Effects on functions of ovine blood mononuclear cells for each of several fatty acids at concentrations found in plasma of healthy and ketotic ewes

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Objective—To assess effects on functions of peripheral blood mononuclear cells (PBMC) obtained from ewes for each of several fatty acids represented in ovine plasma at concentrations mimicking those of ketotic or healthy ewes.

Sample Population—Blood samples obtained from 6 Sardinian ewes.

Procedure—The PBMC were cultured in media that contained oleic (OA), palmitic (PA), stearic (SA), linoleic (LA), or palmitoleic (POA) acid at concentrations similar to those of ketotic or healthy ewes. Synthesis of DNA was stimulated by use of concanavalin A or pokeweed mitogen (PWM). Secretion of IgM was stimulated by use of PWM.

Results—High concentrations (900, 450, and 225 µmol/L) of OA significantly inhibited DNA synthesis and IgM secretion of PBMC. Conversely, low concentrations (56 or 28 µmol/L) of OA significantly enhanced DNA synthesis of PBMC. High concentrations of PA (600, 300, 150, 75, 37.5, or 18.7 µmol/L) and SA (300, 150, or 75 µmol/L) significantly inhibited DNA synthesis of PBMC. High concentrations of PA (600, 300, 150, 75, 37.5, or 18.7 µmol/L) and SA (300, 150, 75, or 38 µmol/L) also significantly inhibited IgM secretion of PBMC. None of the concentrations of LA and POA affected PBMC functions.

Conclusion and Clinical Relevance—Impaired immunoresponsiveness of ketotic ewes is likely associated with an increase of plasma concentrations of OA, PA, or SA and not with that of LA or POA. At physiologic concentrations, single fatty acids are likely to participate in modulation of immunoresponsiveness by exerting suppressive or stimulatory effects on immune cells. (*Am J Vet Res* 2002;63:958–962)

The ketosis-related increase of plasma concentrations of **nonesterified fatty acids** (**NEFA**) is associated with reduced immunoresponsiveness in ewes.[1] Furthermore, it has also been reported that a physiologic mixture of **oleic acid (OA)**, **palmitic acid (PA)**, **stearic acid (SA)**, **linoleic acid (LA)**, and **palmitoleic acid (POA)**, in regard to the relative contribution of each specific fatty acid[2] and at concentrations mimicking those in ewes that have clinical or subclinical ketosis, inhibited lymphocyte functions.[3] These findings led to the hypothesis that immunosuppression in ketotic ewes is likely to be associated, at least in part, with a lipomobilization-associated increase of blood concentrations of NEFA. On the other hand, a series of in vivo or in vitro studies in laboratory animals or humans[4-9] documented that increased serum concentrations of free fatty acids or single fatty acids can interfere with various immune functions in a manner dependent primarily on type and concentration of the specific fatty acid. The objective of the in vitro study reported here was to determine the effects of each of several fatty acids contained in ovine plasma at concentrations mimicking those of healthy ewes or ewes with clinical or subclinical ketosis on DNA synthesis and IgM secretion of sheep **peripheral blood mononuclear cells (PBMC)**.

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Materials and Methods

Animals—Six healthy, nonpregnant, nonlactating, and nonketotic Sardinian ewes were used in the study. Each ewe was approximately 39 months old and weighed approximately 35 kg. Ewes were fed a diet consisting of forages and concentrate that was formulated in accordance with nutritional requirements of sheep provided by Bocquier et al.[10] Blood samples were collected via jugular venipuncture by use of evacuated glass tubes coated with sodium heparin (10 U of heparin/ml).

Preparation of culture media—A solution of each specific fatty acid that was to be added to PBMC culture media was prepared as described elsewhere,[3] and concentrations of each fatty acid were chosen on the basis of composition of plasma concentrations of NEFA in sheep.[2] Briefly, stock solutions of OA[a] (28.4 m*M*), PA[b] (12.7 m*M*), SA[c] (38.6 m*M*), LA[d] (4.2 m*M*), and POA[e] (4.7 m*M*) were prepared by dissolving each fatty acid in 0.1 N KOH at 60 C. Molar ratios of these solutions were 1:3.5, 1:7.8, 1:2.59, 1:2.37, and 1:1.88 for OA, PA, SA, LA, and POA, respectively. Each fatty acid-KOH stock solution, still at 60 C, was neutralized by addition of 1 N HCl and diluted with sterile redistilled water. Solutions of OA, PA, SA, LA, and POA were diluted in RPMI-1640,[f] adjusted to pH 7.4, and sterilized by passage through a filter. Finally, each solution was diluted at concentrations mimicking those of healthy ewes or ewes with clinical or subclinical ketosis.[2,3]

