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Interleukin-1α Production during *Rickettsia rickettsii* Infection of Cultured Endothelial Cells: Potential Role in Autocrine Cell Stimulation

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***Rickettsia rickettsii* infection results in numerous responses by cultured endothelial cells, among them a rapid, transient increase in steady-state levels of tissue factor mRNA (L. A. Sporn, P. J. Haidaris, R.-J. Shi, Y. Nemerson, D. J. Silverman, and V. J. Marder, Blood 83:1527–1534, 1994). In this study, production of interleukin-1 (IL-1) was measured during infection and its potential role in autocrine cell stimulation was investigated. A fivefold increase in levels of IL-1α antigen was measured in cell lysate samples by enzyme-linked immunosorbent assay at 18 h of infection. The majority of IL-1α remained cell associated, as no significant increase was detected in culture medium. No IL-1β antigen was detected in cell lysates or culture medium from either control or infected cultures. A dramatic increase in the levels of IL-1α mRNA occurred following infection, as measured by reverse transcriptase PCR, which revealed the appearance of the expected 421-kb product with RNA extracted from cells infected for 4 h and no detectable product from control cell samples. The presence of functional, cell-associated IL-1α activity in infected cells was confirmed, following disruption, by the ability of the infected cells to induce tissue factor expression in target endothelial cells. Such induction was eliminated by pretreatment of the disrupted cell samples with neutralizing antibodies against IL-1α but not against IL-1β. To investigate whether endogenously produced IL-1 participates in the stimulation of tissue factor expression, neutralizing antibodies against IL-1 or the IL-1 receptor antagonist were added to culture medium during infection. Both anti-IL-1α and the IL-1 receptor antagonist resulted in an approximately 40% inhibition of tissue factor expression, thus implicating IL-1α in autocrine cell stimulation.**

Human infection with *Rickettsia rickettsii* results in an acute, febrile, systemic illness known as Rocky Mountain spotted fever. Pathologic changes associated with this disease include vasculitis, with infiltration of mononuclear cells and neutrophils, and formation of occlusive vascular microthrombi (9, 17–19, 34, 46). Since the vascular endothelium is a primary early target of infection of this obligate intracellular parasite, localized pathologic changes may be influenced or even initiated by proinflammatory and procoagulant responses of this cell type. Indeed, responses of cultured endothelial cells to *R. rickettsii* infection include expression of E-selectin with resultant increased neutrophil adherence (42), increased platelet adherence (38), increased expression of tissue factor (41) and plasminogen activator inhibitor (13), and release of von Willebrand factor from Weibel-Palade bodies (43).

In vitro, endothelial cells produce interleukin-1 (IL-1) in response to injurious stimuli (20) such as bacterial lipolysaccharide (29, 33), tumor necrosis factor (29, 37), or IL-1 (37). In vivo, upregulation of IL-1 production by endothelial cells has been demonstrated for endotoxemia (28), septicemia (28), and retinal ischemia (16). Endothelial cell production of this inflammatory mediator could be central to the local inflammatory and procoagulant responses to *R. rickettsii* infection. Although endogenously produced IL-1 is an autocrine regulator of endothelial cell growth (8), less is known about the role of endogenously produced IL-1 in procoagulant responses of endothelial cells. In this report, we investigate IL-1 production during infection of cultured endothelial cells with *R. rickettsii* and explore the potential of IL-1 for the autocrine cell stimulation that results in tissue factor expression.

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MATERIALS AND METHODS

Endothelial cell culture and infection. Human umbilical vein endothelial cells were cultured as previously described (15, 45) with umbilical cords collected within 48 h of deliveries. Cells were cultured in McCoy's 5a medium (Flow Laboratories, McLean, Va.) containing 20% fetal bovine serum, endothelial cell mitogen (50 μg/ml) (Collaborative Research, Bedford, Mass.), heparin (100 μg/ml) (Sigma Chemical Co., St. Louis, Mo.), and insulin (25 μg/ml) (Sigma). Cells at passage 2 were used in experimental protocols and were plated so as to achieve 80 to 90% confluency after 5 to 7 days in culture. *R. rickettsii* were obtained as a plaque-purified seed stock (1×10^7 to 5×10^7 PFU/cm^2) of *R. rickettsii* (Sheila Smith strain) prepared in Vero cells (African green monkey kidney cells; American Type Culture Collection, Rockville, Md.) (39). Cells were infected with approximately 2×10^4 PFU/cm^2 of cell culture area. Dilutions of seed stock were made in culture medium. Infection was monitored with endothelial cells plated on Thermanox coverslips (Ted Pella Inc., Tustin, Calif.) and stained by immunofluorescence with antibody against *R. rickettsii* (kindly provided by Ted Tzianobos, Centers for Disease Control and Prevention, Atlanta, Ga.) as previously described (41, 45).

