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Extracellular Granzymes A and B in Humans: Detection of Native Species During CTL Responses In Vitro and In Vivo

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Activated CTLs and NK cells induce apoptosis via multiple mechanisms, including that termed granule exocytosis. The latter pathway consists of vectorial secretion of perforin and a family of granule-associated serine proteases (granzymes) to the target cell. To establish whether granzymes are released extracellularly during cytolytic reactions in vivo, ELISAs that measure the native enzymes were developed and were found to specifically detect granzyme A (GrA) and granzyme B (GrB) at picogram concentrations. Low levels of GrA and GrB were present in plasma of healthy individuals (GrA, 33.5 pg/ml (median); GrB, 11.5 pg/ml (median)), whereas significantly higher levels were present in patients with ongoing CTL response, i.e., patients suffering from infections by EBV or HIV type 1. Markedly elevated levels were also noted in synovial fluid of patients with active rheumatoid arthritis. The measurement of soluble granzymes should be useful to assess clinical disorders associated with activated CTL and NK cells. Furthermore, these results suggest that granzymes mediate biologic effects beyond their described role in apoptotic cell death. The Journal of Immunology, 1998, 160: 3610–3616.

Activated CTLs and NK cells induce apoptosis via granule exocytosis, Fas/Apo-1, and TNF-dependent processes (1–3). Lymphocyte granule-mediated cytotoxicity, in particular, appears designed to protect the host from invasion by pathogens and tumor cells. Two distinct mechanisms encompass this phenomenon: 1) perforin (PFN)3-mediated necrosis of the target and 2) PFN/granzyme-induced apoptosis, in which granzyme B (GrB) plays a pivotal role. Following adhesion of the cytotoxic cell to the target, secretory granules are directed to the contact area, and their contents are exocytosed into the intercellular space. In the presence of extracellular Ca²⁺, PFN polymerizes into transmembrane pores (4, 5), facilitating the intracellular delivery of the granzymes. Recently, a new internalization process has been described, in which GrB binds to high affinity binding sites and enters the target cell independently of PFN (6).

Since granzymes and PFN constitute the primary effectors of the granule exocytosis pathway, identification of these proteins by immunohistochemical (7) or molecular biologic techniques (8) has been used to assess the involvement of activated CTL or NK cells in pathologic processes. These methods, however, require the availability of tissue, which severely hampers their clinical utility. Studies in vitro have shown that granzymes may be released extracellularly during cytotoxic cell degranulation (9). Therefore, we hypothesized that levels of extracellular granzymes may reflect endogenous CTL or NK cell activity in vivo. To test this hypothesis we developed ELISAs that accurately identify native human GrA or GrB. We measured the plasma levels of the granzymes in healthy volunteers, in patients with rheumatoid arthritis (RA), and in those suffering infections with EBV or HIV-1. The results show that soluble granzymes are present at low concentrations in the blood of healthy individuals and at significantly higher levels in these disease states.

Materials and Methods

Proteins
GrB was isolated from the human leukemia T cell line YT-Indy by HPLC (10). GrA was purified to homogeneity from IL-2-activated lymphocytes (11). Human neutrophil elastase was obtained from Elastin Products Co., Inc. (Owensville, MO); proteinase 3 was a gift from Dr. K. Koth (Chiron Corp., Emeryville, CA); human mast cell tryptase was a gift from Dr. R. Lutter (Academic Medical Center, Amsterdam, The Netherlands). Biotinylated goat anti-mouse polyclonal Abs were obtained from the Department of Immune Reagents of the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands).

Production and purification of mAbs against native GrA and GrB

BALB/c mice were immunized s.c. with 25 μg of native GrA or GrB suspended in CFA, followed by three to six subsequent injections of 25 μg of the protein suspended in IFA at 2-wk intervals. At the time the animals produced high Ab titers,fusion of the spleen and lymph node cells with mouse myeloma Sp2/0-Ag 14 cells was performed according to standard procedures (12). Conditioned medium from the hybridomas was screened for the presence of anti-GrA or anti-GrB mAb using rat anti-mouse IgG-Sepharose suspensions together with 125I-labeled native GrA or GrB as described previously for anti-C3 mAb (12). The supernatant of Ab-producing hybridomas was concentrated by ammonium sulfate precipitation (50%, w/v) followed by dialysis against PBS, pH 7.4. Thereafter, mAb

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were purified by protein G affinity chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the instructions of the manufacturer. Subclasses of mAb were determined by an isotyping dipstick method (Innogenetics, Antwerp, Belgium). All mAbs were of the IgG1 κ subclass.

