

Fibrinogen mitogenic effect on hemopoietic cell lines: Control via receptor modulation

(cAMP/platelet-activating factor/fibronectin/defined culture media/Arg-Gly-Asp sequence)

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ABSTRACT The possibility that the mitogenic effect of fibrinogen, a major plasma protein (3 mg/ml), is mediated by specific membrane receptors was studied. Specific binding analysis showed that fibrinogen receptors are present only on hemopoietic cell lines that respond to its mitogenic effect. The mitogenic fibrinogen receptor is not recognized by antibodies specific for the platelet fibrinogen receptor or is not competitively blocked by synthetic peptides containing the Arg-Gly-Asp sequence, which is common to fibronectin, fibrinogen, vitronectin, and other cell-attachment proteins. The lymphoma-derived pre-B-cells (Raji) have 149,000 receptors, whereas the lymphoma-derived T cells (JM), which are 3 times smaller, have 54,000 receptors. These receptors have a K_d of 2×10^{-7} M. They are inducible by stimuli specific for the cell lineage: activators of the breakdown of phosphatidylinositol phosphates, such as platelet activating factor for Raji cells, and adenylate cyclase agonists and cAMP analogues for JM cells. The stimuli have no mitogenic effect in the absence of fibrinogen; they do not change the K_d . Each stimulus increases the number of fibrinogen receptors in a dose-dependent manner, which correlates strongly ($r = -0.98$, $n = 5$) with an increased growth rate of cells in the presence of fibrinogen. This correlation concludes that the mitogenic effect of fibrinogen is controlled via receptor modulation.

Hormonal growth factors often have a high affinity for their receptors (1-3); they are present in low concentrations and have a short half-life. Some major plasma proteins such as fibronectin, vitronectin, immunoglobulins, and the third component of complement, C3, may bind to cells via specific receptors (4-6); however, these plasma proteins need not have a high affinity for their receptor in view of their long half-life and their high concentrations in the cellular environment.

We have shown that fibrinogen is mitogenic for certain hemopoietic cell lines grown in a serum-free medium as well as for human bone marrow progenitors in serum-supplemented medium (7). As fibrinogen is not present in serum but is a major plasma protein, we wanted to assess the physiological significance of this mitogenic effect by addressing the following questions. Does fibrinogen bind specifically to the cells that are triggered to proliferate? What are the characteristics of this specific binding? Does fibrinogen bind in this system via the sequence Arg-Gly-Asp, which is common to fibrinogen, fibronectin, vitronectin, and other cell-attachment proteins (8-10)? As fibrinogen is constantly available in large amounts in plasma (3 mg/ml), are the fibrinogen-specific binding sites activated only by specific stimuli? Are these stimuli the same as those that activate the specific binding of fibrinogen to platelet receptors (11-14) during

thrombus formation? Are cell growth rate and activation of specific fibrinogen receptors correlated?

MATERIALS AND METHODS

Reagents. Cell culture media, L-glutamine (200 mM), and penicillin/streptomycin (respectively, 10,000 units and 10 mg/ml of stock solution) were purchased from Aqual-Biochrom (Angoulême, France); fetal calf serum was from GIBCO; ADP, EDTA (disodium salt), dibutyryl cAMP (Bt₂cAMP), dibutyryl cGMP, cholera toxin, transferrin, and gelatin-agarose were from Sigma; ionophore A23187 was from Calbiochem-Behring; ¹²⁵I was from Amersham; synthetic decapeptide Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro was from Peninsula Laboratories (San Carlos, CA); and all other reagents were from Janssen Chimica (Beerse, Belgium). Monoclonal antibodies against platelet glycoprotein IIb-IIIa complex were gifts from B. S. Coller, J. Brochier, and J. McGregor; platelet-activating factor (PAF) was a gift from J. M. Mencia-Huerta. Synthetic hexapeptides Gly-Arg-Gly-Asp-Ser-Pro and Gly-Arg-Gly-Glu-Ser-Pro (GRGDSP and GRGESP, respectively, in the single-letter amino acid code) were a gift from R. Pytela, M. D. Pierschbacher, and E. Ruoslahti.

