Endotoxin-Induced Desensitization of Mouse Macrophages Is Mediated in Part by Nitric Oxide Production

HASSAN FAHMI,¹ DANIEL CHARON,² MICHELLE MONDANGE,² AND RICHARD CHABY^{3*}

Laboratory of Immunology, Faculty of Science, Université Sidi Mohamed Ben Abdellah, Fès, Morocco,¹ and Equipe "Endotoxines," Unité de Recherche Associée 1116 du Centre National de la Recherche Scientifique, and Centre d'Etudes Pharmaceutiques, Université de Paris-Sud, 92290 Châtenay-Malabry,² and Université de Paris-Sud, 91405 Orsay,³ France

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Refractoriness (tolerance) to endotoxin effects, such as induction of tumor necrosis factor alpha (TNF- α) secretion, can be elicited in vitro in macrophages by preexposure of cells to endotoxin (lipopolysaccharide [LPS]) itself. The aim of this study was to determine whether this phenomenon is due to negative feedback mediated by the free radical nitric oxide (NO) produced by cells when they are activated by LPS. Among several efficient inhibitors of NO production, *N*^G-monomethyl-L-arginine did not induce concomitant inhibition of TNF- α secretion. Mouse macrophages that were exposed to LPS in the presence of *N*^G-monomethyl-L-arginine partially maintained the ability to secrete TNF- α in response to a second LPS stimulation, compared with cells preexposed to LPS alone, thus suggesting that NO is involved in part in LPS-induced desensitization of cells. Furthermore, direct exposure of cells to the NO-generating compounds sodium nitroprusside and *S*-nitroso-*N*-acetylpenicillamine mimicked LPS-induced desensitization. However, low concentrations of a synthetic lipid (lipid M4) that is structurally analogous to the reducing end of the lipid A moiety of LPS induced desensitization of mouse macrophages without concomitant production of NO. Taken together, these data suggest that although NO actually takes part in LPS-induced desensitization of mouse macrophages, additional and yet unknown mechanisms must also exist.

There is much suggestive evidence that endotoxin (lipopolysaccharide [LPS]) is the main initiator of various pathophysiological disorders, including septic shock, which follow gram-negative bacteremia (39). Many of these effects are mediated by various cytokines that are secreted by mononuclear phagocytes after LPS-induced activation (34). Under appropriate conditions, LPS pretreatment in vitro can also prime macrophages for enhanced production of reactive oxygen intermediates (37) as well as tumor necrosis factor alpha (TNF- α) and nitric oxide (NO) (53). However, macrophages can also be deactivated by small amounts of LPS. It has been shown, for instance, that LPS is able to suppress antibody-dependent cellular cytotoxicity (28), scavenger receptor activity (48), synthesis of apolipoprotein E (51), and various gamma interferon (IFN- γ)-induced effects, including surface expression of FcR (4) and class II major histocompatibility complex molecules (20) and enhanced H₂O₂ production (13). Small doses of LPS also reduce the sensitivity of an animal to subsequent challenge with larger doses of LPS itself, a phenomenon known as endotoxin tolerance (23).

In vivo, late-phase tolerance is associated with the production of anti-LPS antibodies (29), whereas early-phase tolerance may be due to endogenous mediators such as glucocorticoids (43), cytokines (interleukin 1 α and TNF- α) (15, 50), and growth factors (transforming growth factor β) (46). Refractoriness to endotoxin effects can also be induced in vitro with peritoneal macrophages and macrophage cell lines (49). This desensitization of cells, elicited by LPS itself, can also be partially mimicked by other agents (interleukin 1, interleukin 10, and transforming growth factor β) (5, 7). However, these agents cannot induce a full LPS-tolerant state, suggesting the

existence of other mechanisms. In previous studies with mouse peritoneal macrophages, we have shown that this tolerant state is probably the result of an autocrine mechanism (17) and is not due to downregulation of LPS-binding sites (16). This has also been observed with a human cell line, in which LPS tolerance was established at maximum expression of cell surface CD14 (30). Therefore, the underlying mechanism for LPS-induced desensitization of macrophages remains unclear.