**Competition RIA for the initial characterization of the mAb**

Limiting amounts of Sepharose-coupled mAbs were added to 125I-labeled GrA or GrB (2–4 ng) that had been preincubated for 1 h at room temperature with 2 ng of a second mAb. The mixture was then incubated by head-over-head rotation for at least 4 h at room temperature. Next, the Sepharose beads were washed with PBS/0.05% (w/v) Tween-20, and the amount of bound 125I-labeled GrA or GrB was measured. An mAb was considered to bind to similar or overlapping epitopes when interaction of the second Ab with 125I-labeled GrA or B was inhibited by >50%.

**Biotinylation of Abs**

Purified Abs were biotinylated using long chain biotinyl-N-hydroxysuccinimide ester sulfonic acid (Pierce Chemical Co., Rockford, IL) according to the instructions of the manufacturer.

**Granzyme B ELISA**

Purified mAb GB11 was incubated at a concentration of 2 μg/ml in 0.1 M sodium carbonate/bicarbonate buffer, pH 9.6, for 16 h at 4°C in microtiter plates (Nunc Maxisorb Immunoplate, Nunc, Copenhagen, Denmark; 100 μl/well). The plates were then washed with PBS/0.02% (w/v) Tween-20. An identical washing procedure was performed after each incubation step which consisted of 100 μl, except for the blocking step (150 μl). After coating, residual binding sites were blocked by a 45-min incubation with PBS/2% (v/v) cow milk. All samples to be tested as wells as standards (purified native GrB at various concentrations) were pretreated with 40 μg/ml hyaluronidase (Sigma Chemical Co., St. Louis, MO) for 30 min at room temperature in PBS/0.02% Tween-20. Samples were then appropriately diluted in high performance ELISA buffer (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service) and incubated for 1 h.

Next, the plates were incubated with an excess of biotinylated GB10 mAb (0.5 μg/ml) together with 1% (v/v) normal mouse serum for 1 h, then the plates were incubated for 30 min with streptavidin-polymerized horseradish peroxidase (Department of Immune Reagents, Central Laboratory of The Netherlands Red Cross Blood Transfusion Service), whereafter bound peroxidase was visualized by incubation with a solution of 100 μg/ml 3,3′,5,5′-tetramethylbenzidine (Merck, Darmstadt, Germany) and 0.003% (v/v) H2O2 in 0.11 M sodium acetate buffer, pH 5.5. The reaction was stopped by addition of an equal volume of 2 M H2SO4 to the wells. Finally, the absorbance at 450 nm was read on a Titer-tek Multiscan plate reader (Labsystems, Helsinki, Finland).

**Granzyme A ELISA**

The GrA ELISA was essentially performed as described for that of GrB, except that GA28 (2 μg/ml) was the coating mAb, and biotinylated GA34 (0.5 μg/ml) was used to detect bound GrA.

**Degranulation experiments**

JS-136 cells (a gift from Dr. J. Borst, Dutch Cancer Institute, Amsterdam, The Netherlands) were harvested after 6 days of stimulation with JY cells (13). Cells were washed in Iscove’s supplemented with 0.02% (w/v) BSA and resuspended at a concentration of 1 × 106 cells/ml in Iscove’s modified Dulbecco’s medium (IMDM)/0.02% (w/v) BSA, containing three mAbs directed against CD2 (GB2, 6G4, and Hic 27; Central Laboratory of The Netherlands Red Cross Blood Transfusion Service) each at 1 μg/ml and PMA at a final concentration of 1 ng/ml. Control cells (not stimulated with anti-CD2 and PMA) were resuspended in IMDM/0.02% BSA. Cell suspensions (100 μl/well) were put into 96-well round-bottom microtiter plates (Greiner, Frickenhausen, Germany), and incubated for various times. Aliquots of the supernatant were collected and centrifuged for 8 min at 1500 rpm to remove intact cells. Medium was stored at −20°C until testing.