Cell Lines and Proliferation Assays. Raji, JM, HL60, and U937 cell lines were grown in suspension culture as described (7). Normal rat kidney (NRK) cells were grown in Dulbecco-modified Eagle medium supplemented with 10% fetal calf serum. Cells from serum or serum-free cultures were washed four times in Iscove-modified Dulbecco medium supplemented with 1% penicillin/streptomycin, 1% L-glutamine stock solutions, and transferrin at 5 μg/ml. This medium will be referred to as "defined medium." Cells were seeded at a final density of $2-5 \times 10^3$ cells per ml in a 96-well microtest III Falcon tissue culture plate, and agents were added at the specified concentrations. Cells were counted at the required time with a hemocytometer.

Fibrinogen and Fibronectin. Human fibrinogen was purified from fresh human plasma as described by Kekwick *et al.* (15). The fibronectin fraction was removed by using a gelatin-agarose column (16). Iodination was performed by the chloramine-T method as described by McConahey *et al.* (17) and modified by Marguerie *et al.* (11). NaDodSO₄/polyacrylamide gel electrophoresis as described by Laemmli (18) was used to check the purity of labeled and unlabeled fibrinogen (purity of >99%). Fibronectin was isolated from fresh human plasma as described by Ruoslahti *et al.* (16).

Abbreviations: Bt₂cAMP: N⁶,2'-O-dibutyryl cAMP; GRGDSP, single-letter amino acid code for hexapeptide Gly-Arg-Gly-Asp-Ser-Pro; GRGESP, single-letter amino acid code for hexapeptide Gly-Arg-Gly-Glu-Ser-Pro; NRK, normal rat kidney; PAF, platelet-activating factor.

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Fibrinogen-Binding Assay. Cells were washed three times in defined medium and then were incubated at 20°C with labeled fibrinogen at 50 $\mu\text{g/ml}$ (147 nM), unless otherwise specified, in saturation experiments. After various periods of incubation, 50 μl of the cell suspension was transferred onto a double-layer gradient as described (19). This gradient, composed of an upper sucrose layer and a lower hydrophobic layer, is necessary to obtain reproducible results. Each determination was made in triplicate. After 2 min of centrifugation at $12,000 \times g$, tips of the tubes were cut off with a razor blade, and radioactivity was assayed with a LKB γ counter. Specific binding was determined by calculating the difference between the values obtained in the presence and absence of a 100-fold excess of unlabeled fibrinogen.

Cell-Attachment Assay. NRK, Raji, and JM cells were prepared as described (16). Untreated microwells were coated with fibronectin (10 $\mu\text{g/ml}$) for 2 hr at 37°C. Cells were layered for 1 hr in the presence or absence of fibrinogen, fragment D, or hexapeptides GRGDSP or GRGESP as described by Pierschbacher and Ruoslahti (8).

Statistical Analysis. The theoretical number of specific binding sites and K_d values were calculated by the least-squares-fit curvilinear regression (20). The least-squares fit was obtained by successive iterations derived from the equation $B = N \times U / (K_d + U)$ in which B is the bound ligand, U is the unbound ligand, N is the number of sites, and K_d is the dissociation constant. All data were processed with an Apple IIe computer.

RESULTS

Characterization of the Binding Affinity of Fibrinogen on Cells That Do and Do Not Respond to Its Mitogenic Effect. The kinetics of specific binding on Raji cells is shown in Fig. 1. The binding was achieved within 20 min at 20°C and was reversible; most of the specifically bound fibrinogen could be displaced at 20°C by a 100-fold excess of unlabeled fibrinogen. Similar results were obtained with JM cells, which respond to the mitogenic effect of fibrinogen (not shown).

Unlabeled fibrinogen and ^{125}I -fibrinogen bound to cell lines with the same efficiency (Fig. 2). After cell solubilization with 1% Triton X-100, the membrane-bound ^{125}I -labeled ligand was found to be identical to fibrinogen on NaDodSO₄ gel (Fig. 3).