The role of NO for several characteristics of septic shock remains controversial. Some investigators have observed that myocardial dysfunction (21) and critical hypotension that is resistant to treatment with vasopressors (12) appear to be mediated by NO, whereas others have suggested that hypotension (10) and toxic effects (38) do not correlate with NO production. The massive production of NO observed during endotoxemia is catalyzed by an inducible and calcium-independent form of nitric oxide synthase (iNOS) which is expressed in macrophages and several other cell types (vascular endothelial cells and smooth muscle cells) in response to endotoxin (36). NO that is produced by activated cells is an important intracellular messenger, and it can either activate or inhibit the functions of different cell types. For instance, it has recently been reported that NO increases LPS-dependent production of TNF- α in human neutrophils (47). On the other hand, macrophages, one of the prototypical cell types that can be induced to express iNOS, are themselves targets of NO; in these cells, it induces reduced lifetimes in culture (2, 3), depressed physiological functions (superoxide production and phagocytosis) (1), and even inhibited NOS activity (24). These observations led us to investigate whether NO can induce in mouse peritoneal macrophages an LPS-unresponsive state and therefore be involved in LPS-induced desensitization.

* Corresponding author. Mailing address: Equipe "Endotoxines," URA-1116 du C.N.R.S., Bâtiment 432, Université de Paris-Sud, 91405 Orsay, France. Phone: (33-1) 69417191. Fax: (33-1) 69853715.

FIG. 1. LPS-induced desensitization of macrophages. Thioglycolate-elicited mouse peritoneal macrophages from Swiss mice (10^6 cells per well) were incubated (18 h, 37°C) without or with 0.1, 1, 10, or 100 ng of *B. pertussis* LPS per ml in CM. Cells were washed three times (each incubation, 40 min at 37°C in 0.5 ml CM) and exposed for 18 h at 37°C to various concentrations (50 to 5,000 ng/ml) of *S. typhimurium* LPS. Results are expressed in units of TNF- α produced after the second treatment and are the means $\pm$ SD of three values interpolated from serial dilutions of cell culture supernatants.

MATERIALS AND METHODS

Media and reagents. The culture medium (CM) was RPMI 1640 (Gibco, Grand Island, N.Y.) that contained 2 mM L-glutamine, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 2-mercaptoethanol (5×10^{-5} M). Sodium nitroprusside (SNP) was from Fluka Chemie AG (Buchs, Switzerland). Nicotinamide (NAD) was from Lancaster Synthesis Ltd. (Strasbourg, France). Diphenyleneiodonium (DPI) and N^G -monomethyl-L-arginine (NMMA) were from Biomol Research Laboratories (Plymouth Meeting, Pa.). *N*-Acetyl-D-penicillamine, heparin, actinomycin D, crystal violet, and neutral red were purchased from Sigma Chemical Co. (St. Louis, Mo.). A solution of trypsin in EDTA was

purchased from Biochrom KG (Berlin, Germany). LPSs from *Salmonella typhimurium* LT2 and *Bordetella pertussis* (phase 1 vaccine strain 1414) were prepared in our laboratory by the phenol-water extraction procedure, as described previously (33). Thioglycolate broth and minimal essential medium (MEM) were from Diagnostic Pasteur (Ville d'Avray, France), and fetal calf serum was from Boehringer (Mannheim, Germany).

Cell culture. The murine fibroblast cell line L929- α was from M. Parant (Paris, France). Suspensions of L929- α cells were prepared by treatment (1 min) of confluent cultures (75 cm^2) with trypsin (2 ml; 0.05% in 0.02% EDTA) and subsequent addition of heat-inactivated horse serum (1 ml) to stop reactions.

Swiss mice (4 to 8 weeks old; from R. Janvier, Le Genest Saint-Isle, France) were used as sources of peritoneal macrophages. Peritoneal exudates were harvested 5 days after intraperitoneal injection of 1.7 ml of thioglycolate broth by peritoneal washes with MEM that contained 10 U of sodium heparinate per ml. Suspensions of peritoneal exudate cells (10^6 neutral red-positive cells per well in RPMI 1640 buffered with 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]) were incubated (2 h, 37°C) on plates with 24 wells. Non-adherent cells were removed by four washings with MEM (500 μl per well). The adherent cell population contained 92 to 96% neutral red-positive macrophages.

