Effect of IC14, an anti-CD14 antibody, on plasma and cell-associated chemokines during human endotoxemia

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ABSTRACT. To determine the role of CD14 in lipopolysaccharide (LPS)-induced release of chemokines, 16 humans were injected with LPS (4 ng/kg) preceded (– 2 h) by intravenous IC14, an anti-human CD14 monoclonal antibody, or placebo. LPS elicited increases in interleukin (IL)-8 concentrations in plasma and in lysates of red blood cell (RBC), polymorphonuclear cell and mononuclear cell fractions, which were all reduced by IC14. LPS also induced rises in the plasma and RBC levels of monocyte chemoattractant protein (MCP)-1, which were diminished by IC14. Macrophage inflammatory protein (MIP)-1α and MIP-1β, chemokines that in contrast to IL-8 and MCP-1 can not bind to the Duffy antigen receptor for chemokines on RBCs, were only detected in plasma. IC14 attenuated the LPS-induced release of MIP-1β, but not of MIP-1α. IL-8 and MCP-1, but not MIP-1α and MIP-1β, circulate in RBC-associated form during endotoxemia. LPS-induced chemokine release is, in part, mediated by an interaction with CD14.

Keywords: interleukin-8/erythrocytes/lipopolysaccharide/monocyte chemoattractant protein-1

INTRODUCTION

Chemokines are a family of small proteins that are potent attractants and activators of leukocytes [1, 2]. Based on their structure, chemokines have been divided into several subfamilies, each targeting a distinct group of leukocytes. CXC- and CC-chemokines were the first identified chemokine subfamilies, primarily acting on granulocytes and mononuclear cells, respectively. Most receptors for chemokines bind more than one chemokine. However, CXC receptors only interact with CXC chemokines and CC receptors only bind CC chemokines. The Duffy antigen receptor for chemokines (DARC) represents an exception to this ligand-receptor restriction. DARC is present on erythrocytes and endothelial cells and was initially characterized as a receptor for *Plasmodium vivax* [3]. DARC is also a promiscuous chemokine receptor, binding both CXC and CC chemokines, and it is thought to function as a sink receptor for chemokines present in the circulation [4].

A number of investigations have indicated that in conditions of systemic inflammation, interleukin (IL)-8 circulates in relatively high concentrations in erythrocyte-bound form. Indeed, elevated IL-8 levels have been reported in the red blood cell (RBC) fraction of peripheral blood obtained from patients with sepsis [5], and after administration of IL-1 or IL-2 [6, 7]. In addition, IL-8 could also be recovered from peripheral blood mononuclear cell (PBMC) and polymorphonuclear cell (PMN) fractions from patients with sepsis [5, 8]. These studies have suggested that measurement of cell-associated chemokines together with their plasma levels provides more accurate information on the extent of chemokine production *in vivo*. We recently determined the kinetics of the appearance of IL-8 and other chemokines in peripheral blood cell fractions after intravenous injection of low dose endotoxin (lipopolysaccharide, LPS) in healthy humans [9]. We found that while high concentrations of IL-8 were found in RBCs, PBMCs and PMNs, two other DARC-binding chemokines, monocyte chemoattractant protein (MCP)-1 and growth-related oncogene (GRO) α were present primarily in the RBC fraction. Concentrations of all these chemokines were higher in association with blood cell fractions than their levels measured in plasma.

The cell surface receptor CD14 plays an important role in the recognition of LPS by monocytes, macrophages and granulocytes. After binding LPS, CD14 signals through toll-like receptor 4 leading to cellular LPS responses such as release of pro-inflammatory cytokines and lipid mediators [10]. Knowledge of the role of CD14 in LPS-induced chemokine production *in vivo* is limited. Therefore, in the present study we sought to determine the effect of IC14, an anti-human CD14 monoclonal antibody, on plasma- and cell-associated chemokines in healthy humans injected with a single dose of LPS.

PATIENTS AND METHODS

Study design

The present investigation was performed concurrently with a study examining the effect of IC14 on LPS-induced

cytokine release, granulocyte responses, endothelial cell activation and acute phase protein release, of which the results have been reported elsewhere [11]. The study was approved by the ethics and research committees of the Academic Medical Center, and written informed consent was obtained from all study participants.