Evaluation of DNA synthesis—Evaluation of DNA synthesis was performed as described elsewhere.[2] Briefly, PBMC were isolated from blood samples and then resuspended at a concentration of 2×10^6 cells/ml in enriched RPMI-1640 culture medium. Triplicate cultures were assayed by use of 96-well tissue-culture plates.[g] Each well contained 2×10^5 PBMC in 100 µl of enriched culture medium. Control wells con-

tained 100 µl of PBMC suspension without mitogens or fatty acid soution. Furthermore, we included additional control wells that contained 100 µl of complete RPMI-1640 enriched medium without cells or 100 µl of PBMC suspension without the pyrimidine analogue **5-bromo-2'-deoxyuridine** (**BrdU**).

An optimal concentration of **pokeweed mitogen** (**PWM**; 0.4 µg/ml)[h] and **concanavalin A** (**Con A**; 2.5 µg/ml)[i] was added to plates containing various concentrations of OA (900, 450, 225, 112, 56, 28, 14, and 7 µmol/L), PA (600, 300, 150, 75, 37.5, 18.7, 9.37, and 4.68 µmol/L), SA (300, 150, 75, 38, 19.1, 9.6, 4.8, and 2.4 µmol/L), LA (100, 50, 25, 12.5, 6.3, 3.2, 1.6, and 0.8 µmol/L), or POA (100, 50, 25, 12.5, 6.3, 3.2, 1.6, and 0.8 µmol/L). These concentrations of mitogens and fatty acids were obtained by adding 5 µl of stock solution to plates. In this regard, the highest 3 concentrations of each fatty acid were intended to mimic those of ketotic ewes, whereas the other concentrations were intended to mimic physiologic concentrations.

Plates were incubated in an atmosphere of 95% air and 5% CO_2 for 48 hours at 37 C. We then added 10 µ*M* BrdU in 10 µl of RPMI-1640 to each well, and plates were incubated for an additional 18 hours. Culture medium then was removed from the tissue-culture plates by use of centrifugation (300 × g for 10 minutes), and the plates were dried (60 C for 1 hour).

Cell proliferation was quantitated by use of an ELISA. The assay was performed with a commercial kit[j] that is based on measurement of BrdU incorporated during DNA synthesis in proliferating cells. The ELISA was performed in accordance with the manufacturer's instructions. Values for DNA synthesis were expressed as the **optical density** (**OD**) for test wells minus the OD for control wells that did not contain BrdU. Intra-assay coefficient of variation for the assay was 6.7%.

Evaluation of IgM secretion—Secretion of IgM was determined by growing PWM-stimulated cells (2.0×10^6 cells/well) by use of the same conditions described previously. Cells were cultured (duplicate wells) for 8 days in 24-well tissue-culture plates.[k] At the end of the incubation period, plates were centrifuged at 1,000 × g for 15 minutes, and supernatants were collected and stored at –20 C until analyzed. Analysis was performed within 2 weeks after samples

