Fibrinogen Receptor Exposure and Aggregation of Human Blood Platelets Produced by ADP and Chilling

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ADP-induced platelet aggregation is associated with the specific binding of fibrinogen to receptors. Since fibrinogen is also a cofactor for the spontaneous aggregation of chilled human platelets during rewarming, we examined the ability of these platelets to bind purified $^{125}$I-labeled fibrinogen. Specific binding similar to ADP-induced binding at room temperature was observed after the platelets had been chilled for 20 min at 0–2°C. Aggregation, however, occurred only when the ambient temperature was increased and was followed within 15–30 sec by loss of most of the bound fibrinogen and disaggregation. ADP- and cold-induced fibrinogen binding and aggregation were inhibited by 0.3 μM PGE$_1$, 1 mM dibutyryl cyclic AMP, and 2.5 mM EDTA, unaffected by 208 μM colchicine, and absent from thrombasthenic platelets. Platelets change shape from discs to spiny spheres when they are exposed to ADP or cold. PGE, and dibutyryl cyclic AMP prevented the shape change induced by ADP but not by chilling. Shape change, fibrinogen binding, and aggregation induced by ADP, but not by chilling, were inhibited by incubating platelets with 7.3 μM antimycin A and 4.9 mM 2-deoxy-D-glucose. Fixed platelets bound fibrinogen only if they were exposed to ADP before fixation but they failed to aggregate. Studies with fixed platelets showed that EDTA and a low pH prevent the interaction of fibrinogen with exposed receptors, whereas platelets stimulated with ADP in the absence of divalent cations or at low pH prior to fixation bound fibrinogen at physiologic pH, and this binding was inhibited by EDTA. The present studies provide the following clues about the requirements for fibrinogen receptor exposure and platelet aggregation. (1) ADP and chilling activate platelets by exposing fibrinogen receptors via a common pathway that is blocked by elevating cyclic AMP. (2) Chilling can bypass the seemingly ATP-dependent step for ADP-induced fibrinogen receptor exposure. (3) Receptor exposure by both ADP and chilling is independent of platelet shape. (4) Increased cyclic AMP inhibits the shape change induced by ADP but not that caused by chilling. (5) EDTA and low pH prevent aggregation by interfering with the binding of fibrinogen to exposed receptors. (6) Receptor mobility may be as important as fibrinogen binding in supporting platelet aggregation.

Although fibrinogen has long been recognized as a cofactor in ADP-induced platelet aggregation, its role has only recently been clarified. Fibrinogen specifically binds to stimulated platelets and may subsequently link them into aggregates. The correlation between fibrinogen binding and aggregation is striking. Both are inhibited at low pH, in the presence of EDTA or PGE$_1$, and with platelets that lack the capacity to aggregate either due to the congenital deficiency thrombasthenia or to incubation with EDTA. However, little is known of the mechanism by which fibrinogen binding is initiated or mediates aggregation. Chilled platelets aggregate spontaneously if they are shaken while they are being rewarmed. As with platelets stimulated by ADP. aggregation of platelets by cold requires addition of fibrinogen to the external medium. In contrast, when platelets are stimulated by thrombin or fibrinogen, fibrinogen can be released from the platelet α-granules. It is attractive to postulate that different stimuli induce aggregation through a common pathway, most likely by fluctuations in cytoplasmic calcium levels, which may lead to exposure of fibrinogen receptors.

In the present study, we examined the mechanism of platelet aggregation by comparing the effects of two stimuli—ADP and cold—on fibrinogen receptor exposure and aggregation after increasing platelet cyclic AMP or incubating platelets with metabolic inhibitors. We used prostaglandin E, to stimulate platelet adenylate cyclase and the metabolic inhibitors antimycin A and 2-deoxy-D-glucose to inhibit oxidative phosphorylation and glycolysis, respectively, and thus decrease platelet metabolic ATP levels.

EDTA and low pH also inhibit aggregation. EDTA chelates the necessary external divalent cations, and hydrogen ions may compete for the sites of these cations. We used ADP-treated platelets fixed with formalin in the presence and absence of EDTA and at low pH to determine whether these inhibitors prevent platelets from exposing their fibrinogen receptors or prevent fibrinogen from interacting with exposed receptors.