Measurement of IL-1 antigen. Following infection of endothelial cells cultured in 25-cm^2 flasks, culture medium was collected and cells were washed twice in ice-cold phosphate-buffered saline (PBS) and then harvested by scraping into 1 ml of ice-cold PBS containing 1 μg of leupeptin (Sigma) per ml, 1 mM phenylmethylsulfonyl fluoride (Sigma), and 10 U of aprotinin (Miles, Inc., Kankakee, Ill.) per ml. After centrifugation at $600 \times g$, cell pellets were washed once in the same buffer, lysed in ice-cold PBS containing 0.1% Triton X-100 (Sigma), sonicated for 30 s, and centrifuged at $100{,}000 \times g$ for 1 h at 4°C. Supernatants were stored at −20°C until assayed in triplicate by enzyme-linked immunosorbent assay (ELISA) with human IL-1α and IL-1β Quantikine ELISA kits (R & D Systems, Inc., Minneapolis, Minn.) according to the manufacturer's instructions. Recombinant IL-1α and IL-1β were purchased from Genzyme Corporation, Cambridge, Mass.

IL-1α functional assay. Endothelial cells were cultured in 75-cm^2 flasks, infected or not infected with *R. rickettsii* for 6 h, and then scraped into 4 ml of McCoy's 5a medium plus 20% fetal bovine serum, 200 U of penicillin (Sigma) per ml, 200 μg of streptomycin (Sigma) per ml, and 20 μg of tetracycline (Sigma) per ml. Lysates were kept on ice for 2 h, sonicated briefly, and stored at −70°C.

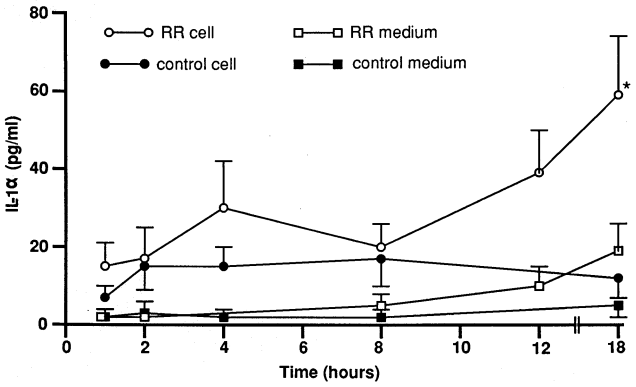

FIG. 1. Measurement of IL-1α antigen (means ± standard errors, $n = 5$) in cell lysate and culture medium samples by ELISA. Endothelial cells were infected for up to 18 h with *R. rickettsii* (RR), and then culture medium was collected and cells were lysed in 0.1% Triton X-100.

Contaminating lipopolysaccharides were removed with polymyxin B agarose (1 mg of immobilized polymyxin B per ml, 50 μl of beads per ml of cell lysate; Sigma). Neutralizing polyclonal antibodies against IL-1α and IL-1β (purified immunoglobulin; R & D Systems, Inc.) were added to lysates at concentrations of 1 and 5 μg/ml, respectively. Cell lysates were then placed on endothelial cells cultured in 12-well plates for 6 h which were then lysed for assay of tissue factor activity.

Tissue factor assay. Following experimental treatment, endothelial cells cultured in 12-well plates were washed twice with TBS (0.05 M Tris, 0.1 M NaCl [pH 7.5]), scraped into 0.16 ml of TBS with 10 mg of bovine serum albumin per ml, and lysed by repeated freeze-thawing. Tissue factor activity of the lysed-cell samples was then determined by a two-stage clotting assay calibrated with a sample of pure human brain tissue factor reconstituted into phospholipid vesicles as previously described (1, 36).

