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Modification of membrane physical properties, biological response and insulin binding in Friend cells by low serum concentration

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The effect of low serum concentration on plasma membrane fluidity and lipid composition, differentiation and insulin binding was investigated in three Friend erythroleukemia clones. Both FLC (clones No. 745) and F(+) (Ostertag F4N) Friend erythroleukemia cells can be induced to differentiate and to produce hemoglobin when exposed to DMSO. Clone R(3) (Ostertag F4-D5-1) is a DMSO-resistant clone when grown under normal conditions (15% serum) but could undergo differentiation with accumulation of protoporphyrin IX upon induction with DMSO when grown in low serum concentration (2.5% serum). Electron spin resonance measurements of the order parameters (S) and $S(T_{\parallel})$ demonstrate that R(3) has a more fluid plasma membrane than the FLC and F(+). The order parameters of the outer hyperfine splittings $S(T_{\parallel})$ at 37°C are 0.60 ± 0.009, 0.62 ± 0.008 and 0.64 ± 0.009 for R(3), F(+) and FLC, respectively. We have used the insulin receptors as a model for a polypeptide hormone receptor associated with the plasma membrane of the Friend clones. Insulin binding studies demonstrated that the receptor of R(3) had a decreased affinity for insulin manifest as a 10-fold increase in the amount of insulin required to compete for half of the tracer binding (18 nM for R(3) vs. 2 nM for FLC and F(+)). Computer-fit Scatchard plot analysis by the negative cooperativity model reveal that R(3) possessed a similar number of sites/cell (about 70000) as the FLC or F(+) cells, with similar high and low affinities. Growing the DMSO-resistant clone R(3) in low serum concentration caused a decrease in receptor number by 35%, and an increase in receptor affinity to that seen with the differentiable clones. Thus, the abnormal properties of the plasma membrane and insulin receptor of the DMSO-resistant clone in our earlier report (Simon et al. (1984) Biochim. Biophys. Acta 803, 39–47) were partially reversed by growing the cells in a low serum concentration, restoring the cellular response to the differentiation agent.

Introduction

The Friend erythroleukemia clones grown in tissue culture can offer a very useful experimental model system for studying the biochemistry and the mechanisms of cellular differentiation of hematopoietic cells [1–4]. These cells can be induced to differentiate by dimethylsulfoxide

Abbreviations: DMSO-dimethylsulfoxide; ESR, electron spin resonance; FLC, Friend erythroleukemia line 745; F(+), FLC Ostertag F4N; R3, DMSO-resistant FLC Ostertag F4-D5-1; (S), order parameter; $S(T_{\parallel})$, order parameter of the outer hyperfine splitting.

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(DMSO) and the other agents [1–4]. Although DMSO must ultimately influence the control of cellular differentiation at the DNA level, the actual primary site of action is unknown. Since DMSO is a penetrant carrier and a cryoprotective agent, the primary effects might be on the plasma membrane [2,5].

The plasma membrane physical properties are an important factor in the regulation of cellular activity of the living cells [6–8]. Studies have shown that change in the membrane fluidity can modify the rate of enzyme activity [8,9], cation permeability [10], methotrexate transport [11], hexose uptake [12], insulin receptors [13,14], growth rate [15], peroxidation [16] and cell differentiation [17,18]. The degree of fluidity of the plasma membrane can be modified altering growth temperature [19], growth in medium supplemented with fatty acids [14,19,20], by growth in different serum concentrations [22] or by growing the cells in different temperatures [21]. We have previously described [13] the membrane physical properties and insulin-binding properties of a clone of FLC that was unable to undergo differentiation. This clone, R(3) (Ostertag clone F4-D5-1) had an alternated membrane lipid composition, increased membrane fluidity, and abnormal insulin receptor. It was most interesting to determine whether change in the physical properties of the cell membrane has any effect on the biological response and membrane organization of the abnormal Friend clone.

The technique of introducing a fatty acid nitroxide free radical as a probe for ESR study has been used in many studies of the physical properties of biological membrane [23–25]. ESR analysis could provide a useful information on the conformational and the dynamic state of the lipids and protein of the plasma membrane. The actual values of the order parameters (S) and $S(T_{\|})$ are only a close estimation [26,27], but the calculated relative values can be used for comparative study of the molecular motion in the biomembrane [23,28,24]. The rapid incorporation of the fatty acid spin probe into the plasma membrane of the intact cells is not toxic to the cultured cells [14,25].