MATERIALS AND METHODS

Materials

The following reagents were used: highly purified fibrinogen labeled with iodine-125 prepared as described elsewhere; unlabeled human fibrinogen (grade L, A.B. Kabi, Stockholm, Sweden) dissolved in water at 10 mg/ml and dialyzed for about 5 hr against 0.01 M sodium phosphate buffer, pH 6.5.

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isotonic saline buffered to pH 7.4 with HEPES (N-2-hydroxyethylpiperazine-N-2-ethane-sulfonic acid) (Calbiochem, San Diego, Calif); ADP (disodium salt, Sigma Chemical Co., St. Louis, Mo.) 0.01 M in isotonic saline stored at −15°C; prostaglandin E1 (PGE1) (Sigma) 2.8 mM in 95% ethanol; stored at −15°C; dibutyl cyclic AMP (dibu-cAMP) (Calbiochem); colchicine (Eli Lilly and Co., Indianapolis, Ind.) prepared from an injectable solution of 0.5 mg/ml with isotonic saline; antimycin A (ANT) (Sigma) 7.3 mM in 95% ethanol; and 2-deoxy-D-glucose (DG) (Calbiochem), 487 mg/ml with 2.8 mM sodium citrate and anesthetic acid2 (Merck and Co., West Point, Pa.). Platelet-piperazine-N-2-ethane-sulfonic acid) (Calbiochem, San Diego, Calif); HEPES-buffered modified Tyrode’s solution without PGE2 and calcium (Sigma) (Sigma) 2.8 mM in 95% ethanol, stored at −15°C; prostaglandin E1 (PGE1), stored at −15°C; dibutyl cyclic AMP (dibu-cAMP) (Calbiochem); colchicine (Eli Lilly and Co., Indianapolis, Ind.) prepared from an injectable solution of 0.5 mg/ml with isotonic saline; antimycin A (ANT) (Sigma) 7.3 mM in 95% ethanol; and 2-deoxy-D-glucose (DG) (Calbiochem), 487 mg/ml in isotonic saline.

Preparation of Gel-Filtered Platelets (GFP)

Platelets were prepared as previously described. Briefly, blood from human volunteers was collected into 0.11 M sodium citrate and acetylsalicylic acid2 (Merck and Co., West Point, Pa.). Platelet-rich plasma (PRP) was obtained by centrifugation (300 g), incubated with 0.1 M PGE1, and acidified to pH 6.5. The platelets were concentrated by centrifugation (1000 g) and resuspended in modified Tyrode’s solution containing 2 mg/ml bovine serum albumin (fraction V, Sigma), no added calcium, and 0.3 M PGE1, buffered to pH 7.4 with 0.01 M HEPES. The platelet concentrate was gel-filtered through a column of Sepharose 2B equilibrated with HEPES-buffered modified Tyrode’s solution without PGE1, and the platelet pellet was resuspended in HBMT to over 500,000/μl

One experiment was carried out with GFP from M.M., a patient with the congenital platelet deficiency, thrombasthenia.21

Preparation of Fixed Platelets

GFP were suspended in HBMT at pH 7.4 with or without 2.5 mM EDTA, or in HBMT at pH 6.5. They were fixed for 60 min at 25°C with an equal volume of fixative containing 4% formalin before and after 1-min exposure to 10 μM ADP, then washed 3 times with cold isotonic saline by centrifugation at 4°C and resuspension. They were finally resuspended at room temperature in HBMT alone, in HBMT containing 2.5 mM EDTA, or in HBMT at pH 6.5. The platelet pellet of the final suspension was adjusted to 300,000/μl.

Fibrinogen Binding to Gel-Filtered Platelets Stimulated With ADP or by Chilling and Rewarming

The amount of fibrinogen associated with the platelets was assessed by measuring the radioactivity in the pellets formed after centrifuging duplicate 0.5-ml samples of GFP through silicone oil. To assess fibrinogen binding during chilling and rewarming, 125I-fibrinogen was added to 4 ml of platelet suspension at a final concentration of 1 mg/ml and 200–400 cpm/μg. Another sample contained 2.5 mM EDTA in addition. A third sample contained labeled fibrinogen and inhibitors in four experiments: 0.3 μM PGE1, in two experiments 208 μg/ml colchicine, and in one experiment 200 μM dibu-cAMP. All suspensions were warmed to 37°C; glass tubes were used to permit more rapid changes of temperature. Similar suspensions, prepared with 1 mg/ml nonradioactive fibrinogen, were used to monitor shape change and aggregation. Shape change was deduced from loss of the characteristic swirl noted when suspensions of disc-shaped platelets are agitated. It was verified by observing fixed aliquots with a phase-contrast microscope.