The eventual loss of cell integrity was measured by lactic dehydrogenase release. This assay was performed with a diagnostic kit, as recommended by the supplier (Sigma Chemical Co.).

Synthetic compounds. Glucosamine-derived glycolipid M4 was synthesized by D. Charon and M. Mondange (9) and complexed with bovine serum albumin in the presence of triethylamine, as previously described (44). *S*-Nitroso-*N*-acetylpenicillamine (SNAP) was synthesized as described by Field et al. (19). A solution of sodium nitrite (10 ml; 69 mg/ml) was added to 40 ml of methanol-1 M HCl- H_2SO_4 (1:1:0.1 [vol/vol]) that contained 956 mg of *N*-acetyl-D-penicillamine over a period of 20 min with stirring. After evaporation of methanol under vacuum, the resulting green crystals were recovered by filtration.

Nitrite determination. The concentrations of NO_2^- in culture supernatants were used as an indicator of NO generated by NOS activity and were measured by the Griess reaction, with sodium nitrite as the standard (14). Briefly, a solution of 1% sulfanilamide-0.1% naphthylethylenediamine dihydrochloride in 2% H_3PO_4 was added to 100 μl in triplicate wells of a 96-well plate. A_{550} was measured by using a Dynatech plate reader.

Estimation of TNF- α secretion. Macrophage culture supernatants were recovered, centrifuged (10 min, $900 \times g$), and rapidly assayed (without storage) for TNF- α activity by determination of the reciprocal of the dilution that killed 50% of actinomycin D-sensitized L929- α cells. Estimations of target cell viability were performed by staining viable cells with crystal violet as described previously (32).

Estimation of macrophage desensitization. The percentage of inhibition of endotoxin-induced production of TNF- α by macrophages desensitized by preliminary treatment was computed by the following formula:

$$\text{Percent desensitization} = [1 - (C_y^x - C_0^x)/(C_y^0 - C_0^0)] \times 100, \quad (1)$$

where C_y^x is the measured mean of the amount of TNF- α produced in response to y μg of *S. typhimurium* LPS per ml by macrophages which had been pretreated with x ng of desensitizing agent per ml. A computer program written in our

FIG. 2. LPS-induced production of NO in unstimulated and LPS-pretreated macrophages. (A) Thioglycolate-elicited peritoneal macrophages from Swiss mice (10^6 cells per well) were first incubated (18 h, 37°C) with 0 to 100 ng of *B. pertussis* LPS per ml, and direct NO_2^- responses were determined. (B) Then cells were washed, and the amounts of NO_2^- produced during a second incubation (18 h, 37°C) in the absence or presence of *S. typhimurium* LPS (50 ng/ml) were determined. Data are the means $\pm$ SD of two determinations.

laboratory (and successfully tested on artificial sets of variables) was used to compute the standard deviations (SD) of the values obtained by this formula.

RESULTS

LPS-induced desensitization of macrophages for TNF- α response to LPS. In preliminary experiments, we established the optimal conditions for in vitro induction and detection of an LPS-hyporesponsive state in mouse macrophages. The multiple LPS-binding sites identified on the macrophage surface (52), which interact with different lipid A substructures (44) and may need additional presenting molecules (45), can probably give rise to intricate mechanisms of desensitization. We reasoned that the use of a simplified system would help to unravel such intricacies. Therefore, we avoided the use of IFN- γ -primed macrophages as well as serum-supplemented media that contained LPS-binding protein (45). Furthermore, in order to restrict analysis to desensitization of receptors that cross-react with a minimal lipid A substructure, the LPS used to elicit an endotoxin-tolerant state (*B. pertussis* LPS) was structurally different (reduced number of fatty acids) (6) from that used to trigger production of TNF- α (*S. typhimurium* LPS).

Therefore, thioglycolate-elicited mouse peritoneal macrophages were exposed for 18 h to various doses (0 to 100 ng/ml) of a purified preparation of *B. pertussis* LPS in the absence of serum and without IFN- γ priming. After cells were washed several times, we analyzed the ability of cells to produce TNF- α in response to various concentrations of another preparation of LPS (from *S. typhimurium*). The results in Fig. 1 show that in this simplified model, desensitization for the TNF- α response to LPS still occurs. Exposure to 10 ng of *B. pertussis* LPS per ml was sufficient to induce a state of endotoxin tolerance in macrophages, as seen by the reduced responsiveness of cells to 50 ng of *S. typhimurium* LPS per ml.

