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EFFECTS OF VARIOUS INHIBITORS ON PLATELET ACTIVATION INDUCED BY TP 82, A CD 9 MONOCLONAL ANTIBODY

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Abstract—TP 82, a monoclonal antibody against CD 9 antigen, induced human platelet activation at concentrations higher than 0.4 μg/mL in terms of aggregation, release of intracellular granule contents, production of arachidonic acid metabolites, and elevation of the intracellular Ca^{2+} concentration. The effects of a competitive inhibitor of ADP, acetylsalicylic acid, EGTA, and GRGDSP which blocks fibrinogen binding to IIb/IIIa complex suggested that each of released ADP, thromboxane A_2, extracellular Ca^{2+}, and close cell contact acts together to potentiate platelet activation induced by TP 82. While each of these inhibitors severely suppressed platelet activation induced by lower concentrations of the antibody (≦0.8 μg/mL), that induced by higher concentrations (≧3.2 μg/mL) was only partially blocked. Intracellular Ca^{2+} elevation was totally dependent upon the production of thromboxane A_2, regardless of the antibody concentrations.

We have previously reported that a monoclonal antibody, TP 82, raised against human platelet membranes, induces irreversible aggregation of platelets accompanied with thromboxane A_2 (TXA_2) formation and the release of intracellular granule contents [1, 2]. This antibody recognizes a 23 KDa glycoprotein that corresponds to the CD 9 antigen designated at the First International Workshop on Human Leukocyte Differentiation Antigens [3]. To the present date, there have been several reports that the monoclonal antibodies that react with 22–24 KDa glycoprotein in platelet membranes have stimulatory effects on platelet functions [1, 2, 4–8], and most of these antibodies have been found to fall in the category of the CD 9 cluster.

Although attempts have been made to elucidate the mechanism through which these antibodies activate platelet functions, the particular signal transduction pathway involved has not been elucidated thus far. This situation stems from the differences in the effects that have been recorded for various metabolic inhibitors on CD 9 cluster-induced platelet activation among reporters. Miller *et al.* [5] and Hato *et al.* [6] reported on almost complete inhibition of serotonin release by extracellular Ca^{2+} depletion, while Boucheix *et al.* found that extracellular Ca^{2+} depletion does not inhibit release of intracellular granule contents [4]. Hato *et al.* [6] found that aspirin inhibited serotonin release (by 60%), while Higashihara *et al.* [1] found only slight inhibitory effect of aspirin (<5%).

Effectiveness of inhibitors is often dependent upon the concentration of platelet activators, including collagen and thrombin [9–12]. We noted that previous studies on the effects of inhibitors on CD 9 cluster-induced platelet activation appeared to deal with only a fixed concentration of the antibody. This was what led us to evaluate effects of various inhibitors on platelet activation induced by different concentrations of TP 82. In the present study, we found that the effects of various inhibitors greatly differ according to the concentration of the antibody, and suggest that the mechanism of platelet activation differs between low dose and high dose of TP 82.

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MATERIALS AND METHODS

Agents. Aequorin was obtained from Baxter-Travenol (Tokyo, Japan). Gly-Arg-Gly-Asp-Ser (GRGDSP), a tetrapeptide that inhibits fibrinogen binding to platelets, was obtained from Peptide Institute (Osaka, Japan). HHT, 12-HETE, Prostaglandin E_1 (PGE_1), and Prostaglandin B_2 (PGB_2) were obtained from Funakoshi (Tokyo, Japan). Phosphocreatinine, creatine phosphokinase, 5′-fluorosulfonylbenzoyladenosine (FSBA), adenosine deaminase, acetylsalicylic acid, ethylene glycol bis(*b*-aminoethyl ether)-*N*,*N*,*N*′-*N*′-tetraacetic acid (EGTA) were purchased from the Sigma Chemical Co. (St Louis, MO). [^{3}H]Serotonin was obtained from Amersham International (Bucks, U.K.). All other laboratory reagents and solvents were of analytical grade or better. Modified Hepes–Tyrode's buffer containing 129 mM NaCl, 2.8 mM KCl, 0.8 mM KH_2PO_4, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 10 mM Hepes pH 7.15, and 5.5 mM glucose, was passed through Millipore filters, and stored at 4° until use.

