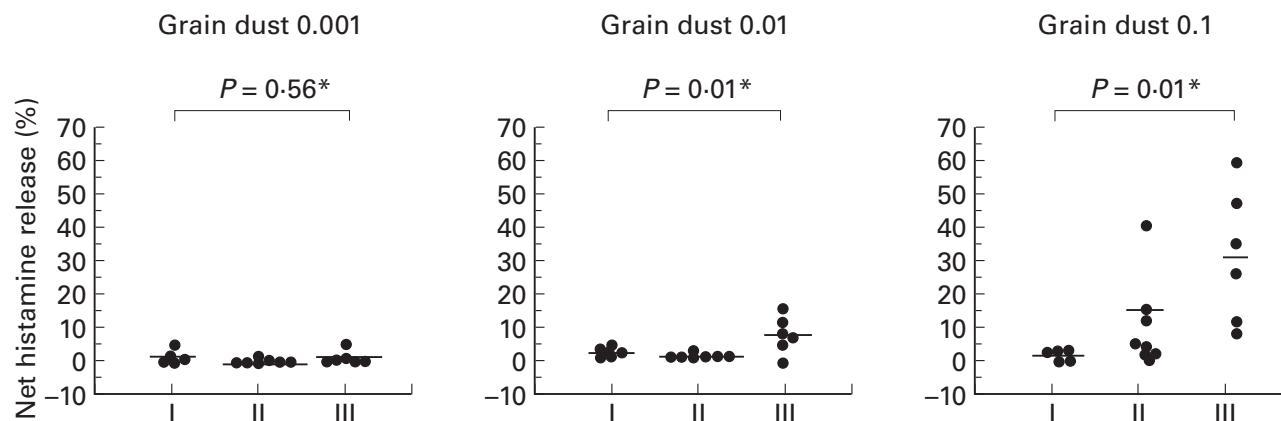


Fig. 2. Comparison of peripheral basophil histamine release by serial additions of grain dust antigens among three groups. Significant differences were noted with additions of 0.01–0.1 mg/mL of grain dust antigens ($P = 0.01$, $P = 0.01$, respectively).

may be one of the responsible mechanism for their asthmatic symptoms [7]. The failure to detect specific IgE antibodies to GD in the sera of the other three workers with positive challenges might indicate an involvement of other immunological or nonimmunologic mechanisms in the induction of asthmatic symptoms. An immunological mechanism involving IgG might have a role in the pathogenesis of asthma [20]. However, in the present study, the result of basophil histamine release by anti-IgG4 antibodies suggest a low possibility for specific IgG4 acting as a sensitizing antibody.

Recently, involvement of neutrophil in the pathogenesis of OA has been suggested. Neutrophilia had been noted in bronchoalveolar lavage fluid in subjects with late asthmatic reaction induced by isocyanate challenge test [21]. Inhalation of grain sorghum dust in normal volunteers could induce an increased peripheral neutrophil count and chemotactic response to GD [10]. An in-vitro study revealed the release of substances in response to GD [22], which manifests NCA by bronchial epithelial cell. To date, the mechanism of neutrophil chemotaxis and the function of neutrophils in GD-induced asthma have not been well documented. Our earlier immunohistochemical study [8] revealed that the number of neutrophil elastase-containing cells and tryptase-containing mast cells was significantly higher in the bronchial mucosa of GD-induced asthma patients than in that of allergic asthma patients, and that no significant differences in pan-T cell or eosinophil (EG2⁺) counts were observed. The present study revealed that serum NCA of group III subjects increased significantly relative to the baseline value at 30 min after GD-BPT, and subsequently decreased significantly at 240 min. This is in contrast to no significant changes of NCA being observed in group II subjects with a negative result on GD-BPT. These findings suggest a possible involvement of neutrophils in the development of GD-induced bronchoconstriction in exposed workers.

The presence of NCA has been demonstrated in sera of individuals with asthma after provocation with allergens. Basically, the NCA may be divided into heat-stable and heat-labile activity [23,24]. The origin of these activities has not clearly been delineated, although the following indirect data indicate the mast cell as the origin of heat-stable activity and the monocyte/macrophage of heat-labile activity [24–26]. The time course of occurrence of the serum heat-labile activity is clearly different from that of heat-stable one: the latter activity showed a quick rise coinciding with or preceding the early reduction of FEV₁, whereas heat-labile activity appeared immediately after asthmatic reaction followed by a second peak coinciding with the late asthmatic response [26]. In the present study, five asthmatic patients with an isolated early asthmatic reaction showed a NCA peak 30 min after the inhalation challenge and NCA decreased significantly at 240 min. In contrast, the patient with dual asthmatic response (indicated by an asterisk in Fig. 1) showed a persistence of enhanced NCA level up to 240 min which coincided with late asthmatic response. Based on the time course of NCA described above, we can speculate that NCA observed in this study might have been made up of two different kinds of NCA.

There have been a few reports suggesting that GD possess a substance that activates mast cells or complements [11–13]. These authors demonstrated that GD releases leukotriene B₄, D₄ and E₄ from lung tissue. Several experiments using lipopolysaccharide revealed that endotoxin was not responsible for histamine release [27,28]. In this study, the histamine release by GD was dose-dependent: the higher the dose of GD, the greater the amount of histamine released. Compared to GD-BPT results, the basophil histamine release by GD was significantly greater in group III subjects than those of group II. Minimal release of histamine was noted in controls. Although we did not attempt to further

explore the mechanism of GD-induced histamine release, we speculate that the degree of basophil histamine release by GD might contribute to the development of airway obstruction in exposed workers. The possibility of endotoxin mediated reaction seemed extremely low in this study. In conclusion, both enhanced serum NCA and basophil histamine release may contribute to the development of airway obstruction caused by GD.

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