Binding was measured initially at 37°C in an untreated portion of each radioactive suspension and also 1 min after adding ADP in another portion of each suspension (final concentration 10 μM). The remaining unstimulated suspensions were then placed in an ice bath, and after 20 min, samples were removed for measuring platelet-associated fibrinogen in the cold. The suspensions were transferred to a 37°C bath; the nonradioactive ones were either immediately shaken and observed for aggregates every few seconds or tested for aggregation using an aggregometer (Payton Associates, Buffalo, N.Y.). Fibrinogen binding was assessed on aliquots of the radioactive suspensions as soon as aggregation occurred in the nonradioactive samples (about 15 sec), and the remainder of the suspensions was left undisturbed at 37°C. The nonradioactive suspensions were observed intermittently for disaggregation and restoration of the discoid shape, and the radioactive suspensions were sampled once more after about 20 min.

The amount of fluid trapped in the pellet, measured in separate samples with 14C-sorbitol, averaged only 0.23 μl/106 platelets in pellets from both unstimulated disc-shaped and stimulated spiny spherical platelets.23

For most of these studies nonspecific binding was defined as the amount of radioactivity associated with the platelets in the presence of 2 mM EDTA. In previous studies with ADP, we showed that this amount of radioactivity could not be displaced by a large excess of unlabeled fibrinogen. This was true for binding in the cold as well. The amount of nonspecifically bound fibrinogen was subtracted from the total amount associated with the platelets, and specific binding was expressed per 106 platelets.

Since the fibrinogen binding observed at low temperatures and during rewarming might result from alterations in the fibrinogen molecule rather than the platelets, suspensions of platelets were incubated as described but without fibrinogen. At each temperature, 1 ml of GFP was added to 0.1 ml of labeled fibrinogen at 22°C. The platelet-fibrinogen mixture was immediately returned to the desired temperature, and binding was assessed 1 min later. Similar studies were carried out in the presence of EDTA.

The kinetics of the fibrinogen interaction with chilled platelets was determined in three experiments using either a constant amount of 125I-fibrinogen (17,000 cpm/25 μl) to which increasing amounts of purified fibrinogen were added or increasing amounts of a mixture of labeled and unlabeled fibrinogen (specific activity 6500 cpm/μg). In two experiments, binding was measured after incubating the platelets with the labeled fibrinogen in the cold for 20 min. In a third experiment, the platelets were chilled for 20 min before adding labeled fibrinogen. Binding was assessed after 1 min and compared to fibrinogen binding induced by 10 μM ADP at room temperature. Nonspecifically bound fibrinogen was determined as the amount bound in the presence of 10 mg/ml unlabeled fibrinogen and was subtracted from the total amount of fibrinogen associated with the platelets. Specific binding was expressed as ng/106 platelets. Saturation curves were constructed and analyzed by the method of Scatchard.

Effect of Metabolic Inhibitors on ADP-Induced Fibrinogen Binding and Fibrinogen Binding Induced by Chilling and Rewarming