Preparation of platelets. Acid citrate dextrose solution was added (1/9, v/v) to fresh blood drawn from

healthy human donors who had not been on any medication for 2 weeks preceding the experiment. The blood was centrifuged at 60 *g* for 15 min to obtain platelet-rich plasma. Platelets were washed twice with Hepes–Tyrode's buffer with 1 μM PGE_1 and resuspended in Hepes–Tyrode's buffer at a concentration of 2×10^5 cells/μL, unless otherwise stated.

Aequorin-loading and measurement of intracellular Ca^{2+} *elevation* ($[Ca^{2+}]i$). Aequorin loading was performed essentially as described by Johnson *et al.* [13]. Platelets in platelet-rich plasma were washed once with Hepes–Tyrode's buffer with 10 mM EGTA, and the cells were resuspended and incubated for 1 hr at 0° in each of the following solutions:

Solution A: NaCl 150 mM, Hepes 5 mM, ATP 5 mM, $MgCl_2$ 2 mM, EGTA 10 mM, aequorin 0.2 mg/mL, PGE_1 1 μM;

Solution B: NaCl 150 mM, Hepes 5 mM, ATP 5 mM, $MgCl_2$ 10 mM, EGTA 0.1 mM, PGE_1 1 μM.

At the end of the second incubation, platelets were separated from unloaded aequorin by Sepharose CL-2B gel filtration using Hepes–Tyrode's buffer as elutant. Aequorin-loaded platelets were resuspended at the concentration of 2×10^5 cells/μL in Hepes–Tyrode's buffer containing 100 μM Ca^{2+}. One milliliter of this suspension was used for measurement of aggregation and aequorin luminescence in a Platelet Ionized Calcium Aggregometer (Chrono-Log, PA, U.S.A.). Calibration of aequorin light signals was essentially as described by Johnson *et al.* [13].

Aggregation. Platelet aggregation was simultaneously measured with aequorin luminescence using the Platelet Ionized Calcium Aggregometer. Maximum aggregation slope (MAS) was determined essentially as described by Ware *et al.* [14].

Analysis of arachidonic acid metabolites. Production of arachidonic acid metabolites from endogenous arachidonic acids was measured by HPLC. We preferred to measure directly the total production of HHT, a cyclooxygenase product, and 12-HETE, a 12-lipoxygenase product, rather than determining relative production of arachidonic acid metabolites from exogenously-incorporated radiolabeled arachidonic acid, since it has been recently shown that exogenous radiolabeled arachidonic acid is unevenly incorporated into different kinds of phospholipids [15]. HHT and 12-HETE production was measured simultaneously with aequorin-detected $[Ca^{2+}]i$. In brief, 10 min after the addition of stimuli, during which $[Ca^{2+}]i$ and aggregation were measured, the cell suspension was added to 4 vol. ethyl acetate. The mixture was acidified by 0.2 M HCl to pH 3.0, and 450 ng PGB_2 was added as an internal standard. Extraction and evaporation were performed essentially as described by Kanaji *et al.* [16], and the evaporated residue was redissolved in 100 μL methanol and subjected to reversed-phase high performance liquid chromatography using a TSK-Gel ODS-80T_M (4.6 × 150 mm, Toyo Soda, Tokyo, Japan); the mobile phase consisted of methanol/water/acetic acid (75:25:0.01, v/v) at a flow rate of 1 mL/min. Column effluent was monitored at 275 nm for PGB_2, and at 235 nm for HHT and 12-HETE. HHT and 12-HETE were identified by the chromatographic behaviors of each authentic sample. The amount of HHT or 12-HETE was quantitated by comparing their peak areas with that of the internal standard.

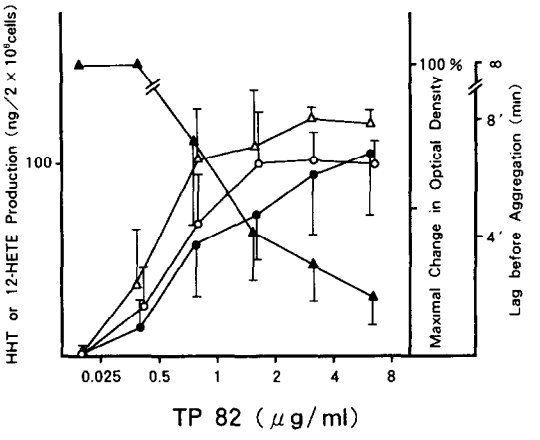

Fig. 1. Dose-dependent activation of platelets induced by TP 82. Various concentrations of TP 82 were added to aequorin-loaded platelet suspensions, and the lag before aggregation (▲—▲), maximal aggregation slope (△—△). HHT production (○—○), and 12-HETE production (●—●) were measured as described in Materials and Methods. The data are presented as the means ± SD of four experiments.