RNA extraction and analysis. Endothelial cells cultured in 75-cm^2 flasks were infected with *R. rickettsii* for 4 h and then lysed with 6.4 ml of Tri Reagent (Molecular Research Center, Inc., Cincinnati, Ohio), and RNA was isolated according to the manufacturer's instructions and dissolved in distilled water. RNA was converted to cDNA in a total reaction mixture volume of 100 μl with a solution containing 10 μg of total cellular RNA, 1,000 U of Moloney murine leukemia virus reverse transcriptase (RT) (Superscript II; Gibco BRL Laboratories, Gaithersburg, Md.), 2.5 μg of oligo(dT) (Gibco BRL), 1 mM (each) all four deoxynucleoside triphosphates (dNTPs) (Gibco BRL), 5 mM dithiothreitol, 75 mM KCl, 3 mM $MgCl_2$, and 50 mM Tris (pH 8.3). Reaction mixtures were incubated at 37°C for 2 h, and then the reactions were stopped by the addition of Tris-saturated phenol-chloroform (1:1). One-tenth volume of the total RT reaction mixture (10 μl), as well as a twofold dilution series, was subjected to PCR with a solution containing 1 μM specific primers, 2.5 U of *AmpliTaq* DNA polymerase (Perkin-Elmer Corporation, Norwalk, Conn.), 200 μM (each) all four dNTPs (Perkin-Elmer), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, and 10 mM Tris-HCl (pH 8.3). PCR was performed for 35 cycles as follows: 94°C for 1.5 min, 55°C for 1.5 min, and 72°C for 2 min. To control for possible amplification of genomic DNA, PCR reactions were carried out in the absence of RT, which in all experiments yielded no PCR product. IL-1α and IL-1β primer pairs were purchased from Perkin-Elmer and spanned exon-exon junctions to further eliminate the possible amplification of contaminating genomic DNA sequences. Primer sequences were as follows: IL-1α sense, 5′-GTCTCTGAATCAGA AATCCTTCTATC-3′, and antisense, 5′-CATGTCAAATTTCACTGCTTCAT CC-3′; IL-1β sense, 5′-AAACAGATGAAGTGCTCCTTCAGG-3′, and antisense, 5′-TGGAGAACACCACTTGTTGCTCCA-3′; and β-actin sense, 5′-GCTGTGCTATGTTGCCCTAGACTTCGA
GC-3′, and antisense, 5′-CGTACTCCTGCTTGCTGATCCACATGTGC-3′. Amplification products were separated on a 1.2% agarose gel containing 0.5 μg of ethidium bromide per ml.

Statistical analysis. *P* values were generated by Student's one-tailed *t* test.

RESULTS

IL-1α and IL-1β antigen were measured by ELISA in cell lysate and medium collected from cultured human umbilical vein endothelial cells at various times after infection with *R. rickettsii* (Fig. 1). *R. rickettsii* organisms were added so as to achieve infection in approximately 80% of endothelial cells within 6 h with 1 to 4 organisms per endothelial cell. IL-1α in cell lysates showed a significant (fivefold) increase only at 18 h ($P = 0.02$, $n = 5$). Slight but statistically insignificant increases in the levels of IL-1α antigen were detected in lysate at 12 h and in culture medium at 12 and 18 h. IL-1α produced as a result of infection was mainly cell associated, since at 18 h, only 32% of the total was present in the culture medium. No IL-1β was detected in cell lysates or culture medium at any time point tested (not shown).

RT-PCR was performed to determine if the observed increase in IL-1α antigen was associated with an increase in steady-state levels of mRNA. With specific primers for IL-1α and for β-actin, total RNAs isolated from the control culture and cultures infected for 4 h were amplified following conversion to cDNA by RT. Figure 2 shows the PCR products of twofold dilutions of the RT reactions. No amplification products were present in control samples with IL-1α primers. However, products corresponding to the expected 421-bp size were seen in samples from infected cells, indicative of increased steady-state IL-1α mRNA levels. Similar amounts of PCR products were generated with β-actin primers, generating the expected 443-bp product, which indicates that equal amounts of total RNA were reverse transcribed in control and infected samples. In agreement with the results of the ELISA, no PCR products were detected with IL-1β primers under any experimental condition.