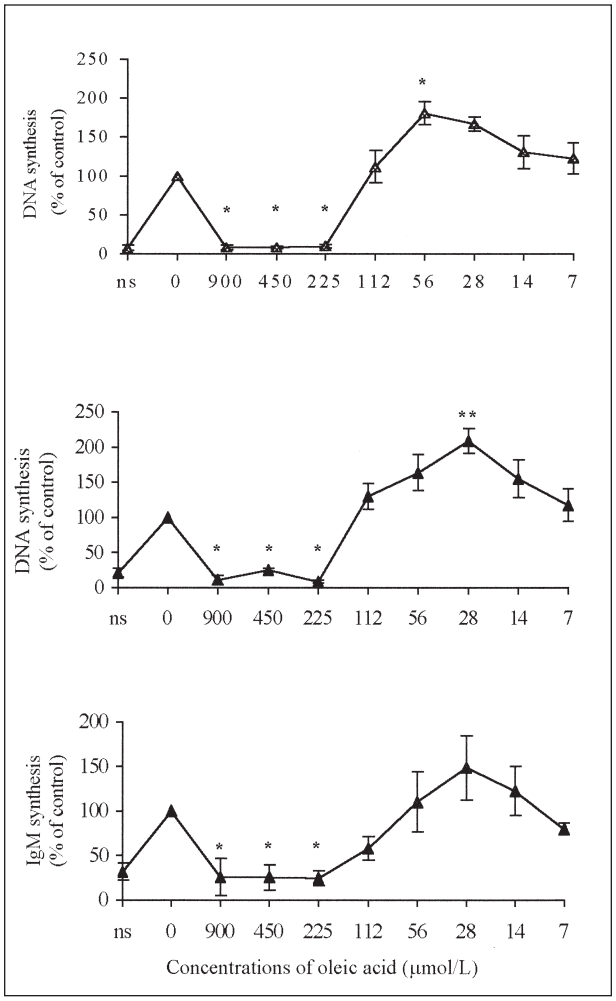

Figure 1—Effects of various concentrations of oleic acid on DNA synthesis stimulated by use of concanavalin A (Con A; top) or pokeweed mitogen (PWM; middle) and IgM secretion stimulated by use of PWM (bottom) in ovine mononuclear cells. Values for DNA synthesis are mean ± SEM percentages comparing optical density of stimulated and unstimulated cells, whereas values for IgM secretion are mean ± SEM percentages comparing concentrations for stimulated and unstimulated cells. Value assigned for the control group was 100%. *Value differs significantly ($P < 0.05$) from value for control group. **Value differs significantly ($P = 0.01$) from value for control group. ns = Not stimulated.

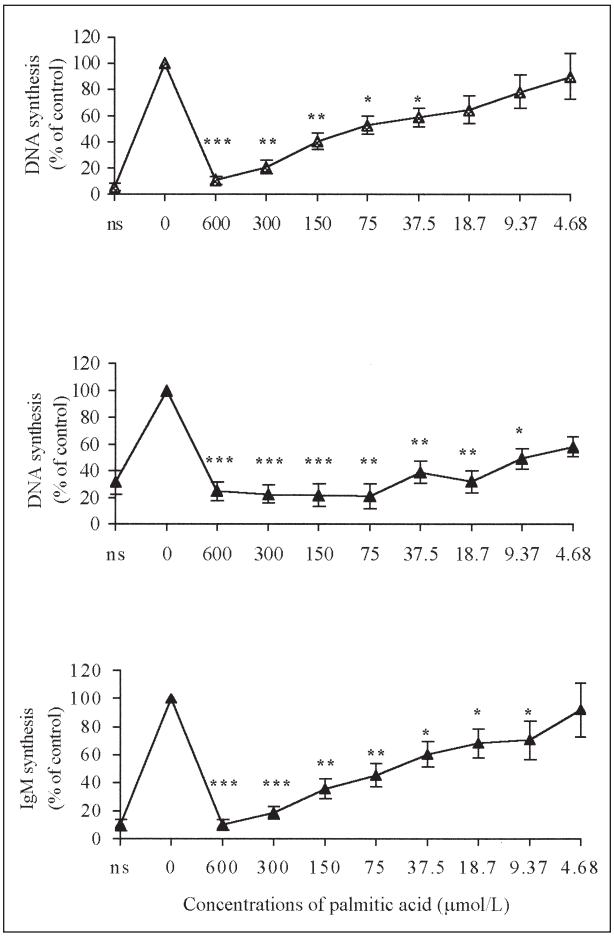

Figure 2—Effects of various concentrations of palmitic acid on DNA synthesis stimulated by use of Con A (top) or PWM (middle) and IgM secretion stimulated by use of PWM (bottom) in ovine mononuclear cells. Values for DNA synthesis are mean ± SEM percentages comparing optical density of stimulated and unstimulated cells, whereas values for IgM secretion are mean ± SEM percentages comparing concentrations for stimulated and unstimulated cells. Value assigned for the control group was 100%. ***Value differs significantly ($P < 0.001$) from value for control group. *See* Figure 1 for remainder of key.

were collected. The amount of IgM released in the culture medium was quantitated by use of a capture ELISA, which was performed as described elsewhere.[3]

Statistical analyses—Data were analyzed by use of a 1-way ANOVA,[1] and differences between values were considered significant at $P < 0.05$. Data were reported as the mean ± SEM expressed as the percentage of control wells (fatty acids were not added), which was set at a value of 100%.