The insulin receptors of the Friend cells are an integral part of the plasma membrane, and was used in this study to reflect the effect of changes in membrane fluidity on protein and membrane organization [14,29]. Other work has examined the effect of temperature [12], fatty acid supplementation [14] and the use of different clones [13] to study the effect of change in membrane fluidity on the properties of insulin receptors. It was found that elevation of temperature and membrane fluidity was associated with a decrease in both affinity and insulin receptor concentration [12]. An increase in polyunsaturated fatty acid of polar lipids was associated with an increase in membrane fluidity and an increase in receptor number and a decrease in receptor affinity [14]. Use of different Friend clones with different sensitivities to DMSO showed abnormal insulin binding of the DMSO-resistant clone with high membrane fluidity [13]. Induction of the Friend cells with DMSO decreased membrane fluidity and the insulin receptor number decreased with no change in receptor affinity [17,30].

In this study we examined the DMSO-inducible and -resistant clones grown under low serum concentration. We now demonstrate that growing the Friend clones in 2.5% serum caused a decrease in membrane fluidity and partially reversed the abnormal insulin binding found in R(3) [13] and its inability to respond to DMSO.

Material and Methods

Cells and cell culture. Three Friend erythroleukemia cell clones were used in this study. The FLC cell line No. 745 was purchased from the Mammalian Cell Repository, Institute for Medical Genetics, Camden, NJ (Catalog No. GM-86) and the F(+) cell lines (F4N) and its DMSO-resistant R(3) (F4-D5-1) were a gift from Dr. W. Ostertag and have been described previously [31]. Cells were grown in suspension culture in Temin's medium (Gibco) supplemented with 2.5% fetal calf serum (Gibco) at 37°C in 5% CO_2 as previously described [14]. The Friend cells were conditioned in 2.5% serum for 1 months, plated at $1.5 \cdot 10^5$ cells/ml and allowed to grow to $1.5 \cdot 10^6$ cells/ml. Viability was determined by Trypan blue dye exclusion and was greater than 95% for the samples used for this study.

Lipid analysis. Harvested cells were washed twice with phosphate-buffered saline containing

1% bovine serum albumin to remove the growth media and any adherent lipid before lipid extraction. The lipids were extracted from the intact cells with chloroform/methanol (2:1, v/v) [32]. The lipids were analyzed as previously described [14].

Electron spin resonance spectroscopy (ESR). ESR measurements were carried out on a varian E-104 spectrometer, as previously described [13,14], using the fatty acid spin label, 5-nitroxystearate (2,3-carboxypropyl-1-4,4-dimethyl-1-2-tridecyl-1-3-oxolidinyloxyl), (Syva, Palo Alto, CA). The observed values of the outer ($2T_{\parallel}$) and of the inner ($2T_{\perp}$) hyperfine splitting (in gauss) were used to calculate the order parameter (S) and the order parameter of the outer hyperfine splitting $S(T_{\parallel})$ [13,14,26,29]. Addition of potassium ferricyanide was used to determine the amount of incorporation of the spin label into the plasma membrane [13,25,29]. Greater freedom of motion of the 5-nitroxystearate in the biomembrane bilayers is associated with the smaller values of the order parameters [23].

Insulin receptor studies. Insulin binding studies were performed on the intact friend clones in the early stationary phase as previously described [13,14]. The binding data were analyzed by a computer program (DERC 2, written in Basic for HP-2000) that curve fits the competition curve to a fourth-order polynomial. The program generated a Scatchard plot of the fitted curve and analyzed the binding data by both the DeMeyts [34] and a two-site model [35].