Table 1 indicates that only unlabeled fibrinogen competes with ^{125}I -fibrinogen. To verify that the small decrease of fibrinogen binding caused by fibronectin is due to unspecific

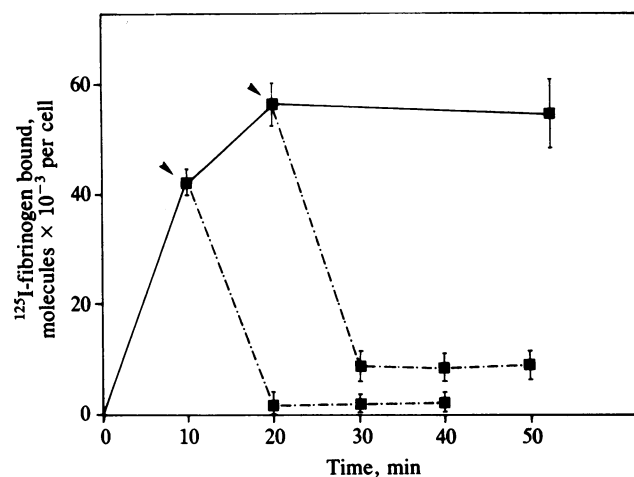


FIG. 1. Displacement of ^{125}I -labeled fibrinogen (^{125}I -fibrinogen) specifically bound to Raji cells. Arrows indicate times at which a 100-fold excess of cold fibrinogen was added (---).

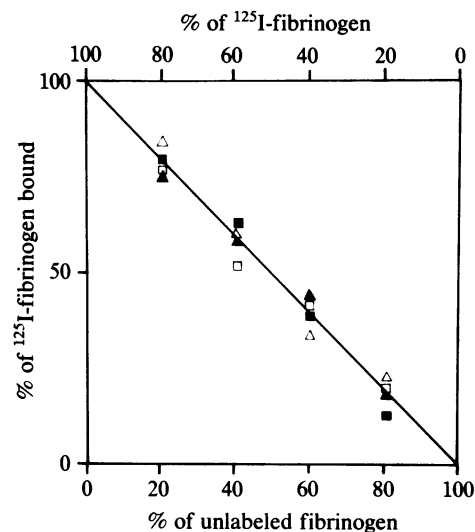


FIG. 2. Relative binding of ^{125}I -fibrinogen and unlabeled fibrinogen. Raji (■), JM (▲), HL60 (□), and U937 (△) cells at 5×10^6 cells per ml were incubated for 20 min at 20°C with a constant total concentration of fibrinogen composed of various concentrations of labeled and unlabeled fibrinogen.

interactions between these two proteins at 20°C (21), we tried to displace labeled fibrinogen with synthetic peptides that contain the cell-attachment Arg-Gly-Asp sequence of fibronectin (8–10). The peptides used (unlike fibronectin) had no interaction with fibrinogen. Although these peptides compete with fibrinogen for binding to its platelet receptor (the glycoprotein IIb–IIIa complex) and with the fibronectin and vitronectin for binding to their specific receptors or to glycoprotein IIb–IIIa complex (5, 8, 9), they do not compete at all with fibrinogen for its binding on Raji and JM cells. In

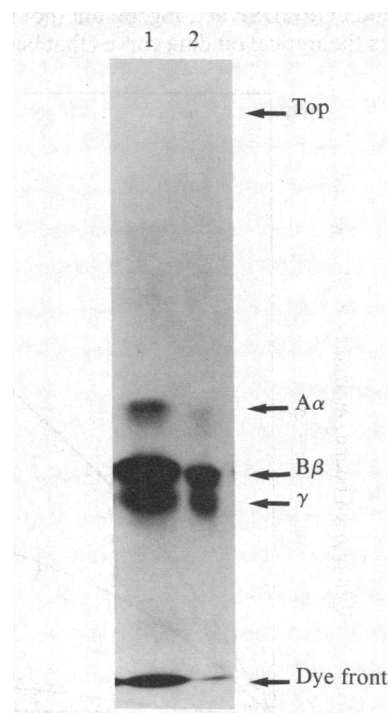


FIG. 3. Autoradiography of ^{125}I -fibrinogen (lane 1) and Raji extract (lane 2) electrophoresis on 7% NaDodSO₄/polyacrylamide gel. The samples were reduced by 2% 2-mercaptoethanol. Positions of the A α , B β , and γ chains were determined from Coomassie brilliant blue staining of unlabeled fibrinogen run in parallel.