Results

Significant differences were not observed among results for PBMC from each of the 6 ewes with regard to the pattern of influence exerted by each specific fatty acid. None of the concentrations of LA or POA significantly affected DNA synthesis or IgM secretion of mitogen-stimulated PBMC (data not shown).

High concentrations of OA (900, 450, or 225 µmol/L) significantly inhibited DNA synthesis and IgM secretion of mitogen-stimulated PBMC (**Fig 1**). Conversely, addition of low concentrations of OA (56 or 28 µmol/L) to PBMC cultures significantly enhanced DNA synthesis of Con A-stimulated ($P < 0.05$) and PWM-stimulated ($P = 0.01$) cells.

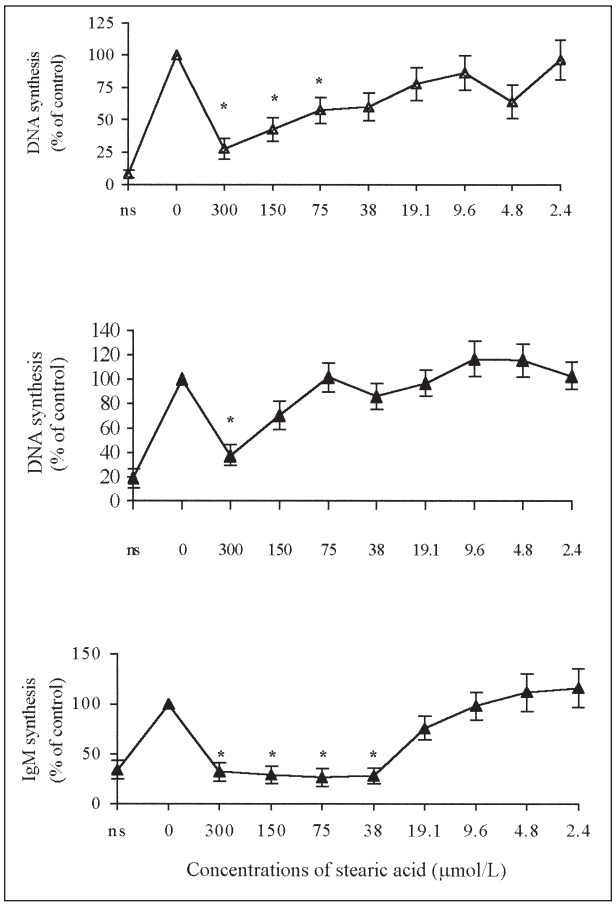

Figure 3—Effects of various concentrations of stearic acid on DNA synthesis stimulated by use of Con A (top) or PWM (middle) and IgM secretion stimulated by use of PWM (bottom) in ovine mononuclear cells. Values for DNA synthesis are mean ± SEM percentages comparing optical density of stimulated and unstimulated cells, whereas values for IgM secretion are mean ± SEM percentages comparing concentrations for stimulated and unstimulated cells. Value assigned for the control group was 100%. *See* Figure 1 for key.

High concentrations of PA significantly inhibited DNA synthesis and IgM secretion of mitogen-stimulated PBMC (**Fig 2**). Addition of PA (600, 300, 150, 75, or 37.5 µmol/L) to the culture media significantly inhibited DNA synthesis of Con A-stimulated PBMC, and the addition of 600, 300, 150, 75, 37.5, 18.7, or 9.37 µmol of PA/L to the culture media significantly inhibited DNA synthesis and IgM secretion of PWM-stimulated PBMC.

High concentrations of SA significantly inhibited the DNA synthesis and IgM secretion of mitogen-stimulated PBMC (**Fig 3**). In detail, the addition of 300, 150, or 75 µmol of SAL to culture media significantly inhibited DNA synthesis of Con A-stimulated PBMC. Furthermore, addition of 300 µmol of SA/L to the culture media significantly inhibited DNA synthesis of PWM-stimulated PBMC, and the addition of 300, 150, 75, or 37.5 µmol of SA/L to the culture media significantly inhibited IgM secretion of PWM-stimulated PBMC.