**Clinical samples**

Plasma samples of healthy volunteers were collected by venipuncture using tubes (Venoject, Terumo Europe N.V., Leuven, Belgium) containing soybean trypsin inhibitor (0.1 mg/ml), 10 mM EDTA, and 20 mM bensamidine (final concentrations). Plasma samples (n = 10), obtained at the Daniel den Hoed Clinic in Rotterdam, were collected in tubes containing 10 mM EDTA and 0.05% (w/v) Polybrene (Janssen Chimica, Beerse, Belgium) (14). Simultaneously, synovial fluid (SF) samples were taken from involved knees using a plastic syringe and immediately transferred into a siliconized Vacutainer tube (Becton Dickinson, Plymouth, U.K.) containing 10 mM EDTA and 0.05% Polybrene. All patients fulfilled the revised American Rheumatism Association criteria for definite RA (15).

Blood from HIV-1-infected patients was collected in tubes (Venoject) containing 15 U of USP heparin. Samples were prepared from specimens sent to the Department of Clinical Viroimmunology, Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, to measure various immune parameters of HIV-1 infection. Diagnoses were based on the presence of Abs against HIV-1 p24 protein in plasma from the patients.

All plasma samples were centrifuged for 10 min at 1300 × g, whereafter supernatants were centrifuged again for 5 min. The final supernatants were stored in small aliquots at −70°C until use.

In addition, serum samples were obtained from 14 patients in various stages of acute EBV infection as determined by immunoblotting for EBV early AgD viral capsid Ag (VCA), or Epstein-Barr nuclear Ag (16). EBV-IgG and IgM levels were further defined by peptide ELISA (17).

**Statistical analysis**

Data are presented as the median and range unless indicated otherwise. For statistical analysis, values below the detection limit were set at 1 pg/ml. The significance of the differences between subject groups was assessed using the nonparametric Wilcoxon-Mann-Whitney test. A double-sided p < 0.05 was considered to indicate a significant difference.

**Results**

**Development of an ELISA for soluble GrB**

BALB/c mice were immunized with native human GrB isolated from the human leukemia cell line YT-Indy. From the fusion experiments three mAbs of the IgG1 κ subclass were obtained that bound 125I-labeled GrB in solution: GB10, GB11, and GB12. Purified mAbs were either coupled to Sepharose beads or biotinylated.

As determined by RIA competition experiments and sandwich ELISA experiments three mAb of the IgG1 κ subclass were obtained: GB10, GB11, and GB12 recognized the same or overlapping epitopes, whereas mAb GB10 appeared to bind to a different epitope. Pilot experiments revealed that the optimal combination was GB11 as the coating Ab and biotinylated GB10 as the detecting Ab. Using this combination, a highly reproducible ELISA was developed in which soluble GrB was detected at a range of 3 ng/ml to 4 pg/ml (Fig. 1). Addition of soybean trypsin inhibitor (100 μg/ml, final concentration), which blocks the active site of GrB, did not affect the detection of GrB in the ELISA (result not shown). Finally, GrB mAb did not cross-react with purified GrA in the ELISA (Fig. 1).

![FIGURE 1. ELISA for soluble human granzyme B. Dilutions of purified granzyme B (○) or purified GrA (●), as a control, were tested. Data indicate the mean ± SD of five experiments. The detection limit of the ELISA (defined as the concentration of GrB showing an absorption twice the background) was 2 to 4 pg/ml.](image-url)
Development of an ELISA for soluble GrA

A procedure similar to that described for GrB mAb was used to raise mAb against purified human GrA. Twenty-six Abs (GA10–GA35), all of the IgG1κ subclass, were obtained. After purification, coupling to Sepharose beads, and biotinylation, mAb were characterized by RIA competition experiments and sandwich ELISAs. Five groups of mAb were identified, each group recognizing one or overlapping epitopes (results not shown). Testing combinations of mAb from the different groups revealed that mAb GA28 as a catching and GA34 as a detecting Ab provided the most sensitive assay for soluble GrA. With this highly reproducible ELISA, GrA was detected at a range from 16 pg to 18 ng/ml (Fig. 2). Addition of benzamidine (up to 100 mM) to the protein, which inhibits the proteolytic activity of GrA, did not affect detection of the protein in the ELISA (result not shown). As can be seen in Figure 2, GrB did not cross-react in this system.