Results

Effect of low serum concentration on growth rate, lipid composition and physical properties of Friend clones

The inducible Friend clone 745 (FLC) and Ostertag F4N (F +) were grown in 2.5% fetal calf serum and the growth rate, lipid analysis and fluidity were compared to those of the DMSO-resistant clone Ostertag F4 D5-1 (R3) grown under identical conditions. After 1 month of conditioning, the three Friend clones divided every 25–30 h in Temin's medium with 2.5% serum (compared to 15–20 h in 15% sera) [14]. The number of cells at stationary phase was similar in 2.5 and 15% serum

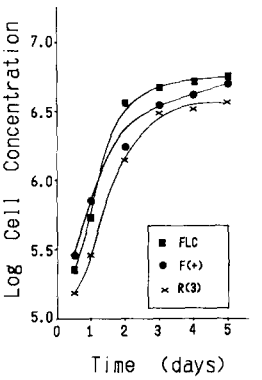

Fig. 1. Growth curves of the Friend clones grown in low serum concentration. The Friend cells were acclimatized to grow in 2.5% serum for 1 month. The cells were placed at $1.5 \cdot 10^6$ cells per ml in Temin's modified Eagle's medium, and at the indicated times triplicate samples were taken and counted in a hemocytometer with Trypan blue dye for viability determination. The data are the mean of two experiments.

($3 \cdot 10^6$ cells/ml) (Fig. 1). The fatty acid composition of the polar lipids were determined at the early stationary phase and found to be similar to all three clones (Table I). Growing the cells in low serum concentration increased the amounts of

TABLE I

FATTY ACID COMPOSITION OF THE POLAR LIPIDS OF FRIEND CLONES

The fatty acid composition was determined by gas-liquid chromatography. The values do not add up to 100% because some of the fatty acids are not listed. Fatty acids are abbreviated as number of carbon atoms: number of double bonds.

Fatty acid composition (%)	FLC	F(+)	R(3)
16:0	17.1 ±1.7	19.7 ±0.9	15.9 ±0.2
18:0	9.3 ±1.5	7.1 ±0.3	6.7 ±1.3
18:1	39.6 ±1.6	49.8 ±0.8	49.6 ±0.9
20:4	6.1 ±1.2	3.5 ±0.2	3.4 ±0.3
Class:			
Saturated	39 ±3 *	29 ±1	27 ±0.5 **
Monoenoic	49 ±3 *	57 ±1	58 ±0.5 **
Polyenoic	20 ±2	11 ±0.5	12 ±0.5
Polyenoic/ monoenoic ratio	0.42 ± 0.06 *	0.18 ± 0.008	0.20 ± 0.01

Statistical significance by unpaired t-test: mean ± S.E., $n = 6$:
* $P < 0.005$ for FLC vs. F (+) and R(3)
** $P < 0.005$ for F(+) vs. R(3)

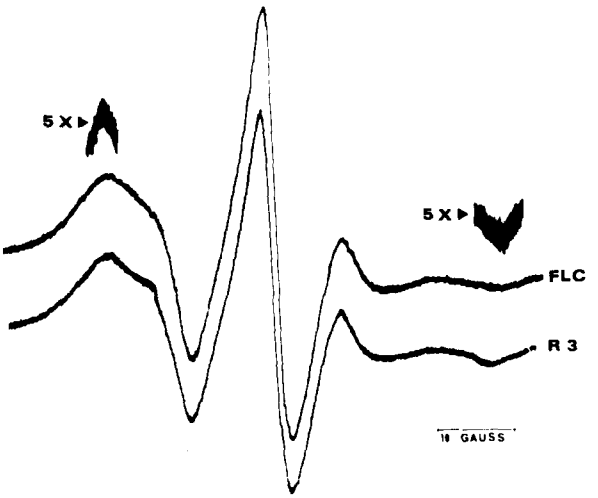

Fig. 2. ESR spectra of 5-nitroxystearate-labeled intact Friend clones grown in low serum concentration. The spectra were taken at 37°C for FLC and R(3) cells. The cells were harvested at $3 \cdot 10^6$ cells per ml, washed with phosphate buffered saline and were used for the experiments as described in Material and Methods. For higher precision measurements of the outer hyperfine splittings, a 5-fold increase of the receiver gain was used at the end points at the $2T_{\parallel}$.

monoenoic fatty acids and lowered the polyenoic when compared to cells grown in 15% serum [13]. The FLC clone was considerably higher in both saturated and polyenoic fatty acids than F(+) and R(3). These alterations in lipid composition would be expected to cause alterations with membrane physical properties.