Release of $[^3H]$*serotonin.* For the incorporation of $[^3H]$serotonin, platelet-rich plasma was incubated with $[^3H]$serotonin (1 μCi/1 mL of PRP, 10–20 Ci/mmol) for 30 min at 37°. At the end of incubation, the cells were washed twice and resuspended at a concentration of 2×10^5 cells/μL in Hepes–Tyrode's buffer containing various concentrations of Ca^{2+}. An appropriate concentration of agonists was added to 0.5 mL of the platelet suspension constantly stirred in an aggregometer cuvette at 37°, and after 10 min, the reaction was terminated with 1% formaldehyde. The samples were rapidly centrifuged and the radioactivity of the supernatants was determined by liquid scintillation counting. The release of $[^3H]$serotonin was quantified by expressing the released label as a percentage of the total radioactivity incorporated into platelets.

RESULTS

Time course of TP 82-induced platelet activation

TP 82, above the concentration of 0.4 μg/mL, induced platelet activation in terms of aggregation, the production of arachidonic acid metabolites, and release of intracellular granule contents. There is typically a lag phase before any change occurs, the length of which is prolonged in inverse proportion to the antibody concentration (Fig. 1).

Figure 2 shows the time course of platelet activation induced by 1.6 μg/mL TP 82. As the optical density of the platelet suspension decreases, indicating the beginning of aggregation, there begins a sharp increase in $[Ca^{2+}]i$, HHT and 12-HETE production, and serotonin release. It is of interest to note that the $[Ca^{2+}]i$ rise is biphasic; just prior to the

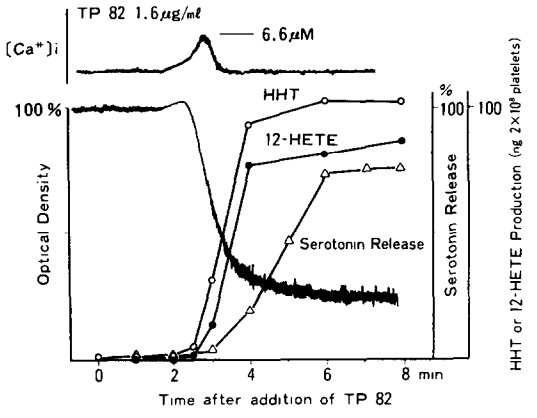

Fig. 2. Time course of TP 82-induced platelet activation. TP 82 at a concentration of 1.6 μg/mL was added to a aequorin-loaded platelet suspension, and $[Ca^{2+}]i$ elevation, aggregation, production of HHT (○—○), and 12-HETE (●—●), and serotonin release (△—△) were measured thereafter. The data are representative of four experiments.

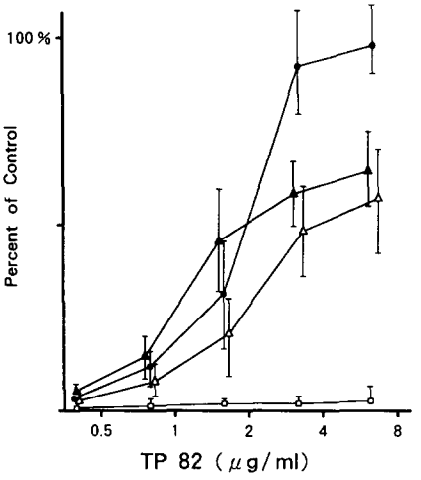

Fig. 3. Effect of acetylsalicylic acid on TP 82-induced platelet activation. Acetylsalicylic acid (0.5 mM) was added to a platelet suspension, and the mixture was incubated for 5 min at 37°. After incubation, various concentrations of TP 82 were added, and $[Ca^{2+}]i$ elevation (□—□), aggregation (△—△), serotonin release (▲—▲), and 12-HETE production (●—●) were measured. The data are presented as the means ± SD of three experiments (percentage to the control data obtained with the corresponding concentration of TP 82 in the absence of acetylsalicylic acid).

overt aggregation, there is a slight increase in $[Ca^{2+}]i$ which is followed by a larger increase during aggregation.