Specificity of the mAb used in the ELISAs

To ascertain that the mAb used in the ELISAs were specific for either GrA or GrB and did not react with other serine proteases homologous to granzymes, we performed additional ELISA experiments. Wells were coated with human neutrophil elastase, mast cell tryptase, proteinase 3, and cathepsin G and incubated with mAb GA28, GA34, GB10, and GB11. GrA mAbs only bound to GrA and not to GrB, neutrophil elastase, tryptase, proteinase 3, or cathepsin G (Fig. 3). Similarly, mAb GB10 and GB11 only bound to GrB and not to neutrophil elastase, mast cell tryptase, proteinase 3, or cathepsin G (not shown).

Degranulation experiments

Since in vitro studies have shown that granzymes may be released during degranulation of cytotoxic cells (9), degranulation experiments were performed, and the medium of the cells was tested for the presence of GrA and GrB. After 6 days of stimulation of the human CTL clone JS 136, degranulation was induced by resuspending the cells in BSA containing IMDM medium supplemented with anti-CD2 mAbs together with PMA. At various times of incubation, samples from the medium were collected and tested for the presence of soluble granzymes. After 30-min incubation, detectable levels of both GrA and GrB were found (Fig. 4). Soluble GrA and GrB levels continued to increase until 5 h of incubation.

Detection of soluble GrA and GrB in plasma and synovial fluid of RA patients

Immunohistochemical and biochemical studies have shown local production of GrA, GrB, and PFN in inflamed rheumatoid joints (8, 20–22). Therefore, plasma and synovial fluid samples of 10 RA patients fulfilling the American Rheumatism Association criteria (15) were tested for the presence of soluble granzymes. Both soluble GrA and GrB were detected in the synovial fluid of the RA patients. In most cases the values markedly exceeded plasma levels detected in normal volunteers (Fig. 5 and Table I). In contrast, plasma levels of GrA were similar to those in controls, while GrB levels tended to be somewhat higher. Since rheumatoid factors present in plasma and SF could lead to spurious results, we...
performed additional specificity control studies. First, we did not observe absorption values significantly above background for ELISAs where the GrA coating Ab was used combined with the biotinylated GrB mAb, and vice versa. Second, rheumatoid factor levels in the samples tested did not correlate with either GrA or B levels. Third, addition of purified rheumatoid factors to the ELISAs did not alter the levels of the granzymes detected. To ascertain whether soluble GrA or GrB was produced locally in the joints, the ratio between SF and plasma samples was calculated for each patient. This ratio exceeded 1.0 in all patients, with a range from 5.66 to 69,554 for soluble GrA and from 4.4 to 3,775 for GrB. Thus, consistent with the finding that RA synovium contains activated CTLs, soluble GrA and GrB are produced locally in the joints of patients with RA.

Circulating levels of soluble GrA and GrB in patients with EBV or HIV-1 infections

Cytotoxic T cells and NK cells are involved in the immune response against a variety of viral infections (23–25). Therefore, we tested blood samples from patients with symptomatic EBV or HIV-1 infection for the presence of soluble GrA and GrB. Serum samples of 14 patients at various stages of EBV infection (Table II) contained elevated levels of both soluble GrA (median, 156; range, 1–5500) and soluble GrB (median, 60; range, 1–4000). Levels of GrA were significantly higher than those in the healthy control group, whereas those of GrB tended to be higher (Fig. 5 and Table I).

Elevated levels of granzymes were also detectable in plasma samples collected from a group of HIV-1-infected patients during the asymptomatic phase: GrA (median, 114; range, 1–1683) and GrB (median, 20; range, 1–74). Analysis of the results obtained with HIV-1- or EBV-infected subjects revealed a significant correlation between soluble GrA and GrB (Fig. 6).