The fluidity of the cell membrane was evaluated by electron spin resonance, noting the freedom of motion of the fatty acid probe 5-doxylstearate (Fig. 2). When measuring fluidity in whole cells, an artifact may occur when the probe is reporting the fluidity of internal membranes. This was not the case in these experiments. The ESR signal was generated almost exclusively from the plasma membrane of the intact cells, since the spin label signal that decayed in the intact cells after 30 min of incubation at 37°C could be restored with the addition of 1 mM ferricyanide. The regenerated ESR signal was 98% of the intensity of the initial signal. The calculated values for the order parameter, S, and order parameter of the outer hyperfine splitting ($T_{\parallel}$) of the various clones are presented in Table II. At all temperatures measured, R(3) cells showed slightly smaller values for the order parameter of the outer hyperfine splitting, $S(T_{\parallel})$, and the order parameter, (S), implying a somewhat greater freedom of motion of the spin label in the bilayer. All the Friend cells that were grown in lower serum concentration had much lower bulk plasma membrane fluidity compared to the cells grown in 15% serum (for example R(3) grown in 15% serum at 37°C [13] $(S) = 0.54 \pm 0.004$ compared to cells grown in 2.5% serum $(S) = 0.57 \pm 0.008$).

It was found that growing the R(3) cells in 2.5% serum partially restored the ability of these cells to undergo differentiation [36]. That is, when grown in 15% serum, the DMSO resistant clone R(3) shows no evidence of differentiation in response to 1.5% DMSO. After growth in a low serum concentration, DMSO induces a significant amount of protoporphyrin in the R(3) cells. The accumulation of protoporphyrin IX (4.45 nM/mg

TABLE II

ORDER PARAMETERS CALCULATED FROM ESR MEASUREMENTS OF FRIEND CLONES

ESR measurements were carried out on a Varian E-104 spectrometer using a 5-nitroxystearate as a fatty acid spin label.

Cell type	Order parameter (S)			Order parameter of the outer hyperfine splitting S ($T_{\parallel}$)		
	16°C	27°C	37°C	16°C	27°C	37°C
FLC	0.74 ± 0.002	0.64 ± 0.007	0.58 ± 0.007	0.83 ± 0.009 [a]	0.73 ± 0.006	0.64 ± 0.009
F(+)	0.75 ± 0.011 [c]	0.64 ± 0.011 [c]	0.58 ± 0.008	0.82 ± 0.009 [c]	0.71 ± 0.003 [c]	0.62 ± 0.008
R(3)	0.72 ± 0.003 [d]	0.63 ± 0.004 [d]	0.57 ± 0.008 [d]	0.79 ± 0.008 [b]	0.68 ± 0.010 [b]	0.60 ± 0.009 [b]

Statistical significance by unpaired t-test: mean ± S.E., $n = 6$. (a) $P < 0.005$ for FLC vs. F(+). (b) $P < 0.005$ for FLC vs. R(3). (c) $P < 0.01$ for FLC vs. F(+). (d) $P < 0.05$ for FLC vs. R(3).

protein) in cells was due to a lack of enzyme ferrochelatase in the R(3) cells. This enzyme is needed for the conversion of protoporphyrin IX into hemoglobin.

Effect of low serum concentration on insulin receptors

The insulin receptor binding of the three Friend clones was used as a model of functional protein associated with plasma membrane [14,30]. Fig. 3 demonstrates the Scatchard plots that were derived from insulin binding of the Friend clones. The curvilinearity of the Scatchard plots has been ascribed to either negative cooperativity [39] or to two independent classes of binding site [40]; the interpretation of the binding data is given in Table III.

The specific insulin binding of R(3) is similar (0.90) to FLC or F(+) (1.09 and 1.15). All Friend clones demonstrate a curvilinear plot with maximal binding of 7.5 ng/10^7 cells, corresponding to about 75 000 sites/cell. The affinity of the unoccupied form, $\bar{K}_e$, was slightly lower for R(3) (0.026 nM^{-1}) than f(+) (0.033 nM^{-1}) or FLC (0.05 nM^{-1}). The linear Scatchard plot found for R(3) grown in 15% serum [13] was reversed to a curvilinear one (Fig. 3). The affinity of the occupied form, $\bar{K}_f$, was similar in all clones (0.01 nM^{-1}). When the data were analyzed assuming a two-site