Platelets were incubated at 37°C for 30 min with 7.3 μM ANT and 4.87 μM DG. The final ethanol concentration in the platelet suspensions never exceeded 0.1%. GFP were not used in these studies because they lost much of their responsiveness to ADP after 30 min at 37°C, although their aggregability was quite stable at room temperature for up to 2 hr. Instead, the platelets were separated by bringing platelet-rich plasma to pH 6.5 with citric acid and centrifuging at 1000 g for 20 min. The platelet pellet was resuspended in a small volume of isotonic saline to minimize the possibility of spontaneous aggregation during resuspension and immediately brought to 300,000–500,000 platelets/μl with HBMT. This method separated platelets from most of the fibrinogen in plasma since they did not aggregate with ADP alone, and the amount of ADP-induced fibrinogen binding observed even at very low fibrinogen concentrations (50 ng/ml) did not differ markedly from that observed with GFP. However, since the precise fibrinogen
FIG. 1. Amount of fibrinogen specifically bound to gel-filtered platelets initially at 37°C, following stimulation with 10 μM ADP in the cold, and during and after rewarming. Mean ± SEM of 4 experiments; ADP, 3 experiments. Fibrinogen binding during chilling and rewarming in the presence of 208 μM colchicine (●), 2 experiments.
spiny spheres with pseudopods and clumped when shaken during rewarming like ethanol-treated control platelets. There was no significant difference by analysis of paired samples between the amount of fibrinogen bound to control platelets and to platelets treated with ANT and DG (Table 1).

**Fibrinogen Binding to Formalin-Fixed Platelets**

Platelets fixed prior to stimulation with ADP were disc-shaped and failed to bind fibrinogen specifically. Platelets fixed after ADP-treatment resembled spiny spheres with pseudopods and took up considerably more fibrinogen. The increase of radioactivity was abolished in the presence of a large excess of unlabeled fibrinogen but not by bovine serum albumin. Fixed ADP-treated platelets failed to aggregate when shaken with fibrinogen.

The curve for specific fibrinogen binding to fixed ADP-treated platelets was similar to that noted in earlier studies on unfixed platelets, but at saturation,

![Fibrinogen Saturation Curve](image)

**Table 1. Effect of Antimycin A (ANT) and 2-Deoxy-D-Glucose (DG) on Platelet Aggregation and Fibrinogen Binding Induced by ADP and Chilling**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Treatment</th>
<th>Temperature (°C)</th>
<th>Percent Bound per 10⁶ Platelets</th>
<th>Aggregability*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>0.1% Ethanol</td>
<td>22</td>
<td>3.99 (3.98, 4.0)†</td>
<td>+</td>
</tr>
<tr>
<td>ADP</td>
<td>7.3 μM ANT + 4.9 mM DG</td>
<td>22</td>
<td>1.0 (1.0, 1.0)</td>
<td>+</td>
</tr>
<tr>
<td>Cold</td>
<td>0.1% Ethanol</td>
<td>4</td>
<td>5.52 ± 1.56†</td>
<td>−</td>
</tr>
<tr>
<td>Cold</td>
<td>0.1% Ethanol</td>
<td>15</td>
<td>4.68 ± 3.76</td>
<td>+</td>
</tr>
<tr>
<td>Cold</td>
<td>0.1% Ethanol</td>
<td>37</td>
<td>0.36 ± 0.02</td>
<td>−</td>
</tr>
<tr>
<td>Cold</td>
<td>7.3 μM ANT + 4.9 mM DG</td>
<td>4</td>
<td>5.62 ± 2.84</td>
<td>+</td>
</tr>
<tr>
<td>Cold</td>
<td>7.3 μM ANT + 4.9 mM DG</td>
<td>15</td>
<td>5.10 ± 2.08</td>
<td>+</td>
</tr>
<tr>
<td>Cold</td>
<td>7.3 μM ANT + 4.9 mM DG</td>
<td>37</td>
<td>0.48 ± 0.28</td>
<td>−</td>
</tr>
</tbody>
</table>

*With 1 mg/ml unlabeled fibrinogen.
†Mean and range of two experiments.
‡Mean ± SEM of five experiments.
Fig. 3. Comparison of specific fibrinogen binding to ADP-treated fixed and unfixed platelets (specific activity of fibrinogen, 3000 cpm/μg). Unfixed platelets (upper curve), from results of previous experiments.9 Fixed platelets (lower curve), mean ± SEM of 5 experiments.

Fixed platelets bound approximately 30% less fibrinogen than unfixed platelets (p < 0.05) (Fig. 3).

Specific fibrinogen binding to ADP-treated fixed platelets was significantly reduced in the presence of 2.5 mM EDTA (Fig. 4 A and B) (p < 0.05 for paired samples). In contrast, the presence of EDTA during stimulation of unfixed platelets with ADP did not significantly decrease (p > 0.1) the amount of fibrinogen bound to the platelets after they had been fixed and washed (Fig. 4D).