Table 1. Competition of various proteins for the fibrinogen-specific binding site of Raji cells

Added proteins	Final concentration, μM	^{125}I -fibrinogen molecules specifically bound per cell
None	—	42,000
Unlabeled fibrinogen	15	5,100
	3	8,400
Fibronectin	3	30,000
RGDSPASSKP	2000	41,000
GRGDSP	2000	43,000
GRGESP	2000	41,000
Insulin	15	37,000
Epithelial growth factor	5	40,000
Transferrin	15	36,000
Thyroglobulin	8	40,000
Bovine serum albumin	220	43,000
IgG	3.3	40,000

^{125}I -fibrinogen ($50 \mu\text{g/ml}$; $0.15 \mu\text{M}$) was added to 5×10^6 cells per ml in the presence of competing unlabeled proteins at concentrations 20- to 10,000-fold higher. Specifically bound ^{125}I -fibrinogen was estimated after a 20-min incubation at 20°C . RGDSPASSKP, single-letter amino acid code for the decapeptide Arg-Gly-Asp-Ser, Pro-Ala-Ser-Ser-Lys-Pro.

cell proliferation experiments, the peptides containing the Arg-Gly-Asp sequence had no mitogenic effect alone at any concentration, and they did not inhibit the mitogenic effect of fibrinogen or its D fragment (not shown). In the cell-attachment assay, JM and Raji cells did not adhere in the presence of fibronectin at 10 – $50 \mu\text{g/ml}$, whereas NRK cells did. We confirmed that, as previously described (8), the hexapeptide GRGESP at 1 mg/ml in these experiments did not compete with fibronectin for NRK cell attachment, whereas hexapeptide GRGDSP at 1 mg/ml did (not shown).

Fig. 4 represents theoretical binding curves that best fit the

experimental points and provide the theoretical number of specific binding sites and the fibrinogen concentration for which half of these binding sites are saturated (K_d). As calculated by the computer program, Raji and JM cells, which respond to fibrinogen, have a K_d of 3.5×10^{-7} and $2.0 \times 10^{-7} \text{ M}$ and 149,000 and 54,000 binding sites per cell, respectively. For HL60 and U937 cells, which do not respond to fibrinogen, no saturation of specific binding sites could be observed.

Effect of Bt_2cAMP , PAF, and Other Platelet Stimuli on Fibrinogen-Specific Binding and Mitogenic Effect. Bt_2cAMP at concentrations between 10 and $100 \mu\text{M}$ increased up to 2 times the number of fibrinogen receptors on JM (Fig. 5). The increase in receptor number was well correlated ($r = -0.918$, $n = 6$) with a corresponding increase in cell proliferation when fibrinogen was added. The highest concentration (1 mM) of Bt_2cAMP was toxic for cell growth. At the optimal concentration of Bt_2cAMP ($30 \mu\text{M}$), the doubling time (17 hr) was the shortest ever observed with this cell line, whatever the culture conditions, with or without serum. There was no effect of Bt_2cAMP on growth when fibrinogen was not added. Sodium butyrate and dibutyl cGMP used under the same conditions were ineffective, but isoproterenol, 3-isobutyl-1-methylxanthine, and cholera toxin had a similar effect (data not shown). Bt_2cAMP did not activate fibrinogen receptors on Raji cells or platelets.

We compared results with various stimuli of the platelet receptor for fibrinogen to see whether they similarly increased the number of fibrinogen receptors on Raji and JM cells (Table 2).