Sixteen healthy male volunteers (mean ± SE: 24 ± 1 years) were enrolled in this double-blind, randomized placebo-controlled trial. Medical history, physical examination, routine laboratory examination, and electrocardiogram were all normal. Tests for HIV-infection, hepatitis B and C were negative. The participants did not smoke, had not used any medication, had not had any febrile illness in the month preceding the study and had never received monoclonal antibody therapy before. The subjects fasted overnight before LPS administration. On the study day, two intravenous canulas were inserted, one for LPS administration and blood collection, the other for infusion of IC14 or placebo. Eight of the volunteers received IC14, and eight were given placebo. All participants were challenged with LPS one hour after the end of the IC14 or placebo infusion.

The study drug IC14 was supplied by ICOS Corporation, Bothell, WA, USA. A dose of 1 mg/kg in a solution of 150 ml 0.9% NaCl was administered intravenously over 1 hour through a 0.22 m m low protein binding filter. The placebo solution consisted of the dilution fluid and was administered in an identical manner. The *Escherichia coli* endotoxin preparation used in this study, lot G (UPS, Rockville, MD, USA) was administered intravenously over 1 minute at a dose of 4 ng/kg, 2 hours after the initiation of the IC14 or placebo infusion. The study was performed in a special research unit under the continuous supervison of physicians, with emergency and resuscitation equipment immediately available. Blood pressure, pulse rate, oral temperature and symptoms were assessed every 30 minutes during the first 4 hours after LPS challenge.

Blood was drawn from ante-cubital veins by separate venipunctures before infusion of IC14 or placebo (– 2 h), directly before LPS administration (0 h) and 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10 and 24 hours after LPS. EDTA plasma for chemokine measurements was obtained by centrifugation at 1500 g for 20 min.

Blood cell fractions

Bood cell fractions were harvested from peripheral blood drawn at -2, 3 and 6 h using methods described previously [9]. In brief, RBCs, PBMCs and PMNs were isolated from heparinized blood layered on an equal volume of Polymorphprep (Nycomed Pharma AS; Oslo, Norway), and centrifuged at 500 g for 30 minutes at 20°C. The harvested PBMC and PMN fractions were diluted 1:2 in 0.5 N RPMI 1640 (Bio Wittaker, Verviers, Belgium), in order to restore normal osmolality and were spun at 400 g for 10 minutes at 20°C. Remaining erythrocytes were lysed using ice-cold isotonic NH_4Cl solution (155 mM NH_4Cl, 10mM $KHCO_3$, 0.1 mM EDTA, pH 7.4) for 10 minutes. The cell fractions were spun again at 400 g for 10 minutes at 4°C, and the pellet was resuspended in 1N RPMI containing 5% normal human serum (Bio Wittaker) to the original blood volume. Purity of the cell fractions was checked using a 0.1% eosin stain and was found to be above 98%. All three cell fractions were spun at 400 g for 10 min at 4°C. Thirty ml of ice-cold isotonic NH_4Cl solution (as described above) was added to the pellet of RBC fraction. It was incubated on ice for 10 min; 300 m l lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1% Triton X-100, and Pepstatin A, Leupeptin and Aprotinin (all 20 ng/ml; pH 7.4) were added to the pellet of PBMC and PMN fraction, and incubated on ice for 15 min. Lysed fractions were resuspended in 1 N RPMI (RBC to 40 ml and PBMC and PMN to 1 ml). In separate experiments, both lysing buffers were shown not to influence ELISA measurements.

Assays

Chemokine concentrations were measured by ELISA. IL-8 was measured according to the instructions of the manufacturer (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, the Netherlands). MCP-1 was measured using purified monoclonal mouse anti-human MCP-1 (2 μg/ml; PharMingen, San Diego, CA, USA) as coating antibody, biotinylated rabbit anti-human MCP-1 (1 μg/ml; PharMingen) as detecting antibody, and human recombinant MCP-1 (PharMingen) as standard. For determination of macrophage inflammatory protein (MIP)-1α and MIP-1β levels, purified monoclonal mouse anti-human MIP-1α and anti-human MIP-1β were used as coating antibodies (4 μg/ml; R&D Systems, Abingdon, United Kingdom), biotinylated affinity purified goat IgG anti-human MIP-1α and MIP-1β (20 ng/ml; R&D Systems) as detecting antibodies, and recombinant human MIP-1α and MIP-1β as standards (R&D Systems). Detection limits were 1.7 pg/ml (IL-8), 8.2 pg/ml (MCP-1) and 15.6 pg/ml (MIP-1α and MIP-1β).