To determine whether the cell-associated IL-1α produced in response to *R. rickettsii* infection was functional, an assay system was devised whereby cultures of infected endothelial cells were exposed to lysates of infected cells and then expression of tissue factor in target cells was used as a measure of cell stimulation. To ensure that the tissue factor activity measured originated from target cells and not from infected cell lysates, monolayers were extensively washed to remove residual cellular debris. Minimal functional IL-1α was present in control cell lysates, as the presence of these lysates did not result in increased tissue factor expression in target cells (not shown). Exposure of target endothelial cells to recombinant IL-1α at concentrations ranging from 0.05 to 0.25 ng/ml (diluted into control cell lysate to mimic experimental conditions) resulted in the concentration-dependent expression of tissue factor (Fig. 3A; $n = 3$). Control and 6-h-infected cultures were harvested by scraping, briefly sonicated, and then placed on target cultures for 6 h. Infected endothelial cell lysates induced a significant increase in tissue factor activity ($P = 0.0005$, $n = 12$), averaging 3.3 times that produced with lysates of control

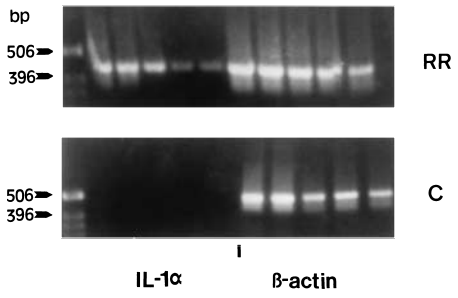

FIG. 2. Steady-state levels of IL-1α mRNA as measured by RT-PCR. One-microgram amounts of total RNA, extracted from control endothelial cells (C) and endothelial cells infected for 4 h with *R. rickettsii* (RR) and converted to cDNA, as well as 1:1, 1:2, 1:4, and 1:8 dilutions of this cDNA, were amplified with specific primer pairs for IL-1α and β-actin. Amplification products were analyzed with a 1-kb ladder on 1.2% agarose gels containing 0.5 μg of ethidium bromide per ml and photographed under UV illumination.

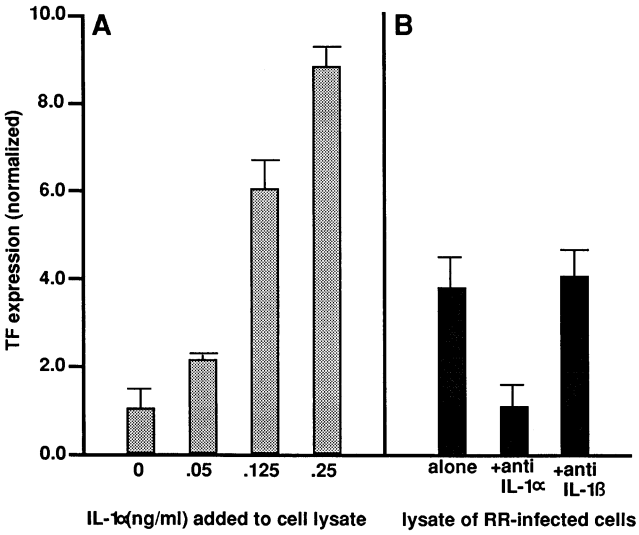

FIG. 3. Assay for biological activity of IL-1α in cell lysate samples. (A) Known amounts of IL-1α were diluted into samples of control cell lysate and then placed on target endothelial cells for 6 h, at which time tissue factor (TF) activity in target cells was assayed. (B) Control endothelial cells and endothelial cells infected for 6 h with *R. rickettsii* (RR) were scraped into culture medium and sonicated prior to placement on target cells in the presence or absence of neutralizing antibodies against IL-1α (1 μg/ml) or IL-1β (5 μg/ml). Results shown (means ± standard errors) were normalized to values obtained with control cell lysate in the absence of added IL-1α.

endothelial cells (Fig. 3B). The response of target cells was not due to infection by residual, viable organisms, as no intracellular organisms were present after immunofluorescence staining of cells cultured on coverslips and treated in parallel (not shown). The addition of neutralizing antibody against IL-1α, but not IL-1β, to infected cell lysates produced a significant decrease in cell stimulation ($P = 0.0002$, $n = 6$) to a level not statistically different from that induced by control lysates ($P = 0.09$, $n = 6$) (Fig. 3B). On the contrary, preincubation with neutralizing antibodies against IL-1β resulted in no decrease in tissue factor expression (Fig. 3B). These results suggest that functional IL-1α was present in infected endothelial cells within 6 h, before IL-1α antigen could be detected by ELISA (Fig. 1).