Persistence of NO production after removal of LPS. To determine whether production of NO by macrophages can influence the responsiveness of cells to further stimulation, we first examined the extent of NO production during and after first exposure to LPS. The results in Fig. 2B show that macrophages which had been washed after first exposure to LPS and reincubated for 18 h in the absence of LPS produced about as much NO (25 to 35 μ M) as they did during the first incubation (Fig. 2A). Moreover, the presence of LPS (50 ng/ml) during the second incubation did not reduce but rather enhanced production of NO during that period. A similar observation of enhanced NO responses upon restimulation with LPS has been also reported by Zhang and Morrison (53). Note that the smallest dose of LPS that was able to trigger NO synthesis (1 to 10 ng/ml) (Fig. 2A) is similar to that which elicited desensitization for LPS-induced TNF- α production (Fig. 1). It was therefore tempting to see if desensitization of macrophages to endotoxin effects can be mediated by NO.

Direct effects of inhibitors of NO production on LPS-induced secretion of TNF- α . The role of NO in macrophage desensitization can be demonstrated by using an inhibitor of NOS activity. It was first necessary, however, to establish that this inhibitor cannot directly block the LPS-induced effect (TNF- α secretion). To select an inhibitor that was suitable for our study, we first examined the influence of three inhibitors, NAD (25), DPI (42), and NMMA (40), on direct, LPS-induced production of NO and TNF- α . At 30 mM, 1 μ M, and 0.2 mM, respectively, all three inhibitors completely blocked LPS-induced production of NO (Fig. 3A). However, the results in Fig. 3B show that DPI and NAD also blocked LPS-induced production of TNF- α . Therefore, these two inhibitors cannot be

FIG. 3. Influence of pharmacological agents on NO production (A) and TNF- α secretion (B). The amounts of NO₂⁻ and TNF- α produced by thioglycolate-elicited mouse peritoneal macrophages were determined after the incubation (18 h, 37°C) of cells in medium alone or in the presence of NMMA (0.2 mM), DPI (1 μ M), or NAD (30 mM). Data are the means $\pm$ SD of two determinations.

used to analyze LPS-induced desensitization for the TNF- α response to endotoxin. On the other hand, the third inhibitor, NMMA, did not inhibit the direct TNF- α response to LPS (Fig. 3B) and was therefore selected for further studies.

Partial inhibition of LPS-induced desensitization by NMMA. Thioglycolate-elicited peritoneal macrophages were exposed to increasing concentrations (0 to 100 ng/ml) of LPS in the presence or absence of NMMA (0.2 mM). The doses of LPS and NMMA used were those demonstrated to be effective for macrophage desensitization (Fig. 1) and inhibition of NO production (Fig. 3A), respectively. Then the magnitude of LPS-induced desensitization was estimated by the reduced ability of cells to secrete TNF- α in response to a second treatment with LPS (5 μ g/ml). The results in Fig. 4 clearly show that macrophage desensitization elicited by first exposure to LPS can be partially overcome by adding NMMA to the incubation medium and suggest that NO may contribute to LPS-induced desensitization. Very similar results were obtained when the experiment was carried out in the presence of serum. In CM supplemented with 10% fetal calf serum, the restorations of TNF- α secretions elicited by NMMA at LPS concentrations of 2 and 10 ng/ml (27% $\pm$ 4% and 33% $\pm$ 4%, respectively) were not significantly different from those observed in the absence of serum (25% $\pm$ 6% and 37% $\pm$ 4%, respectively).