Role of thromboxane$_2$ (TXA_2) in TP 82-induced responses

TXA_2, which is synthesized via the cyclooxygenase pathway in platelets, is known to play a pivotal role in platelet activation induced by various agonists. We evaluated the role of TXA_2 in TP 82-induced platelet activation by the use of acetylsalicyclic acid, a cyclooxygenase inhibitor. Preincubation with 0.5 mM acetylsalicylic acid for 5 min prior to TP 82 stimulation severely suppressed all parameters of platelet activation induced by low concentrations of the antibody (≦0.8 μg/mL). However, aggregation and serotonin release were partially overcome by higher concentrations of the antibody, and the production of 12-HETE, a metabolite of arachidonic acid, was induced at an almost same magnitude as that of the control. In great contrast with other parameters, a rise in $[Ca^{2+}]i$ was never observed in the presence of acetylsalicylic acid at all the concentrations of TP 82 tested in the present study (Fig. 3). These findings suggest that the production of TXA_2 is a prerequisite for a rise in $[Ca^{2+}]i$, while the release of intracellular granule contents, aggregation, and phospholipase A_2 activation, though to a lesser degree, were partially dependent upon TXA_2 in platelet activation induced by higher concentrations of TP 82.

Role of released ADP

ADP, released from dense granules of platelets, is known to potentiate platelet response to platelet agonists. Thus, the effect of released ADP was evaluated by blocking ADP receptors on platelet membranes. Briefly, aequorin-loaded cells were incubated at room temperature for 30 min with 100 μM FSBA, an ADP inhibitor which forms a covalent binding with ADP receptors, and 2 units/mL adenosine deaminase to scavenge contaminating adenosine [17]. After incubation, platelets were washed twice and resuspended in the Hepes buffer at a concentration of 2×10^8 cells/mL. The control consisted of the platelets incubated without FSBA but otherwise treated the same. Platelets thus processed showed no aggregatory response to 10 μM ADP, while thrombin- (0.5 units/mL) induced aggregation was well preserved. FSBA-treated cells showed weaker responses to TP 82, especially that of HHT production. However, in contrast with inhibition of TXA_2 production which almost completely suppressed platelet response with lower concentration of TP 82, FSBA partially blocked platelet activation induced by the corresponding concentrations of the antibody (Fig. 4).

The combined use of phosphocreatine 5 mM and creatine phosphokinase 40 units/mL, which scavenge released ADP, gave essentially the same results with FSBA, except that the degree of inhibition was greater than that of FSBA (data not shown). This may be attributable to the inhibitory effect of this combination unrelated to its ADP-scavenging property [18]. Nonetheless, these findings suggest that released ADP is not a prerequisite for TP 82-induced platelet activation, although it serves to potentiate platelet responses.

Role of extracellular Ca^{2+}

Depletion of extracellular Ca^{2+} by 1 mM EGTA severely suppressed all parameters of platelet activation induced by lower concentrations of TP 82, while its inhibitory effect was less obvious with higher concentrations of the antibody (Fig. 5). Since $[Ca^{2+}]i$ elevation induced by lower concentrations of the

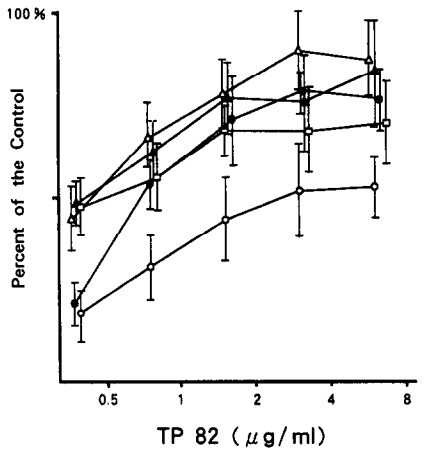

Fig. 4. Effect of an ADP receptor blocker on TP 82-induced platelet activation. A platelet suspension was incubated with FSBA (100 μM) and adenosine deaminase (2 units/mL) for 30 min at room temperature. After incubation, the cells were washed once, and resuspended in a Hepes buffer at a concentration of 2×10^8 cells/mL. Various concentrations of TP 82 were added, and $[Ca^{2+}]i$ elevation (□—□), aggregation (△—△), serotonin release (▲—▲), and production of HHT (○—○) and 12-HETE (●—●) were measured. The data are presented as the means ± SD of three experiments (percentage to the control data obtained with the corresponding concentrations of TP 82). The control cells were treated the same, except that FSBA was absent during incubation.

antibody was almost completely suppressed by extracellular Ca^{2+} depletion, Ca^{2+} influx from the extracellular fluid appears to contribute to a large part of $[Ca^{2+}]i$ elevation. In contrast, the absence of extracellular Ca^{2+} did not fully inhibit $[Ca^{2+}]i$ elevation when higher concentration of TP 82 were used. $[Ca^{2+}]i$ elevation induced by higher concentrations of TP 82 appears to consist of Ca^{2+} mobilization from intracellular Ca^{2+} storage sites and Ca^{2+} influx from the extracellular fluid.