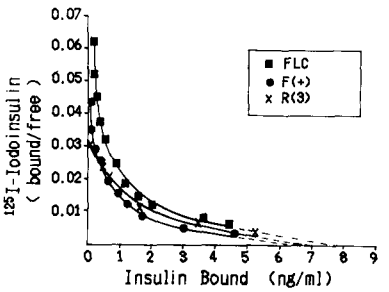

Fig. 3. Scatchard plot of insulin binding of Friend clones grown in low serum concentration. Intact cells were used for the insulin binding, 20 pM [^{125}I]iodoinsulin and unlabeled insulin was added so that the final concentration of the insulin varied from 20 pM to 0.18 μM. Incubation was carried out at 15°C and was terminated by addition of gold buffer. R_0 = the total receptor concentration was obtained by extrapolation of the Scatchard plot to the abscissa. The data of three determinations were analyzed and fitted by a computer program.

model (two separate classes of receptors) there was little difference between the high-affinity and the low-affinity receptor of the inducible clones and R(3). These data suggest that growth in 2.5% sera caused the marked increase in the low-affinity form of the receptor found in R(3) cells compared to cells grown in high serum concentrations [13].

Not all the binding properties were restored by growing R(3) in low serum concentration. We studied the kinetics of dissociation of [^{125}I]-iodoinsulin from the insulin receptor by dilution

TABLE III

SCATCHARD PLOT ANALYSIS OF INSULIN-BINDING PARAMETERS OF FRIEND CLONES

The Friend clones were grown in 2.5% serum for 4 days and were harvested at $3 \cdot 10^6$ cells/ml. They were washed with phosphate-buffered saline and were used for insulin binding as described in Materials and Methods.

Scatchard parameters	Cell types		
	FLC	F(+)	R(3)
Specific binding/10^6 cells (pg)	1.09 ± 0.04 [a]	1.25 ± 0.01	0.90 ± 0.06 [b]
I_{50} (nM)	1.84 ± 0.10	1.79 ± 0.12	17.9 ± 0.70 [b]
Sites/cell	79200 ± 14978	76000 ± 329	72400 ± 3250
Two-sites model			
High-affinity sites			
Number of sites/cell	5100 ± 400	3400 ± 228	5300 ± 465 [b]
K_1 (nM^{-1})	0.57 ± 0.06	0.51 ± 0.05	0.22 ± 0.02
Low-affinity sites			
Number of sites/cell	74200 ± 1510	73700 ± 1000	67200 ± 6585
K_2 (nM^{-1})	0.14 ± 0.003	0.011 ± 0.008	0.015 ± 0.003

Statistical significance: mean ± S.E. of three separate experiments. (a) $P < 0.005$ for FLC vs. F(+). (b) $P < 0.005$ for R(3) vs. F(+).

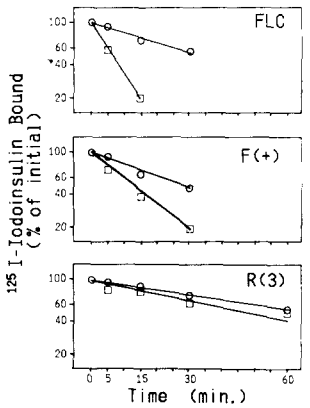

Fig. 4. Negative cooperativity of the insulin receptors of Friend clones grown in low serum concentration. The intact cells were incubated with 20 pM [^{125}I]iodoinsulin for 3 h at 15°C. 100 μl aliquots were diluted in 10 ml buffer (dilution) (○), or 20 ml buffer with unlabeled insulin (1 μg/ml) (□). The dilutions were incubated again at 15°C and at the indicated periods, triplicate tubes were sedimented at 15000 × g for 2 min. The supernatants were aspirated and the tubes counted in a Beckman Gamma 300 counter for 10 min. The data are a mean of three experiments.

in the presence or absence of excess insulin (Fig. 4). In the R(3) cells insulin had little influence on the dissociation rate constants of the ^{125}I-labeled insulin. In the FLC and F(+) cells, insulin caused a 2–3-fold increase in the dissociation rate. Thus, although the R(3) demonstrated a curvilinear Scatchard plot, kinetically it did not show negative cooperativity.