Leukocyte counts and differentials were assessed by a Stekker analyzer (counter STKS, Coulter counter, Bedfordshire, United Kingdom).

Statistical analysis

Values are given as mean ± SEM. Changes of parameters in time were tested using one-way ANOVA. Differences between IC14 and placebo treatments were tested by analysis of variance (ANOVA) for repeated measures. a for all tests was set at 0.05.

RESULTS

Effect of IC14 on blood cell counts

LPS injection was associated with profound changes in the different cell populations present in peripheral blood, characterized by a neutrophilic leukocytosis, lymphocytopenia and monocytopenia; the number of RBCs in peripheral blood did not change after LPS administration (Table 1). At certain time points, at which blood was obtained for preparation of cell fractions, leukocyte counts and differentials differed between the placebo and IC14 groups (Table). In particular, IC14 treated subjects had lower PMN counts at 6 h post-LPS, and higher lymphocyte counts at 3 and 6 h post-LPS than subjects who received placebo. Considering that IC14 influenced the number of PMNs and PBMCs present in peripheral blood, the concentrations of cell-associated chemokines are expressed per 10^6 cells to allow direct comparison of the placebo and IC14 groups.

Table 1
Cell fractions in peripheral blood before and after LPS injection

Time (hours)	–2		3		6	
	Placebo	IC14	Placebo	IC14	Placebo	IC14
RBC (10^{12}/L)	5.0 ± 0.2	4.8 ± 0.1	4.8 ± 0.2	4.6 ± 0.1	4.7 ± 0.1	4.5 ± 0.1
WBC (× 10^9/L)	6.1 ± 0.4	7.2 ± 1.0	7.2 ± 0.8	10.0 ± 0.7	15.3 ± 1.3	11.0 ± 0.8*
Neutrophils (× 10^9/L)	2.89 ± 0.24	3.68 ± 0.61	6.68 ± 0.76	7.62 ± 1.20	14.60 ± 1.20	9.79 ± 0.69*
Monocytes (× 10^9/L)	0.60 ± 0.05	0.45 ± 0.08	0.04 ± 0.01	0.06 ± 0.02	0.36 ± 0.07	0.45 ± 0.07*
Lymphocytes (× 10^9/L)	2.35 ± 0.24	2.30 ± 0.39	0.46 ± 0.07	0.91 ± 0.22*	0.28 ± 0.04	0.66 ± 0.01*

Data are means ± SE of eight healthy subjects per treatment group. * $P < 0.05$ versus placebo.

Effect of IC14 on DARC-binding chemokines in plasma and blood cell fractions

LPS injection was associated with a transient rise in plasma IL-8 levels peaking at 1.8 ± 0.4 ng/ml after 2 hours ($P < 0.001$). The increase in plasma IL-8 was accompanied by transient increases in cell-associated IL-8 recovered from all three cell fractions. RBC- and PBMC-associated IL-8 peaked after 3 h (0.07 ± 0.02 and 1060.52 ± 405.02 pg/10^6 cells, respectively; both $P < 0.01$), while PMN-associated IL-8 reached maximum concentrations after 6 h (218.00 ± 24.17 pg/10^6 cells ($P < 0.01$) (Figure 1). IC14 strongly attenuated the rise in plasma IL-8 (peak: 0.3 ± 0.1 ng/ml; $P < 0.001$) (Figure 1). In addition, IC14 decreased LPS-induced rises in IL-8 associated with RBCs (peak: 0.03 ± 0.01 pg/10^6 cells; $P < 0.005$), PBMCs (peak: 84.26 ± 25.36 pg/10^6 cells; $P < 0.001$) and PMNs (peak: 60.43 ± 30.69 pg/10^6 cells; $P < 0.05$) (Figure 1).