To investigate the possibility that IL-1 participates in an autocrine fashion in the induction of tissue factor expression during infection, endothelial cells were infected in the presence of IL-1 receptor antagonist or neutralizing antibodies targeted against IL-1 and then assayed for tissue factor activity. The presence of these agents did not inhibit infection of the endothelial cells (not shown). The IL-1 receptor antagonist, which blocks the interaction of both IL-1α and IL-1β with the type I IL-1 cell surface receptor (10–12, 22, 31), inhibited tissue factor expression induced by recombinant IL-1α and IL-1β nearly completely. However, the receptor antagonist inhibited tissue factor expression induced by *R. rickettsii* infection by only 37% ± 9% (Fig. 4). The neutralizing antibodies resulted in nearly complete inhibition of stimulation by their respective recombinant antigens, with minimal cross-reactivity, whereas neutralizing antibodies against IL-1α or IL-1β resulted in 43% ± 5% and 10% ± 3% inhibition of tissue factor expression, respectively, in *R. rickettsii*-infected cells (Fig. 4).

DISCUSSION

In this study, we provide evidence that cultured human umbilical vein endothelial cells respond to *R. rickettsii* infection by increasing the synthesis of functionally active IL-1, the majority of which remains cell associated. This expression of IL-1 appears to contribute to the tissue factor response of infected endothelial cells, but inhibition studies suggest that such autocrine stimulation accounts for at most only 40% of observed tissue factor activity.

IL-1α production by endothelial cells increased early in the course of *R. rickettsii* infection. While uninfected endothelial cells contained no detectable IL-1α mRNA by RT-PCR, infection resulted in amplification products as early as 4 h later, indicating a rapid increase in the steady-state level of IL-1α mRNA. Since cultured endothelial cells possess a type I IL-1 receptor (29), occupation of which by IL-1α or IL-1β results in the expression of tissue factor (4, 44), a biological assay for the presence of IL-1 in cell lysate was developed, with tissue factor expression in target endothelial cells as an endpoint. Lysates of endothelial cells after only 6 h of infection indeed resulted in tissue factor expression in target cells that was completely inhibitable with neutralizing antibodies against IL-1α but not against IL-1β (Fig. 3B). A clear-cut increase in the level of IL-1α antigen was detected in cell lysates only after 18 h, and an increase in the level of IL-1β antigen was not detected in cell lysate or culture medium at any time point. Statistically significant increases in IL-1α levels were not detected by ELISA in medium containing control or infected cells at any time point, indicating that IL-1α antigen was mainly associated with the cells.

Other studies have demonstrated cell-associated IL-1 following stimulation of monocytes (32), endothelial cells (24), B cells (14, 25), macrophages (23, 27), and dendritic cells (35). IL-1α is the predominant cell-associated form (3, 7, 26), and in endothelial cells, this form is mainly an IL-1α precursor (37).