Discussion

Serum concentration in cell culture can play an important role in modulating the extent of cell proliferation [40,41], or can modify the response of cells to differentiate like the neural cells [42] or the myoblast cells [43]. In our current work, we have demonstrated that reducing fluidity by growing cells in 2.5% serum, resulted in both a decreased in receptor number and restoration R(3) binding properties.

Growth of the Friend clones in low serum concentration cause an increase in the amount of monoenoic fatty acids and a decrease in the polyenoic fatty acid (compared to cells grown in 15% serum) [13]. In all three friend clones the fatty acid composition of the polar lipid similar and the small alteration could not account for the dramatic fluidity changes that were found between the FLC, F(+), and the resistant clone R(3). The change in the order parameter of the ESR spectra could be dependent upon other factors affecting plasma membrane organization, such as different types of phospholipid, ratio of phospholipid to cholesterol, ratio of lipid to protein or different protein organization [5,7,9,17,24].

Others have examined the effect of temperature [12], fatty acid supplement [14] and the use of different clones [13] to study the effect of change in membrane fluidity on the properties of insulin receptors. It was found that elevation of temperature and membrane fluidity was associated with a decrease in both affinity and insulin receptor concentration [12]. An increase in polyunsaturated fatty acid of the polar lipids was associated with an increase in membrane fluidity and an increase in receptor number and a decrease in receptor affinity [14]. Induction of the Friend cells to differentiate with DMSO decreases membrane fluidity and the insulin receptor number decreases with no change in receptor affinity [17,30]. Use of different Friend clones with different sensitivities to DMSO also showed increased receptor number and decreased affinity of the DMSO-resistant mutant with high membrane fluidity [13]. Our finding with the R(3) mutant appeared to be compatible with our previous observations [14] when the Friend cells were grown in media enriched with unsaturated fatty acids.

Growing the Friend clones in low serum concentration revealed that the abnormal insulin receptor found for the R(3) cells grown in 15% serum were partially reversed [13]. Competitive binding assays demonstrated a shift to the right in the displacement curve for R(3) with about 10-fold increase in the amount of insulin necessary for the 50% displacement, still not similar to FLC or F(+) but lower than the 40-fold increase found in R(3) cells grown in high serum [13]. The Scatchard plot (Fig. 3) approached curvilinearity and was similar in all three Friend clones, suggesting two classes of receptors or negative cooperativity, and the abnormal linear plot found in R(3) cells grown in high serum [13] was changed. A similar effect of decreased serum concentration has been demonstrated in other cell systems. When hepatoma cells

(H-35 and HTC) were grown in 10% serum, a linear Scatchard plot for insulin binding was obtained [44]. When grown in 2.5% serum [45] the Scatchard plot was curvilinear. Treatment of cells with wheat-germ agglutinin or other lectins also led to a linearization of the Scatchard plot [46].

Not all the binding properties were restored by growing the R(3) cells in low serum concentration. When the dissociation kinetics of R(3) were studied (Fig. 4), unlike the FLC and the F(+) cells, there was still no increase in dissociation rate, even though it was lower than that found in high serum concentration [13].

The increased membrane fluidity found in the R(3) clone [13] may be been directly related to its inability to undergo erythropoietic differentiation [2,5,13,17,18,35,37]. The process of differentiation was associated with the induction of 'membrane active' substances that were similar to DMSO in being cryoprotective agents [2,5]. The process of differentiation is associated with major changes in membrane fluidity [17,18] and in the promyelocytic leukemia cells (HL-60), it was found that the differentiation-resistant clones showed more fluid membranes than the clones that were inducible [47]. The decrease in membrane fluidity of the DMSO-resistant clones R(3) by growing the cells in low serum concentration was associated with the partial restoration of the ability to differentiate. That is, when R(3) were grown in 15% serum, these cells showed no biochemical evidence of differentiation in response to 1.5% DMSO. After growth in 2.5% serum, DMSO induced significant amount of accumulation of protoporphyrin IX inside the R(3) cells [36].

It was found that the Friend clones could provide a useful system in studying the complex regulation and organization of the plasma membrane before and during erythrocyte differentiation. From this study it is suggested that the biological 'trigger' of the Friend cells differentiation resides in the plasma membrane.

Acknowledgements

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