Discussion

In recent studies,[1,3] it was indicated that the ketosis-associated increase of plasma concentrations of NEFA is likely to be associated with alterations of cellular and humoral immune responses in sheep. Analysis of results of the study reported here supported this hypothesis and indicated that among fatty acids at concentrations mimicking those of ketotic ewes, only OA, PA, and SA should be considered responsible for impairment of PBMC functions.

Furthermore, our results also indicated that fatty acids represented in ovine plasma are capable of interfering with in vitro PBMC functions in a dose-dependent manner and that the effects depend on type and concentration of each fatty acid, because the same concentrations of several fatty acids exerted disparate effects (inhibition, stimulation, no effect) on the same PBMC function.

To our knowledge, effects of each of the fatty acids OA, PA, SA, LA, or POA on PBMC functions have not been assessed in ruminants. Conversely, the influence of these fatty acids on lymphocyte functions has been studied extensively in vivo and in vitro in laboratory animals and humans.[9,11-14]

Our results in sheep are in agreement with those obtained for other species, which documented negative effects of high concentrations of OA, PA, and SA on lymphocyte functions.[15-18] Several studies[7,19,20] have been performed in other species to ascertain the mechanisms through which high concentrations of fatty acids impair lymphocyte functions. Addition of OA, PA, and SA to media for culture of lymphocytes induces modifications in cell membrane phospholipids.[17,21,22] Lipid composition and architecture of cell membranes play an important role in many aspects of cellular physiologic processes, such as fluidity of membranes, transport of ions and substrates into cells, activity of membrane-associated enzymes, receptor expression, and distribution of cytoskeletal proteins, such that their modification can result in changes in cellular functions.[23-26] Furthermore, it has been documented that fatty acid-induced biophysical

changes of cell membranes can be responsible for changes of signal transduction pathways[12,27,28] or regulation of gene transcription.[8,25,29]

It has been reported[17,21] that OA can reduce lymphocyte functions by modulating **protein kinase C (PKC)**, although the mechanisms of such modulation have not been elucidated. In this regard, it has been suggested that OA can decrease synthesis or increase degradation of PKC, decrease generation of the second messengers inositol-1,4,5 triphosphate and diacyl glycerol, and block entry of calcium into cells by acting directly on receptor-operated calcium channels.[14,18,21] On the other hand, inhibition of 1 or more of these essential events of the cascade leading to cell activation may also represent a mechanism through which OA may alter production of **interleukin (IL)**-2 or expression of receptors (ie, the α-subunit of IL-2 receptors or transferrin receptors) that are crucial for proliferation or antibody secretion of activated lymphoid cells.[14,17,19,26,30-32]

Comparing dose-response curves of PBMC after addition of various concentrations of OA, PA, SA, LA, and POA to culture media, the results of the study reported here were in agreement with results for other researchers[23,33] in that saturated fatty acids are more detrimental to PBMC functions of sheep, compared with unsaturated fatty acids. In our study, physiologic concentrations of PA or SA continued to exert inhibitory effects on lymphocyte functions, whereas the same or analogous concentrations of OA, LA, or PA did not have any effect or, in the case of OA, acted in a stimulatory manner.

Other investigators have indicated that the effects of saturated fatty acids are not attributable to inhibition of PKC activity[21] or IL-2 production[18] or to reduced expression of IL-2 or transferrin receptors.[17] In addition to the hypothesis that the negative effects of saturated fatty acids depend on their incorporation into cell membranes with a consequent decrease in membrane fluidity,[23] other studies[8,20] indicate that a mechanism through which saturated fatty acids could cause immunosuppression may be the induction of cell death via apoptosis or necrosis. Addition of PA and SA may decrease DNA and IgM synthesis through induction of cell death via dissipation of the mitochondrial trasmembrane potential, regulation of anti-apoptotic protooncogenes such as Bcl-2, or induction of de novo synthesis of ceramide.[34] Furthermore, researchers reported that high doses of saturated fatty acids can cause preferential necrosis by generating reactive oxygen species and that this phenomenon can be partially prevented by addition of antioxidants to culture media.[8,35] In light of the latter hypothesis, it may be interesting to assess the in vivo capacity for prevention of negative effects of saturated fatty acids on immune functions of sheep by the use of diets enriched with antioxidant substances.