PAF activated fibrinogen receptors on Raji but not on JM cells (Table 2 and Fig. 6). As with the effect of Bt_2cAMP on JM cells, there was a strong correlation ($r = -0.978$, $n = 5$) between the increase in receptor number and the reduction in doubling time when fibrinogen was added together with increasing PAF concentrations (Fig. 6). At the optimal concentrations of PAF (0.001 to $0.10 \mu\text{M}$), the doubling time for Raji cells was the shortest we have ever observed in the

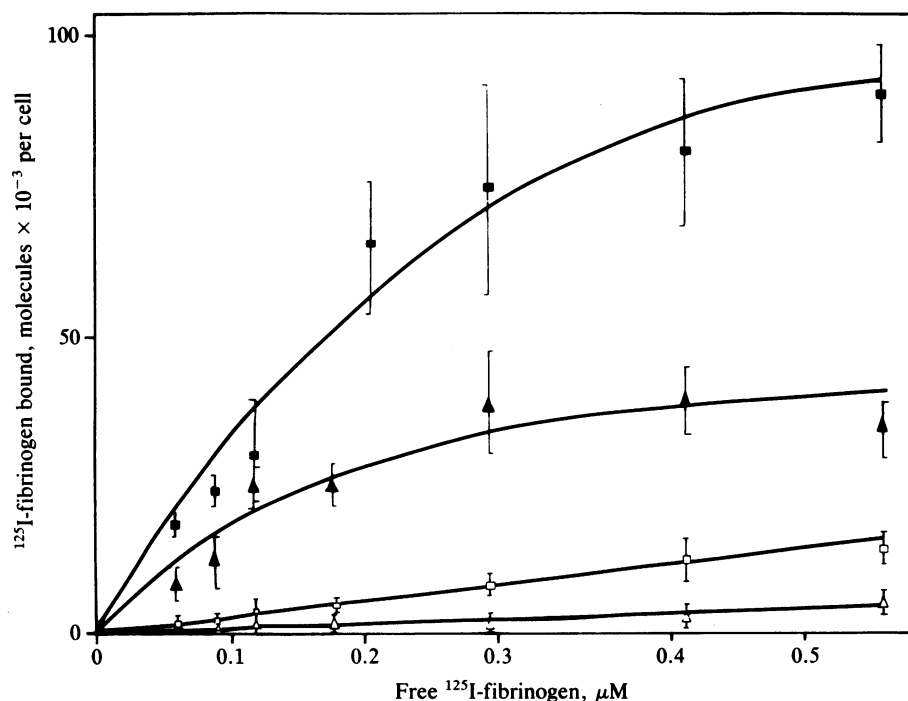


FIG. 4. Specific binding of ^{125}I -fibrinogen to various hemopoietic cell lines as a function of ^{125}I -fibrinogen concentration. Raji (\blacksquare), JM (\blacktriangle), HL60 (\square), and U937 (\triangle) cells were incubated for 20 min at 20°C with various concentrations of ^{125}I -fibrinogen.

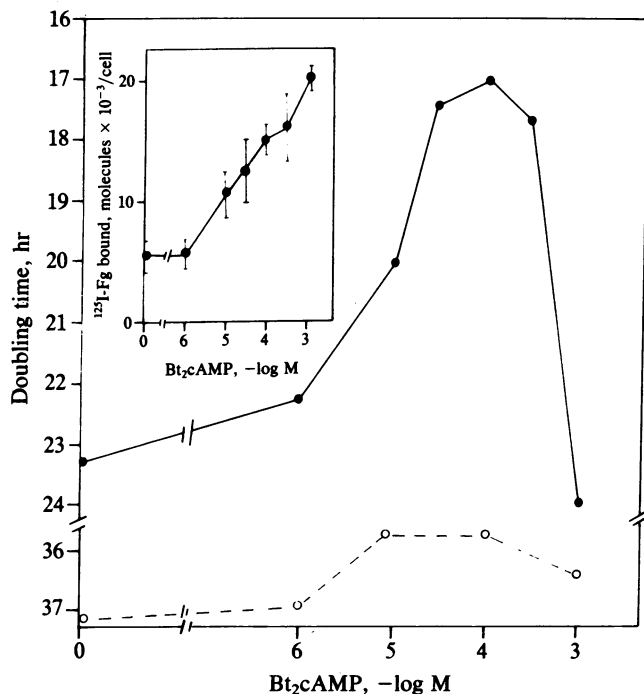


FIG. 5. Effect of various concentrations of Bt_2cAMP on JM cells: doubling time in the presence of fibrinogen at $10 \mu g/ml$ (●) or in the absence of fibrinogen (○). Cells were seeded at a final density of 2×10^5 cells per ml in defined medium with Bt_2cAMP . Cells were counted 24 hr later. (Inset) Binding assay of ^{125}I -fibrinogen (^{125}I -Fg). After counting, we washed the cells once in defined medium and processed them for specific binding at $20^\circ C$ for 30 min.

presence or absence of serum. There was no effect of PAF on growth when fibrinogen was not added.