LPS induced a transient rise in plasma MCP-1 levels peaking at 92.54 ± 16.22 ng/ml after 4 hours ($P < 0.001$). The rise in plasma MCP-1 was accompanied by a transient increase in RBC-associated MCP-1, peaking after 3 hours (27.85 ± 9.22 pg/10^6 cells, $P < 0.01$) (Figure 2). MCP-1 concentrations measured in PMN and PBMC fractions were very low, both before and after LPS injection (data not shown). Treatment with IC14 attenuated both the LPS-induced increase in plasma levels of MCP-1 to 28.68 ± 12.02 ng/ml ($P < 0.001$), and the LPS-induced rise in RBC-associated MCP-1 (10.22 ± 3.39 pg/10^6 cells; $P < 0.05$) (Figure 2).

Effect of IC14 on plasma and cell-associated chemokines that do not bind DARC

LPS injection was associated with transient rises in the plasma concentrations of MIP-1α (0.46 ± 0.08 ng/ml) and MIP-1β (24.39 ± 3.03 ng/ml), peaking at 2 hours. IC14 attenuated the LPS-induced increase in the plasma levels of MIP-1β (peak: 9.22 ± 1.63 ng/ml, $P < 0.001$), but did not significantly affect MIP-1α release (peak: 0.35 ± 0.08 ng/ml) (Figure 3). MIP-1α and β were undetectable in RBCs and PMNs, and were low in PBMCs prior to LPS administration, and did not increase in any of the cell fractions thereafter in either treatment group (data not shown).

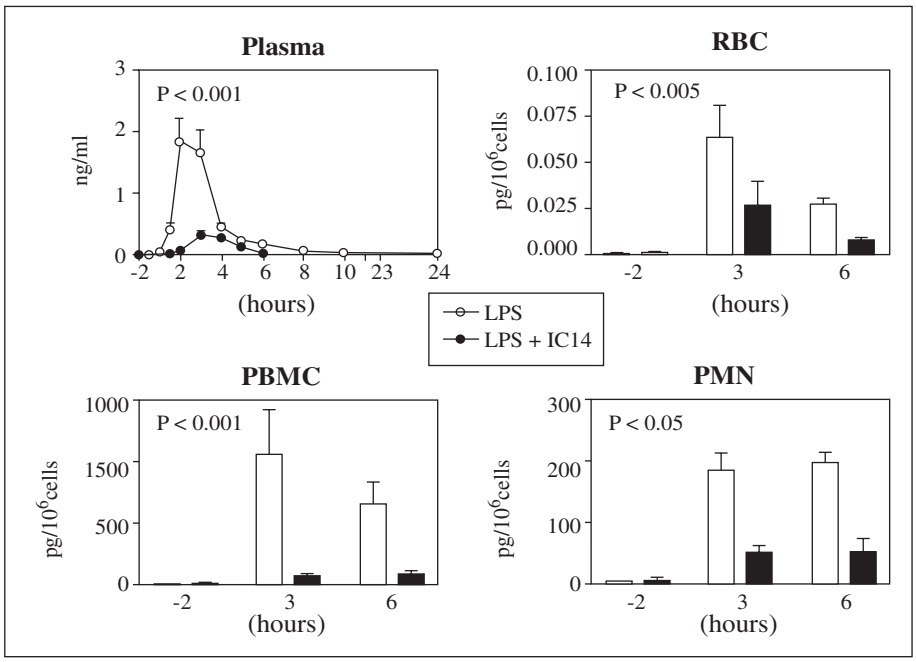

Figure 1
Anti-CD14 treatment inhibits LPS-induced increases in plasma and cell-associated IL-8 concentrations. Mean ± SEM IL-8 concentrations in plasma and in isolated RBCs, PBMCs and PMNs after LPS administration (4 ng/kg) to healthy humans. Placebo (white) or IC14 (1 mg/kg i.v., black) was given 2 hours prior to LPS challenge as a 1-hour infusion. P-values indicate differences between treatment groups.