Longitudinal studies in EBV- or HIV-1-infected subjects

To establish whether levels of soluble granzymes fluctuated with the stage of viral infection, levels were measured in plasma samples taken during the acute and convalescent phases. Longitudinal plasma samples from four EBV-infected and one HIV-1-infected patients were available for analysis (Fig. 7). Although both GrA and GrB levels were increased during the acute phase of the infection, levels of GrA were consistently higher than those of GrB (not shown). Moreover, increases in GrA were associated with markers of early viral infection, namely IgM anti-VCA in EBV infection and p24 Ag in HIV-1 infection (Fig. 7). In the period following the acute infection, the granzyme levels decreased to the (high) normal range. Thus, during viral infection the levels of both GrA and GrB increased during the acute phase and then declined during resolution.
Discussion

Granzyms have been identified in supernatants during cytotoxicity assays in vitro. The significance of this observation, however, has not been evaluated due to the lack of techniques that allow accurate measurement of the native species. Although a mAb recognizing human native GrB has been described (26), we have generated mAbs against disparate epitopes of native GrA and GrB that allow the development of ELISAs that detect soluble granzymes at picogram levels. The anti-granzyme mAbs were specific, lacking cross-reactivity with nonhomologous serine proteases (see Fig. 3). Using these sensitive assays we show here for the first time that free granzymes can be detected during states of CTL and NK cells activation both in vitro and in vivo.

The mechanisms that contribute to the increase in extracellular granzymes is uncertain. It is commonly accepted that the secretion of the granzymes is induced by target cells during the cytotoxic cell response. Evidence indicates, however, that various chemokines may also elicit exocytosis of the granzymes independently of target cells (27). Furthermore, cytokytic cells have been reported to constitutively secrete the granzymes following activation (28). In addition to an enhanced secretion, an impaired clearance of granzymes during a cytotoxic response can contribute to an increase in extracellular granzymes. Unfortunately, the precise clearance mechanisms of circulating granzymes are not known. We have observed in 30 patients with varying degrees of renal dysfunction no correlation between creatine clearance and circulating granzyme levels (result not shown). Thus, apparently kidney function does not influence the clearance of circulating granzymes. However, as long as the precise clearance mechanisms are not known, it remains to be established to what extent impaired clearance may have contributed to an increase in circulating granzymes.

The release of granzymes during NK or CTL degranulation has been documented previously using either esterolytic assays or immuno-blotting techniques. BLT ((benzyloxycarbonyl) lysine thio-benzyl ester) esterase activity (substrate for GrA) and asp-ase activity (substrate for GrB) have been detected in supernatants during cytotoxicity assays (29–33), while GrA has been detected using an immunoblotting technique (34). These approaches, however, fail to accurately measure enzymatically active forms of the granzymes. We show that CTL stimulated to undergo granule exocytosis secrete biologically active granzymes (Fig. 3). Furthermore, the release of granzymes has been observed during standard cytotoxicity assays and after activation of NK cells or CTL by such cytokines as IL-2 (results not shown). Although the signals that regulate the degranulation and constitutive secretion of the granzymes and the contribution these processes make to increase extracellular levels await further characterization, it is clear that granzymes are positioned to mediate extracellular biologic effects independently of PFS.

Detectable levels of extracellular granzymes were present in the plasma of healthy donors. It remains to be established whether these low plasma levels are due to degranulation or constitutive secretion of the serine proteases from cytotoxic cells (28). Since normal peripheral blood contains a small percentage of activated, granzyme-positive lymphocytes, the levels in normal subjects may reflect the constitutive release of the proteases from these activated cells (18, 35, 36). During testing of normal plasma, an occasional sample was identified with levels that exceeded the upper limit of normal (>200 pg/ml). Dose-response curves of such samples did not parallel those generated with purified granzymes. After pretreatment with hyaluronidase, a strategy used to enhance reliable measurement in SF, much lower levels were detected and those response curves parallel the internal standard. Since we have not observed hyaluronidase sensitivity among patient samples, we decided to treat all samples and standards routinely with hyaluronidase to prevent the detection of false positive signals and ensure that the measured samples parallel the internal standards. The basis