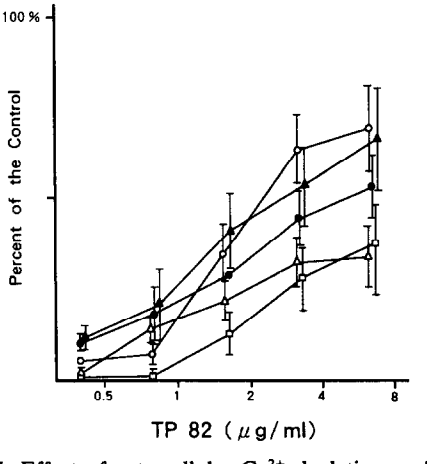

Fig. 5. Effect of extracellular Ca^{2+} depletion on TP 82-induced platelet activation. EGTA (1 mM) was added to a platelet suspension to chelate extracellular Ca^{2+}. After 3 min, various concentrations of TP 82 was added to the suspension, and $[Ca^{2+}]i$ elevation (□—□), aggregation (△—△), serotonin release (▲—▲), and the production of HHT (○—○) and 12-HETE (●—●) were measured. The data are presented as the means ± SD of three experiments (percentage to the control data obtained with the corresponding concentrations of TP 82 in the presence of 100 μM Ca^{2+}).

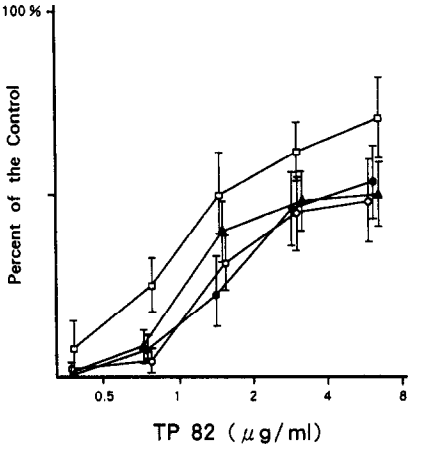

Fig. 6. Effect of GRGDSP on TP 82-induced platelet activation GRGDSP (100 μM) was added to a platelet suspension, and the mixture was incubated for 5 min at 37°. After incubation, various concentrations of TP 82 were added to the cell suspension, and $[Ca^{2+}]i$ elevation (□—□), serotonin release (▲—▲), production of HHT (○—○) and 12-HETE (●—●) were measured. The data are presented as the means ± SD of three experiments (percentage of the control data obtained with the corresponding concentrations of TP 82 in the absence of GRGDSP).

Role of cell contact

In platelet activation induced by weak agonist, cell contact via the binding of fibrinogen to IIb/IIIa complex potentiates the response [19]. We therefore evaluated the effect of GRGDSP, a tetrapeptide which blocks the fibrinogen binding site of IIb/IIIa complex, on platelet activation induced by TP 82. At our hand, 100 μM GRGDSP effectively blocked platelet aggregation induced by 0.5 units/mL thrombin. GRGDSP at this concentration completely blocked platelet aggregation induced by TP 82 over the concentration range tested in the present study. Platelet activation was severely suppressed in the presence of 100 μM GRGDSP when TP 82 was used below the concentration of 0.8 μg/mL. With higher concentrations of TP 82, GRGDSP reduced platelet responses by approximately 50% (Fig. 6). In contrast with depletion of extracellular Ca^{2+}, $[Ca^{2+}]i$ elevation appears to be least affected among other parameters of platelet activation.

DISCUSSION

In platelet activation, thromboxane A_2 formation, released ADP, extracellular Ca^{2+} and cell contact mediated by the binding of fibrinogen to IIb/IIIa complex are known to be important factors, while the requirement for these factors are different among agonists and among functional responses. In the present study, we attempted to determine which of

these factors is involved in platelet activation induced by various concentrations of TP 82.