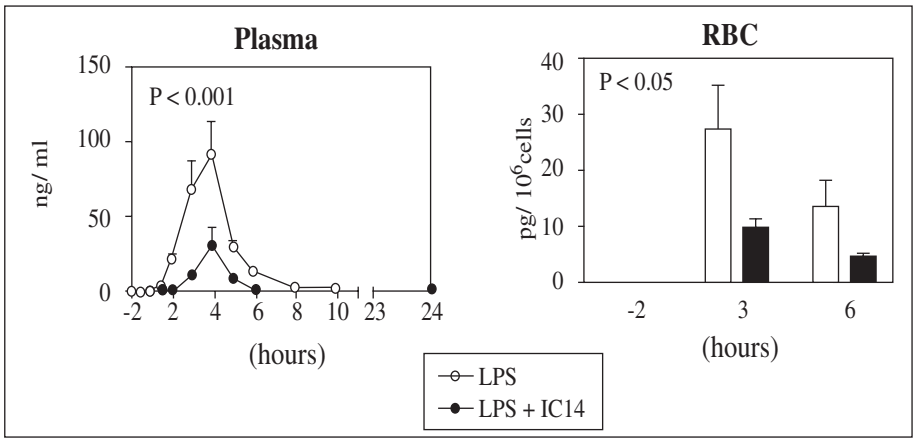

Figure 2
Anti-CD14 treatment inhibits LPS-induced increases in plasma and RBC-associated MCP-1 concentrations. Mean ± SEM MCP-1 concentrations in plasma and in isolated RBCs after LPS administration (4 ng/kg) to healthy humans. Placebo (white) or IC14 (1 mg/kg i.v., black) was given 2 hours prior to LPS challenge as a 1-hour infusion. P-values indicate differences between treatment groups.

DISCUSSION

Inflammation and infection are accompanied by enhanced production of chemokines. Increased release of IL-8 in these conditions is reflected not only by elevated concentrations of this prototypic CXC chemokine in plasma, but also in cell fractions from peripheral blood [5-8]. The association of IL-8 with RBCs has been linked to its capacity to bind DARC, a promiscuous receptor expressed at the surface of RBCs. Intravenous administration of LPS induced increases in the plasma and cell-associated concentrations of not only IL-8 but also of other DARC-binding chemokines [9]. In the present study we examined the role of CD14 in this LPS effect in humans *in vivo*. Firstly we confirmed that chemokines known to interact with DARC (IL-8 and MCP-1) circulate in increased quantities in a cell-associated form during endotoxemia, whereas chemokines that do not bind DARC (MIP-1α and MIP-1β) could only be detected in plasma. In addition, we found that inhibition of the LPS-CD14 interaction by infusion of an anti-human CD14 mAb attenuated LPS-induced increases in the plasma and cell-associated concentrations of IL-8 and MCP-1, as well as the secretion of MIP-1β into plasma, but did not influence MIP-1α release.

CD14 is a pattern recognition receptor that recognizes a number of bacterial antigens including LPS [12]. After the interaction between CD14 and LPS, cells become activated via toll-like receptor 4 [10]. The significance of CD14 in LPS-mediated effects *in vivo* is illustrated by findings that CD14 gene-deficient mice are highly resistant to LPS toxicity [13] and that anti-CD14 antibody treatment protected monkeys and rabbits against the lethal consequences of gram-negative bacteremia and endotoxemia [14, 15]. Recently, we found that IC14 markedly reduced a number of inflammatory responses to intravenous administration of LPS in normal humans, including the release of proinflammatory cytokines and granulocyte activation [11]. Some LPS effects, however, were not inhibited by IC14 or to a much lesser extent. These responses included the release of soluble tumor necrosis