All other factors that activate the fibrinogen receptor on platelets were without effect or exerted a negative effect on the fibrinogen receptor of JM or Raji cells (Table 2). Ca^{2+} removal, thrombin, or ADP were ineffective. Ionophore A23187, which increased fibrinogen binding on platelets, decreased by half the number of receptors both on Raji and JM cells.

DISCUSSION

Our results support the idea that the mitogenic effect of fibrinogen on two human lymphoma cell lines is mediated by a specific receptor that is inducible. The binding of fibrinogen to this receptor is specific, reversible, and saturable. K_d values are $2.0 \times 10^{-7} M$ for JM cells and $3.5 \times 10^{-7} M$ for Raji cells. This low affinity, as compared with other hormone-receptor interaction, is not surprising in view of the high concentration of fibrinogen in the plasma (3 mg/ml).

Table 2. Effect of various stimuli of fibrinogen receptors on lymphoma-derived cell lines and comparison with results of other studies made on platelets (14)

Effectors	Final concentration, μM	^{125}I -fibrinogen-bound molecules per cell		
		JM	Raji	Platelets
None	—	19,000	43,400	600
Ionophore A23187	2	10,000	22,100	19,000
PAF	0.010–0.040	19,000	78,000	23,300
Thrombin	(2 milliunits/ml)	17,500	36,700	38,700
ADP	10	17,900	42,500	27,100
ADP/EDTA	10/10,000	16,000	38,900	300

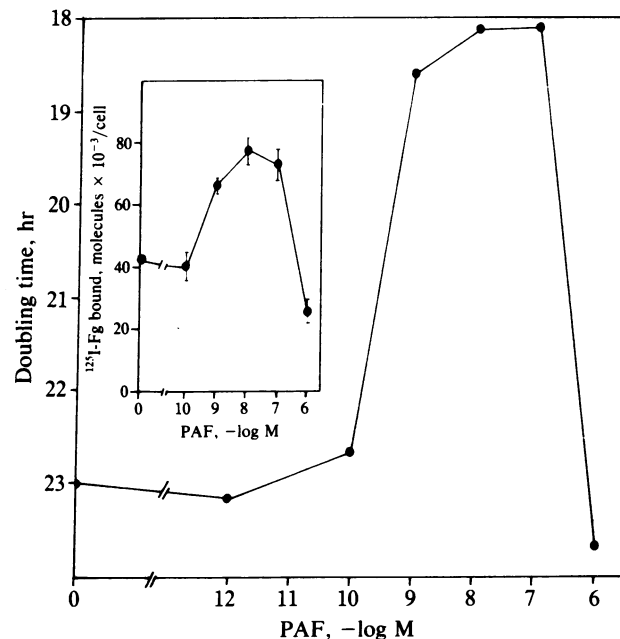


FIG. 6. Effect of various concentrations of PAF on Raji cells: doubling time in the presence of fibrinogen at $10 \mu g/ml$ (●). Cells were seeded at a final density of 2×10^5 cells per ml in defined medium with PAF. Cells without fibrinogen died. Cells were counted 24 hr later. (Inset) Binding assay of ^{125}I -fibrinogen (^{125}I -Fg). After counting, we washed the cells once in defined medium and processed them for specific binding at $20^\circ C$ for 30 min.

We previously eliminated the possibility that the mitogenic effect of fibrinogen is due to a contaminant (7). This is further ascertained by the fact that 90% of the cell-bound radioactivity is specifically bound (Table 1) and behaves as pure fibrinogen on NaDodSO₄/polyacrylamide gel electrophoresis (Fig. 3).