Analysis of the study reported here indicated that SA was less immunosuppressive than PA. Such observation is novel and difficult to explain. However, in light of the fact that such a result was particularly evident for DNA synthesis of PWM-stimulated PBMC, we can hypothesize that it was attributable to the potential for B lymphocytes but not T lymphocytes[15] to convert SA to OA, and OA was not inhibitory at low concentrations.

It also is interesting that SA inhibited Con A-induced DNA synthesis more markedly than it inhibited PWM-induced DNA synthesis. These results were in agreement with in vivo and in vitro studies[34,36] in which it was documented that T and B lymphocytes are differentially sensitive to the inhibitory effects of fatty acids in that they have differing abilities to incorporate and metabolize fatty acids or to tolerate changes in membrane composition. In particular, as reported previously, the deficiency of stearoyl-CoA desaturase in T cells, which converts SA to OA, could cause an accumulation of toxic amounts of distearoylphosphatidylcholine,[15] which could be responsible for the higher toxicity of SA toward T lymphocytes.

Furthermore, we observed that the addition of SA to PWM-stimulated PBMC cultures inhibited IgM synthesis more markedly than it inhibited DNA synthesis. This result, even though difficult to explain, may have been related to a differential effect of fatty acids on T-lymphocyte classes.[14,18] It has been reported[37-39] that T-helper-1 lymphocytes, the class of lymphocytes that produce interferon-γ (a cytokine that modulates development and differentiation of B cells), are more sensitive than T-helper-2 lymphocytes to the effects of fatty acids.[14]

Finally, our results are in agreement with findings obtained for nonruminant species[8,14,15,19] with regard to the fact that physiologic concentrations of OA can stimulate PBMC functions. In light of the effects of fatty acids on organization and physical properties of cellular membranes and on the role of unsaturated fatty acids as intracellular messengers,[17,40] we suggest that OA concentrations can be immunostimulatory by enhancing mechanisms involved in activation and subsequent proliferation of cells.

Data reported here reinforce our hypothesis[1,3] that the lipomobilization-related increase of plasma concentrations of NEFA is potentially detrimental to immune responses of sheep. These results represent progress in the identification of the mechanisms through which ketosis alters the immune functions of ruminants. Additionally, these results may also represent a contribution to the studies in cattle and help to explain the association between intense lipomobilization and the impairment of functions of nonimmunologic cells (hepatocytes and cells of the reproductive tract).[41,42] Our results also are interesting from an immunopharmacologic point of view, because they may represent a contribution to identification of substances capable of down-regulating the immune responses that could be used in pathologic circumstances in which such down-regulation is necessary.

As indicated elsewhere for plasma concentrations of NEFA,[1,3] results of the study reported here also suggest that plasma concentration of OA, PA, or SA may represent biochemical indicators for the reactivity of the immune system of sheep. Finally, supporting our hypothesis reported elsewhere,[43] we suggest that these fatty acids at physiologic concentrations are likely to participate, similar to the situation reported for other nutrients or metabolites,[44] in the subtle regulation of

the immune response by participating in establishing the physiologic equilibrium between up- and down-regulation.

[a]Oleic acid, Sigma Chemical Co, Milano, Italy.
[b]Palmitic acid, Sigma Chemical Co, Milano, Italy.
[c]Stearic acid, Sigma Chemical Co, Milano, Italy.
[d]Linoleic acid, Sigma Chemical Co, Milano, Italy.
[e]Palmitoleic acid, Sigma Chemical Co, Milano, Italy.
[f]RPMI 1640 medium, Sigma Chemical Co, Milano, Italy.
[g]96-Well plate flat-bottom, Corning Glass Works, Corning, NY.
[h]Pokeweed mitogen, Sigma Chemical Co, Milano, Italy.
[i]Concanavalin A, Sigma Chemical Co, Milano, Italy.
[j]Biotrak, APB, Milano, Italy.
[k]24-Well plate flat-bottom, Corning Glass Works, Corning, NY.
[l]Instat, GraphPad Software Inc, San Diego, Calif.

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