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<th>Sample</th>
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* Z, zebra (BZLF<sub>1</sub>) protein; IM, infectious mononucleosis; EA<sub>D</sub>, early diffuse Ag (BMRF<sub>1</sub> + BALF<sub>2</sub>); VCA, viral capsid Ag (BFRF<sub>3</sub> + BdRF<sub>1</sub>).
for this hyaluronidase sensitivity is unclear. We are now evaluating whether this behavior is due to interaction of glycosylated granzyme with circulating hyaluronic acid followed by disproportionate binding of the complexes to the microwells.

The role of granule-mediated killing as a host defense against certain viral infections is well established (2, 37–39). Although the release of granzymes by CTL of patients suffering from viral infections has been documented by insensitive enzymatic assays (40, 41), we show here that blood samples of EBV- or HIV-1-infected patients contain elevated levels of both native GrA and GrB. These levels varied dramatically from virtually none to 100-fold more than normal. The marked differences in the levels are probably related to the interindividual variation in the intensity of the CTL/NK response as well as the stage of infection when the samples were obtained (see Fig. 7). Importantly, during the acute phase, granzyme levels increased simultaneously with early markers of infection. Therefore, our results suggest that elevated levels of soluble granzymes in plasma may indicate the presence of an acute viral infection. Preliminary results suggest that this association may also exist during Dengue fever and CMV infection after renal allograft transplantation.

The potential extracellular functions of the granzymes in antiviral immunity have not been extensively evaluated. GrA induces the production of IL-6 and IL-8 in fibroblasts and epithelial cells (42) and stimulates IL-6, IL-8, and TNF production in monocytes by a mechanism that does not involve activation of the thrombin receptor (43). GrB in the absence of PFN has been reported to induce apoptosis in targets infected with viruses entering cells via endocytosis (e.g., adenovirus) (6). This suggests that soluble GrB may mediate a novel form of antiviral cell-mediated cytotoxicity (44). Therefore, extracellular granzymes may control viral infections through both cytotoxic and noncytotoxic mechanisms (37).

Granzyme-positive cells have been identified in the synovial tissue (21, 22) as well as in the SF of patients with RA (20). Although RA plasma samples contained slightly elevated levels of GrA (4/10) and GrB (7/10), strikingly higher levels of both granzymes were detected in paired SF samples. Since the ratio of plasma to SF levels consistently exceeded 1.0, the data strongly suggest that the granzymes are produced and secreted within the affected joint. The significance of markedly increased SF levels in the pathogenesis of RA is unknown. GrA and GrB have been reported to degrade extracellular matrix proteins in vitro (45, 46). GrB, in particular, is able to cleave cartilage aggrecan with an efficiency comparable to that of stromelysin (6). Hence, our findings suggest a potential role for the granzymes in the tissue destruction of the rheumatoid joint.

In summary, we have developed sensitive ELISA assays that are suitable for the accurate detection of extracellular granzymes. High
levels of both GrA and GrB were detected in the plasma of patients experiencing viral infections as well as in the joints of patients with active RA. The assays will provide invaluable tools for study of the involvement of CTL and NK cells in human disease, allo-media-
tion, and graft-vs-host disease and should stimulate interest in identifying additional functions for this novel family of granule-associated serine proteases.

References

1. Lowin, B., M. Hahne, C. Mattman, and J. Tschopp. 1994. Cytolytic T-cell cy-
totoxicity is mediated through perforin and Fas lytic pathways. Nature 370:650.
totoxic T cells deficient in both functional Fas ligand and perforin show residual cytolytic activity yet lose their capacity to induce lethal acute graft-vs-host disease. Exp. Med. 183:657.
pONENT of complement (C3) results in the exposure of neoepitopes also present in activation products of C3. J. Immunol. 141:1602.
ization and diagnostic application of immunodominant domains of the BFRF3-
Corrections


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The ninth author’s middle initial is missing. The correct name is William G. Fairbrother.

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The fifth author’s name should have been published as J. A. Kummer.

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