Similar results were obtained when examining the effect of low pH. Fibrinogen binding was low when fixed ADP-treated platelets were exposed to fibrinogen (p < 0.05) at 6.5 (Fig. 4C), but was not significantly reduced (p > 0.1) when the platelets were stimulated with ADP at low pH before fixation and washing and incubated with fibrinogen at pH 7.4 (Fig. 4E).

DISCUSSION

This study indicates that chilling platelets for 20 min induces specific fibrinogen receptors that are similar both in number and affinity to receptors exposed by ADP at room temperature. However, chilled platelets, whether or not they have been treated with ADP, fail to aggregate when shaken in the presence of fibrinogen unless they have been warmed.11,12,21

Platelets in chilled suspensions aggregated about 15 sec after the suspensions were transferred to 37°C and shaken. Much but not all of the fibrinogen bound in the cold had dissociated when binding was measured in parallel unshaken samples shortly afterwards, but about 6300 molecules of fibrinogen remained associated per platelet. This is similar to the amount of fibrinogen bound by platelets stimulated with ADP at the lowest fibrinogen concentration necessary to support aggregation.9 Disaggregation was rapid, paralleling the dissociation of fibrinogen rather than return to the discoid shape.

About 20% of the specifically bound fibrinogen remained associated with the platelets after rewarming at 37°C for 20 min. Similarly, about 20% of the fibrinogen bound 1 min after the platelets were exposed to ADP could not be removed by adding EDTA or destroying the ADP with the enzyme apyrase.9 Similar observations were made by others.24 Possibly this fibrinogen had been internalized.

Fibrinogen binding during chilling and rewarming is similar whether platelets are chilled and warmed in the presence of ligand or whether fibrinogen is added to the chilled platelets at various temperatures 1 min before measuring binding. The crucial step is the chilling of platelets for 20 min, which argues against the possibility of fibrinogen binding resulting from changes in the fibrinogen molecule itself.

Binding to both chilled and ADP-treated platelets is inhibited by PGE1 and dibu-cAMP and failed to occur with thrombasthenic platelets, suggesting that the same fibrinogen receptors are exposed. Neither platelets treated with PGE1 or dibu-cAMP, nor thrombasthenic platelets, aggregate when shaken at 37°C with ADP21,25 or during rewarming in the presence of fibrinogen. The severe deficiency in surface membrane glycoproteins IIb and IIIa in thrombasthenic platelets26 is presumably associated with their failure to bind fibrinogen.
Although chilling platelets causes them to change from discs to spiny spheres\textsuperscript{22,24,27} as well as to bind fibrinogen, shape change cannot be equated with exposure of fibrinogen receptors. Thus, thrombasthenic platelets, like normal platelets in the presence of EDTA, change their shape in the cold but fail to bind fibrinogen. Fibrinogen dissociates very rapidly from chilled platelets as they are warmed, although they remain as spiny spheres for some time thereafter. Colchicine does not prevent the dissociation of fibrinogen bound in the cold, although it prevents the chilled platelets from resuming their discoid shape. Since colchicine\textsuperscript{28} and chilling\textsuperscript{27} cause platelets to become spiny spheres by inhibiting microtubule polymerization, dissolution of microtubules thus does not result in the appearance of specific fibrinogen receptors. The shape change induced by ADP appears to have a different basis as it is inhibited by cytochalasin D (unpublished data), PGE\textsubscript{1}, and dibu-cAMP, which do not inhibit the shape change induced by cold. However, ADP-induced shape change can also not be equated with fibrinogen binding since ADP induces the appearance of fibrinogen receptors even when cytochalasin B has prevented the platelets' shape change from discs to spiny spheres.\textsuperscript{29}

Stimulus-response coupling in platelets has been attributed to an increase in the concentration of cytoplasmic calcium ions.\textsuperscript{16} Exposure of fibrinogen receptors is probably part of this response, inasmuch as receptor exposure by both ADP and chilling is blocked by PGE\textsubscript{1} and dibu-cAMP. PGE\textsubscript{1} elevates the level of cyclic AMP in platelets,\textsuperscript{17,30} which in turn can activate a system for ATP-dependent sequestration and hence reduction of cytoplasmic calcium.\textsuperscript{16}