The effects of four inhibitors or inhibitory procedures, each of which blocks one of the factors listed above respectively, were generally dependent upon the concentration of TP 82. With lower concentrations of TP 82 (0.4–0.8 μg/mL), all the parameters of platelet activation were severely suppressed by any of these inhibitory procedures, except for FSBA, a competitive blocker of ADP, which reduced platelet activation only by approximately 50%. Nonetheless, these findings suggest that thromboxane A_2 formation, released ADP, the presence of extracellular Ca^{2+}, and cell contact act together to fully activate platelets with lower concentrations of TP 82, and the defect of any of these factors severely suppresses the final responses of platelets. The absolute requirement for all these four factors for full activation is reminiscent of that induced by epinephrine, ADP, and low concentrations of collagen [9, 10, 12]. It may be that a part of the activation mechanisms employed by lower concentrations of TP 82 is also shared by that induced by so-called "weak agonists".

In contrast with platelet activation induced by lower concentrations, that induced by higher concentrations of TP 82 (3.2–6.4 μg/mL), was only partially inhibited by each of the four inhibitory procedures. Thus, although thromboxane A_2 formation, released ADP, extracellular Ca^{2+}, and cell contact act synergistically to potentiate platelet activation, the defect of any one of these factors does not completely inhibit platelet activation in terms of phospholipase A_2 activation, release of intragranule contents, $[Ca^{2+}]$i elevation, and aggregation, except for the complete cessation of $[Ca^{2+}]$i elevation by a thromboxane A_2 inhibitor. The difference in the inhibitory effects of various inhibitors between platelet activation induced by lower concentrations of TP 82 and that of higher concentrations suggests that certain additional mechanisms are operative in platelet activation induced by higher concentrations of TP 82. At the concentration range of 2 to 3 μg/mL of TP 82, almost all the CD 9 antigens on platelet membranes are bound with the antibody (unpublished data). It may be that platelet activation induced by TP 82 at subsaturating concentrations is susceptible to various inhibitors, while that with suprasaturating concentrations of TP 82 is not. At present we have no knowledge of molecular or biochemical mechanisms that may account for the differences. However, this phenomenon is not confined to platelet activation induced by TP 82. The dependence of inhibitory effects on the agonist concentration has been demonstrated with collagen and thrombin activation [9–11].

It is of interest to note that $[Ca^{2+}]$i elevation induced by TP 82 was totally dependent upon the cyclooxygenase pathway regardless of the antibody concentrations. This finding suggests that thromboxane A_2 formation is a prerequisite for $[Ca^{2+}]$i elevation induced by TP 82. Similarly, collagen-induced $[Ca^{2+}]$i has also been shown to be totally dependent upon thromboxane A_2 [20–22]. Favier *et al.* in a recent paper [23] showed that $[Ca^{2+}]$i elevation induced by two monoclonal antibodies against CD 9 (ALB6 and VI-PL3) was only partially blocked by aspirin (0.1 mM). Their findings combined with ours suggest that there may be differences in the mechanism for $[Ca^{2+}]$i elevation among CD 9 monoclonal antibodies. While inhibition of thromboxane A_2 production completely abolished $[Ca^{2+}]$i elevation induced by TP 82, suppression of three other parameters of platelet activation was only moderate when higher concentrations of TP 82 were used. This suggests that certain signal transduction pathways other than thromboxane A_2 is operative in induction of aggregation, release of intracellular granule contents, phospholipase A_2 activation induced by high concentrations of TP 82.

While CD 9 molecules, with which TP 82 reacts, are one of the major glycoproteins on platelet membranes [7], the physiological role of this glycoprotein has not been elucidated. However, a limited body of evidence suggests that it is involved in platelet activation induced by other agonists; Fab of a CD9 antibody, which lacks the platelet-stimulating activity, has been shown to inhibit platelet aggregation induced by collagen [24], and recently, the complex formation between CD9 and IIb/IIIa glycoprotein, which is a physiological fibrinogen-binding site, has been demonstrated [25]. Similarity between TP 82 and collagen, i.e. the presence of an overt lag phase before activation, total dependence of $[Ca^{2+}]$ elevation upon thromboxane A_2 production and the agonist-dose-dependent effects of various inhibitors, along with the inhibitory effect of CD9 Fab on collagen-induced aggregation, implicates that certain signal transduction pathways may be shared by collagen-induced activation and that of CD9 antibodies.

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