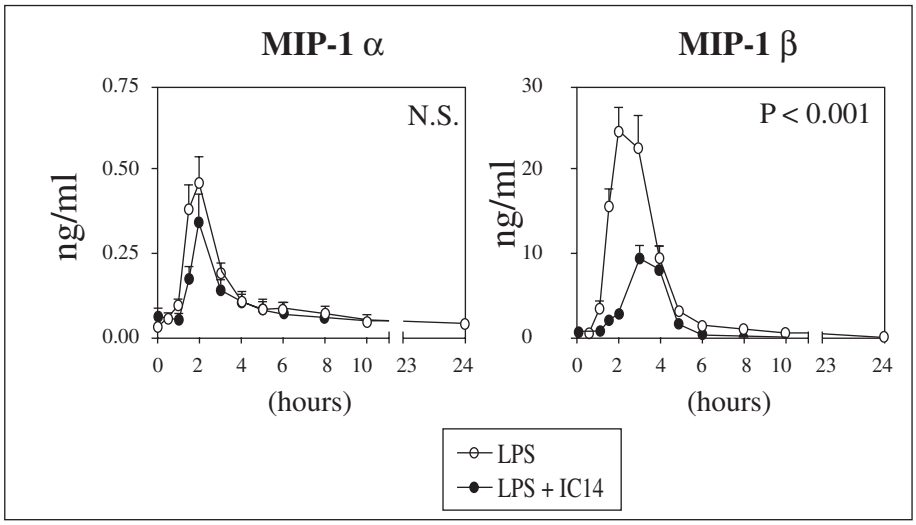

Figure 3
Anti-CD14 treatment inhibits LPS-induced release of MIP-1β, but not of MIP-1α. Mean ± SEM MIP-1α and MIP-1β concentrations in plasma, after LPS administration (4 ng/kg) to healthy humans. Placebo (white) or IC14 (1 mg/kg i.v., black) was given 2 hours prior to LPS challenge as a 1-hour infusion. P-values indicate differences between treatment groups. N.S. = nonsignificant.

factor (TNF) receptors, acute phase protein release and endothelial cell activation.

We also here report that the release of chemokines is not uniformly inhibited by IC14. In particular, whereas IC14 significantly reduced LPS-induced IL-8, MCP-1 and MIP-1β release, the rise in MIP-1α concentrations remained unaltered. Although our study does not elucidate the mechanisms underlying the different effects of IC14 on chemokine release, several possible explanations are conceivable. Firstly, some of the LPS effects measured in this investigation could, in part, be mediated via a CD14, independent pathway. In line with this hypothesis are findings in CD14 deficient mice in which some LPS responses, such as the acute phase protein response, were completely intact [16]. Alternatively, since saturation of surface CD14 on circulating monocytes and granulocytes by IC14 was not complete (albeit > 90%) [11], very low doses of LPS might have been sufficient to induce MIP-1 α release. Finally, different cell types with different sensitivities for CD14 blockade and/or different roles for CD14 in the induction of a cellular response could be involved in the production of different chemokines during endotoxemia.

Cell-associated IL-8 and MCP-1 concentrations were expressed per 10^6 cells. We chose this approach since IC14 influenced PMN and PBMC counts after LPS administration, which would have hampered comparison of cell-associated chemokine levels expressed per ml blood in IC14 and placebo-treated subjects. However, when cell-associated IL-8 and MCP-1 concentrations were adjusted to the numbers of RBCs, PMNs and PBMCs present in one ml of blood, the differences between the IC14 and placebo groups remained qualitatively similar (data not shown). Considering that chemokines that are not able to bind DARC cannot be detected in the RBC fraction after LPS injection [[9] and the present investigation], it is highly likely that the association of IL-8 (and MCP-1) with RBCs is mediated by a specific interaction with DARC. It is less clear how IL-8 becomes associated with PMN and PBMC fractions. Patients with sepsis demonstrated elevated IL-8 mRNA levels in peripheral blood leukocytes [17], suggesting that at least some of the PMN-associated IL-8 is produced by these cells. An alternative explanation could be that IL-8 produced by other cell types binds IL-8 receptors on PMNs and PBMCs, a possibility that is supported by the observation that recombinant IL-8 rapidly associates with RBCs, PMNs and PBMCs after addition to whole blood [5].

The association of IL-8 with RBCs has been proposed to serve two possible goals. First, it may prevent accumulation of this chemokine in blood in a soluble active form, or second, it may provide a reservoir, preventing rapid clearance from the circulation and from which IL-8 can be released [4]. It is conceivable that such mechanisms also hold true for other DARC-binding chemokines such as MCP-1. Nonetheless, it seems clear that measurement of DARC-binding chemokines in the RBC fractions may provide more relevant information on the extent of chemokine production than the mere measurement of plasma concentrations. LPS injection into healthy humans elicits profound rises in plasma and RBC-, PMN- and PBMC-associated levels of IL-8, and in plasma and RBC-associated concentrations of MCP-1, which, at least in part, is mediated by an interaction between LPS and CD14.

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