ADP-induced aggregation and exposure of fibrinogen receptors was markedly inhibited in platelets in which the intracellular concentration of metabolic ATP had been reduced by incubation with metabolic inhibitors. It is not clear whether ATP is consumed during exposure of fibrinogen receptors and aggregation or whether it is necessary to maintain the platelets in readiness to respond to a stimulus.\textsuperscript{18} It was reported that more prolonged incubation of platelets with metabolic inhibitors was necessary to inhibit aggregation induced by cold than by ADP.\textsuperscript{11} Similarly, we found that platelets incubated with these inhibitors for 20 min showed markedly diminished aggregation and fibrinogen receptor exposure after stimulation with ADP but no decrease in these responses after stimulation by chilling. Thus, platelet activation due to chilling appears to bypass an ATP-dependent step.

Extracellular calcium (or possibly magnesium) is essential for both fibrinogen binding and aggregation. ADP-treated fixed platelets were used to determine whether this calcium was necessary for fibrinogen receptor exposure or fibrinogen binding to exposed receptors. Formalin fixation of ADP-treated platelets caused only a moderate reduction in fibrinogen binding. By comparing the effect of adding EDTA or lowering the pH before and after fixing ADP-treated platelets, we established that external calcium is not required for exposure of fibrinogen receptors, but rather is necessary for fibrinogen to combine with exposed receptors.

There are several possible explanations for this. Although calcium is not taken up by platelets during stimulation with ADP,\textsuperscript{31,32} it has recently been demonstrated that platelets from thrombasthenic subjects or platelets incubated with EDTA for 8 min at 37°C, pH 7.8, showed a striking deficiency in their ability to take up calcium following mild calcium deprivation.\textsuperscript{32,33} This suggests that part of the platelet surface calcium may normally be associated with fibrinogen receptors and form bridges to fibrinogen. It is also possible that the calcium maintains the receptor in a conformation favorable for combining with its ligand. This possibility seems unlikely as calcium can restore the fibrinogen-binding ability of receptors on fixed platelets, which probably cannot undergo a conformational change. Still another explanation for the inability of fixed ADP-treated platelets to bind fibrinogen in the presence of EDTA or low pH is that these conditions affect the fibrinogen molecule. This also seems unlikely since EDTA prolongs the clotting time of fibrinogen only after incubation,\textsuperscript{34} but inhibits binding of this protein to platelets at once.

Fixed ADP-treated platelets, like chilled platelets and chilled ADP-treated platelets,\textsuperscript{23} fail to aggregate when shaken in the presence of sufficient fibrinogen. Thus, fibrinogen binding is necessary but not sufficient for platelet aggregation. Perhaps the fibrinogen molecules must assume a specific orientation once bound to the platelet receptor in order to support aggregation. This orientation may be altered in the presence of EDTA, at a low pH, or even by binding to fixed ADP-treated platelets. On the other hand, membrane fluidity or receptor mobility may be essential for platelet aggregation. Membrane fluidity is reduced at low temperatures,\textsuperscript{35} and the crosslinking of membrane proteins with formalin\textsuperscript{36,37} may prevent receptor movement in the lipid bilayer. Perhaps fibrinogen receptors must be able to cluster to permit a number of interplatelet fibrinogen bridges to form close together, or fibrinogen, a dimeric molecule, crosslinks receptors in a phenomenon like patching,\textsuperscript{38} which in turn causes the platelets to become sticky and adhere to each other.

The present studies have provided some new
insights into the mechanism of fibrinogen receptor exposure and platelet aggregation. Both ADP and chilling activate platelets (i.e., expose fibrinogen receptors) via a common pathway that is blocked by elevating cAMP and is independent of platelet shape. Chilling, however, can bypass a seemingly ATP-dependent step necessary for ADP-induced fibrinogen receptor exposure. Once exposed, the receptors will bind fibrinogen unless EDTA is present or the pH is at 6.5. Although failure to bind fibrinogen seems to result in the inability of platelets to aggregate, fibrinogen binding alone is not sufficient to support aggregation. Receptor mobility, as well as the proper orientation of the fibrinogen molecule on its receptor, may be of significance.

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REFERENCES


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