Only cells with specific receptors respond to the fibrinogen mitogenic effect. The T lymphoma-derived JM cells have 54,000 receptors, and the B lymphoma-derived Raji cells, which are 3 times their size, have 149,000 receptors per cell. Hemopoietic cell lines such as HL60 or U937 or fibroblast cell lines such as CCl 39 or NRK cells have no receptors and do not respond to fibrinogen.

Specific stimuli increase the number of fibrinogen receptors in a dose-dependent manner that correlates strongly with an increased cell growth rate in the presence of fibrinogen. The stimuli have little or no effect on the doubling time in the absence of fibrinogen. This correlation strongly suggests that the receptor mediates the mitogenic effect of fibrinogen.

Various growth factors (22–24) trigger the hydrolysis of phosphatidylinositol. PAF could act via a similar pathway through diacylglycerol formation and the activation of protein kinase C (25). On the other hand, Bt_2cAMP and cAMP agonists, which inhibit membrane phospholipase C activity on platelets (26), could activate phosphorylations at other levels such as the phosphorylation of the 40S ribosomal protein S6, which facilitates the initiation process of protein synthesis in early G₁ stage (27). The fact that PAF activates a B-cell lymphoma- but not a T-cell lymphoma-derived cell line and that Bt_2cAMP does the reverse offers a situation of specific control by different cell types, which can be further analyzed with molecularly defined culture media.

The main stimuli of the platelet fibrinogen receptor (glycoprotein IIb–IIIa complex) have no effect on the mitogenic fibrinogen receptor on Raji and JM cells. The glycoprotein IIb–IIIa complex on platelets is strongly dependent on Ca^{2+} (28, 29), whereas the fibrinogen receptor on lymphoma cells

is not (Table 2). Platelet fibrinogen receptors are mainly activated by ADP, the secretion of which is promoted by thrombin and ionophore A23187 (14), whereas lymphoma cell fibrinogen receptors are still activated after ADP removal (Table 2).

These observations are in agreement with unpublished results from our laboratory, which do not favor the hypothesis of identical fibrinogen receptors on platelets and on lymphoma cells. Specific antibodies against the platelet glycoprotein complex IIb-IIIa (30, 31) do not block the binding of ¹²⁵I-fibrinogen on Raji or JM cells (data not shown). In addition, indirect immunofluorescence studies with the same antibodies gave negative results on these cells. Moreover, the fibrinogen binding on the glycoprotein IIb-IIIa complex is competitively blocked by the hexapeptide GRGDSP (9), whereas the fibrinogen binding on JM and Raji cells is not (Table 1). The 25% inhibition of the fibrinogen binding when fibronectin is added in excess could be explained by interaction between fibronectin and fibrinogen at binding-assay temperatures <20°C. Fibronectin was previously named "cold insoluble globulin" because of this interaction with fibrinogen at low temperatures (21).

The mitogenic receptor for fibrinogen does not recognize synthetic peptides that contain the Arg-Gly-Asp sequence; D fragments that do not contain this sequence (32) retain the mitogenic effect of the whole fibrinogen molecule (7). These observations suggest the existence of another fibrinogen receptor that does not belong to the family of receptors that recognize the Arg-Gly-Asp sequence (5, 9, 10).

We have shown that Raji cells form polykaryons without fibrinogen and enter cytokinesis in suspension culture when fibrinogen or its D fragment are added (7). A similar effect on cytokinesis was demonstrated by Orly and Sato (33) with fibronectin, but in this case the cells flattened on a solid support on which fibronectin is anchored. Hemopoietic progenitors, unlike most normal cells, can grow in semi-solid medium with low-anchorage requirements. We suggest the possibility that fibrinogen acts as a floating extracellular matrix for cells of the hemopoietic and immune system that express receptors for it. Fibrinogen would act as a "permissive" factor (34). Other major plasma proteins such as component C3 of complement have a permissive effect on growth of specific cells (6; 35). That such proteins have important sequence homology with known growth factors (5, 6) suggests some common functions during evolution.

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