Induction of desensitization by NO donors. To determine if direct exposure of macrophages to NO can mimic the desen-

FIG. 4. Influence of NMMA on LPS-induced desensitization. Thioglycolate-elicited mouse peritoneal macrophages (10^6 cells per well) were preincubated (18 h, 37°C) with 0 to 100 ng of *B. pertussis* LPS per ml in the absence or presence of NMMA (0.2 mM). Cells were washed three times (each incubation, 40 min at 37°C in 0.5 ml CM) and exposed (18 h, 37°C) to *S. typhimurium* LPS (5 $\mu\text{g/ml}$). Results are expressed in units of TNF- α secreted during the last incubation and are the means $\pm$ SD of two determinations.

sitizing effect of LPS, we incubated macrophages (18 h, 37°C) with 10 and 50 μM of the NO-generating compounds SNP and SNAP. The concentrations of NO_2^- generated at that time (6 ± 1 and 16 ± 1 μM with 10 and 50 μM , respectively, of SNP and 4 ± 1 and 10 ± 1 μM with 10 and 50 μM , respectively, of SNAP) were similar to those found when macrophages were exposed to 3 to 5 ng of LPS per ml (Fig. 2A). Then the abilities of SNP-treated cells and SNAP-treated cells to respond to LPS were examined. In both cases, TNF- α responses to LPS were considerably reduced (Fig. 5). A similar reduction in macrophage responsiveness to LPS was not observed with macrophages that had been preincubated in the presence of NO_2^- ; the amounts of TNF- α secreted by macrophages that had been preincubated (18 h, 37°C) in the absence or presence of sodium nitrite (100 μM) or *B. pertussis* LPS (100 ng/ml) and then restimulated with *S. typhimurium* LPS (10 $\mu\text{g/ml}$) were 1,300, 1,450, and 0 U/ml, respectively. Furthermore, we found that exposure of cells to SNP and SNAP did not compromise cell membrane integrity and viability, as assessed by the absence of lactic dehydrogenase release (data not shown).

Stimulation time required for NO-dependent desensitization. Since the results mentioned above strongly suggested that NO contributes to downregulation of TNF- α production and since secretion of NO (as a result of activation of iNOS) is normally undetectable for 8 to 12 h, we expected a shorter exposure to LPS to elicit incomplete and NO-independent desensitization. This is actually what we observed (Fig. 6) when we compared the influence of NMMA on the refractory states to LPS elicited by short (3-h) and prolonged (18-h) preincubations with LPS. The results show that desensitization obtained after a stimulation period of 3 h did not exceed 40 to 50% and was not significantly affected by NMMA (Fig. 6), whereas that obtained by stimulation for 18 h with LPS reached 100% and was clearly (though only partially) reduced in the presence of NMMA (Fig. 6). This clearly shows that NO

FIG. 5. Influence of pretreatment with NO donors on TNF- α responses to LPS. After incubation (18 h, 37°C) in CM that contained 10 or 50 μM SNP (A) or SNAP (B), mouse macrophages were washed three times (each incubation, 40 min at 37°C in 0.5 ml CM) and exposed (18 h, 37°C) to *S. typhimurium* LPS (1 ng/ml to 10 $\mu\text{g/ml}$). Then the amounts of TNF- α secreted were determined. Data are the means $\pm$ SD of two determinations.

is one factor in LPS-induced desensitization, but not the only one.

Desensitization and NO production induced by synthetic lipid M4. In a previous study (16), we observed that compound M4, a synthetic lipid that is structurally related to the reducing moiety of lipid A, can desensitize mouse macrophages but, unlike LPS, does not downmodulate the expression of LPS receptors. This observation prompted us to compare the abilities of LPS and compound M4 to induce NO production and/or cell desensitization. The results in Fig. 7A show that at 0.1 and 1 $\mu\text{g/ml}$, LPS induced NO production, whereas lipid M4 did not. However, with either amount, the latter clearly induced desensitization of macrophages (Fig. 7B). This result shows that NO is not involved in desensitization induced by the lipid A analog and is thus an additional argument to suggest that desensitization elicited by LPS itself is only partially due to NO production.

DISCUSSION

In the first part of this study, we have shown that NO can downregulate LPS-induced production of TNF- α by mouse macrophages, and in the second part, we have shown that this effect alone cannot explain the mechanism of macrophage desensitization by pretreatment with LPS.

As to the first point, the possibility that NO may inhibit macrophage responsiveness to LPS should not be surprising in view of the pleiotropic nature of NO function. Our observation adds to several reports on the immunoregulatory role of NO.