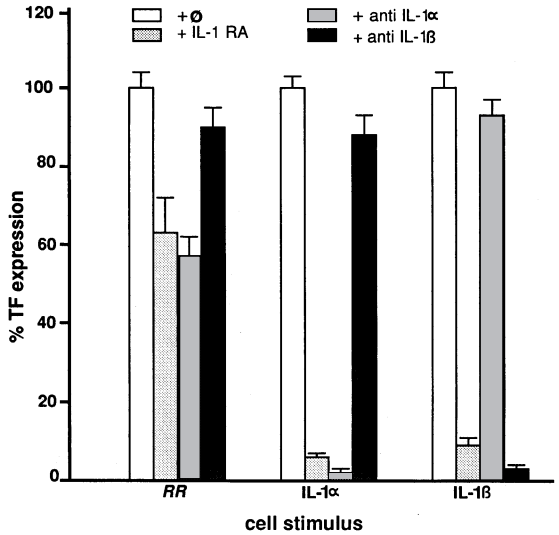

FIG. 4. IL-1 antibody and receptor antagonist inhibition of tissue factor (TF) expression. Endothelial cells were infected or treated with IL-1α (0.5 ng/ml) or IL-1β (0.5 ng/ml) for 6 h, alone (Ø) or in the presence of 100 ng of IL-1 receptor antagonist per ml, neutralizing antibody against IL-1α (1 μg/ml), or antibody against IL-1β (5 μg/ml). After 6 h, cells were lysed and assayed for tissue factor activity. Results (means ± standard errors) are expressed as percent tissue factor activity expressed in infected or IL-1-stimulated endothelial cells cultured in the absence of blocking agents (Ø) (neutralizing antibodies or receptor antagonist [RA]).

This precursor form of IL-1 possesses biological activity, as demonstrated by its ability to bind to the type I IL-1 receptor; however, it is likely anchored to the cell surface via fatty acid acylation (2) or through a lectin-like mechanism (5). The cell-associated IL-1α activity found in *R. rickettsii*-infected endothelial cells may represent both cytosolic and membrane-bound forms, as only a fraction of total cell-associated IL-1α has been shown to be membrane bound (2).

We utilized the neutralizing antibodies against IL-1α and IL-1β as well as the IL-1 receptor antagonist to study potential autocrine stimulation of endothelial cells during infection. Tissue factor expression was chosen as a response, since *R. rickettsii* infection results in rapid and dramatic increases in tissue factor expression (41). Indeed, the presence of these soluble inhibitors, which block the interaction of IL-1 with the type I cell surface receptor, reduced tissue factor expression in the same culture, albeit by only 40%. Since little soluble IL-1 was found in culture medium of infected endothelial cells and since culture medium conditioned by infected cells contains little cell-stimulatory activity (41), this inhibitable IL-1α was likely membrane bound.

Others have demonstrated the involvement of IL-1 in autocrine cell stimulation. E-selectin expression in cytomegalovirus-infected endothelial cells is nearly completely inhibited by the presence of neutralizing antibodies against IL-1 (40). Endotoxin-induced endothelial cell production of nitric oxide is mediated in an autocrine fashion by endogenously produced IL-1α and tumor necrosis factor alpha (6). IL-1 is also a negative autocrine regulator of endothelial cell growth, likely due to down-regulation of the fibroblast growth factor receptor (8). Blocking endogenously produced IL-1 with neutralizing antibodies relieves growth inhibition, and culture of endothelial cells with an IL-1α antisense oligomer extends the in vitro lifespan (15).

Blocking the interaction of endogenous IL-1 with its cell-surface receptor resulted in only partial inhibition of tissue factor expression (Fig. 4), a result which has several possible explanations. Most likely, the intracellular presence of *R. rickettsii* directly stimulated tissue factor expression, which is merely enhanced by IL-1α. It is also possible that endogenous IL-1 plays a larger role in the induction of tissue factor expression but that the ability of antibodies or receptor antagonist to block activity is reduced because of a tight membrane association of IL-1. For example, membrane-bound IL-1 is highly active and higher concentrations of IL-1 receptor antagonist are needed to block cell stimulation than are needed to block the action of soluble IL-1 (21). Concentrations of IL-1 receptor antagonist fivefold higher (500 ng/ml) than those required to inhibit stimulation by soluble IL-1, however, did not result in any further inhibition of tissue factor expression in *R. rickettsii*-infected endothelial cells (data not shown). IL-1α can also stimulate cells through an intracellular signalling pathway, requiring transport to the nucleus via its nuclear localization sequence, and nuclear localization of IL-1α correlates with its ability to impair cell growth and IL-1α-inducible gene expression (30). This pathway, if operative in stimulating tissue factor expression in response to infection, would be inaccessible to soluble inhibitors.

In summary, *R. rickettsii* infection of cultured endothelial cells results in increased expression of IL-1α, which participates in autocrine stimulation, resulting in tissue factor expression. Thus, endothelial cell production of IL-1 may play a key role in the localized procoagulant and inflammatory responses which occur in the course of rickettsial disease.

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