FIG. 6. Influence of NMMA on desensitization obtained after short or prolonged exposure to LPS. Thioglycolate-elicited mouse peritoneal macrophages (10^6 cells per well) were preincubated at 37°C for 3 or 18 h with 0 to 100 ng of *B. pertussis* LPS per ml in the absence or presence of NMMA (0.2 mM). Cells were washed three times (each incubation, 40 min at 37°C in 0.5 ml CM) and exposed (18 h, 37°C) to *S. typhimurium* LPS (5 $\mu\text{g/ml}$). Results are expressed in units of $\text{TNF-}\alpha$ secreted during the last incubation and are the means $\pm$ SD of two determinations.

What is surprising are the differences in regulation that are induced by NO in different cell types. For instance, NO can suppress the function of lymphocytes (26, 35), dendritic cells (27), Langerhans cells (11), and cardiac myocytes (41); on the other hand, it can induce $\text{TNF-}\alpha$ secretion by human peripheral blood mononuclear cells (31) and increase $\text{TNF-}\alpha$ production by human neutrophils (47). In this study, we have shown that NO secreted by LPS-stimulated macrophages has an autocrine effect and can partially inhibit LPS-induced $\text{TNF-}\alpha$ production by these cells. These opposite effects of NO on LPS-mediated $\text{TNF-}\alpha$ production in neutrophils and macrophages deserve closer scrutiny in view of the roles that are played by these two cell types and these two mediators in the pathophysiological manifestations of endotoxemia and septic shock (12, 21, 39). The differential effects of NO on macrophages and neutrophils are not due to the presence of an

NO-sensitive guanylate cyclase in the latter since the ability of NO to increase LPS-induced $\text{TNF-}\alpha$ production in neutrophils is cyclic GMP independent (47). Although the underlying mechanisms for these opposite effects of NO are unknown, our observations may indicate that different cell types have differences in their LPS signaling pathways, which in turn may reflect differences in expression of certain LPS receptors (8). The differences in responses of mouse macrophages and human mononuclear cells to NO are also noteworthy. In contrast to the finding of Lander et al. (31) of direct induction by NO of $\text{TNF-}\alpha$ secretion in human peripheral blood mononuclear cells, this study with mouse macrophages shows that LPS-induced $\text{TNF-}\alpha$ production was decreased by preexposure of cells to NO. The inductive effect of NO on human peripheral cells observed by Lander et al. may either occur secondarily to the action of different mediators ($\text{IFN-}\gamma$) that are produced by

FIG. 7. NO production (A) and desensitization (B) induced by LPS and lipid M4. Thioglycolate-elicited mouse peritoneal macrophages were incubated with 1 ng to 10 μg of *B. pertussis* LPS or lipid M4, and direct NO_2^- production was determined. Desensitization (inhibition of $\text{TNF-}\alpha$ responses to 10 μg of *S. typhimurium* LPS per ml) induced during incubation with 0.1 and 1 μg of agent per ml was determined after three washings of cells, as described in Materials and Methods. Data are the means $\pm$ SD of two determinations.

other cells (T lymphocytes) in their heterogeneous preparation or reflect an intrinsic difference between the responses of human monocytes and mouse macrophages to NO and LPS. In support of the latter possibility, it has been shown, for instance, that these two types of cells give different responses to analogs of lipid A substructures (18, 22).

With regard to the role of NO in LPS-induced endotoxin tolerance, our finding that NMMA partially inhibits desensitization of mouse macrophages suggests that the large amounts of NO produced by these cells after activation with LPS exert a negative feedback effect on the synthesis of TNF- α in response to further exposure to LPS. It is clear, however, that this autocrine effect is not the general mechanism that accounts for LPS-induced desensitization since in human monocytes LPS induces desensitization (30) without detectable production of NO. In line with this remark, our findings of NO-independent desensitization after a brief stimulation with LPS (Fig. 6) and of desensitization induced by lipid M4 without synthesis of NO (Fig. 7) also show that LPS tolerance involves more than NO-mediated feedback. This is in line with our previous observations (17) that a factor that is released from macrophages early after stimulation with LPS is involved in desensitization. Since cytokine-mediated feedback alone cannot explain the refractory state to LPS (15), a conjunction of NO and cytokines may be required. On the other hand, we have previously shown that the loss of LPS receptors does not explain the phenomenon of desensitization (16). Therefore, a more detailed analysis of interrelations among the various activation pathways of macrophages will be essential to understanding the mechanism of endotoxin tolerance.

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