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Fibrinogen and its fragment D stimulate proliferation of human hemopoietic cells *in vitro*

(defined media/growth factors/plasma/low cell density culture/bone marrow precursor cells)

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ABSTRACT Purified fibrinogen at concentrations of 3–30 nM has been found to stimulate continuous growth of human lymphoid and myeloid cell lines under serum-free conditions. A strong proliferative response resulted from the synergism elicited by the addition of fibrinogen to transferrin-supplemented medium. This effect was observed with the pre-B-cell line Raji, the T lymphoma-derived JM, and the monocytic cell line U 937, either at high or low cell densities. With the promyelocytic cell line HL 60, fibrinogen did not shorten the doubling time of cultures seeded at high cell densities (2×10^5 cells per ml). However, at cell densities lower by 2 orders of magnitude and in the same medium, it promoted growth with a doubling time similar to that obtained at high cell concentrations. Fibrinogen also was found to increase the plating efficiency and colony size when human bone marrow cells were cultured in semisolid medium containing serum. In long-term bone marrow liquid cultures without fibrinogen, colony-forming cells were no longer detected after 6 weeks. In those cultured with fibrinogen, ≈50 granulocyte–macrophage colonies per 10^5 cells were obtained after 6 weeks, and 10, after 12 weeks. Purified fibrinogen fragment D possessed a stimulating activity similar to that of the intact fibrinogen molecule. This fragment cannot form fibrin, thus eliminating fibrin as a source of the mitogenic effect.

The use of serum in tissue culture hinders the study of the regulation of cellular proliferation (1–6) because the roles and interactions amongst various growth factors are difficult to analyze in nondefined media. Sato and his group (for a review see ref. 7) have contributed to the development of the concept of hormonally defined media and have shown how useful these media can be for analyzing the controls of proliferation and differentiation at the molecular level. Iscove and co-workers (8–10) have applied this concept to the culture of murine hemopoietic cells.

A minimal medium is composed of defined factors that are all necessary in a particular combination for the growth of a particular cell type; removal of each factor individually prevents growth. These media facilitate the study of each growth requirement and its effects. By designing minimal media containing purified plasma components, we have analyzed their possible roles in the control of human hemopoietic cell proliferation (11).

Fibrinogen is a major component of plasma, where its concentration is about 3 mg/ml. Blood clotting results from the polymerization of fibrinogen into fibrin (12). Here we present evidence that fibrinogen may play another role by stimulating the proliferation of certain human hemopoietic cells.

MATERIALS AND METHODS

Reagents. Reagents were obtained from the following sources: thrombin from Roche; horse and fetal calf serum from Flow Laboratories; RPMI 1640, Iscove's modified Dulbecco's medium (IMD medium), stock solutions of L-glutamine (200 mM), penicillin/streptomycin at 10,000 units/ml and 10,000 μg/ml, respectively, from GIBCO; and monoiodinated pig insulin from Commissariat Energie Atomique. Plasminogen and plasmin were prepared as described (13). Other reagents were from Sigma.

Fibrinogen. Purified fibrinogen was provided by G. Marguerie. This material was more than 99% pure (14). Pure fibrinogen fragments D and E were gifts from R. F. Doolittle.

Purified fibrinogen, mixed or not with radiolabeled insulin, was clotted by a 30-min treatment with thrombin (1 unit/ml), after which fibrin was extracted and washed with an equal volume of saline before being centrifuged on a Beckman Microfuge B, and the supernatant was collected.

Cell Lines. Raji, a pre-B-cell line (15); JM, derived from a human T-cell leukemia (16); U 937, a monocytic cell line (17); and K 562, an erythroid cell line (18), were provided by G. Lenoir (International Agency for Research on Cancer, Lyon, France). HL 60, a promyelocytic cell line (19), was provided by R. Gallo (National Cancer Institute, Bethesda, MD). Cell lines were maintained in suspension in RPMI 1640 medium with 2 g of sodium bicarbonate per liter, 1% penicillin/streptomycin and 1% L-glutamine stock solutions, and 10% heat-inactivated fetal calf serum in Corning TM 25 flasks. Defined media were prepared as described (11). Doubling times were estimated only when cultures contained more than 98% viable cells as determined by trypan blue exclusion.

Long-Term Human Bone Marrow Culture and Colony-Forming Cell Assays. Culture initiation was performed as described by Gartner and Kaplan (4) with the following exceptions. Growth medium consisted of IMD medium supplemented with 1 μM hydrocortisone, 15% fetal calf serum, 10% horse serum, 1% penicillin/streptomycin and 1% L-glutamine stock solutions, 10 nM transferrin, 50 μM 2-mercaptoethanol, and 3 g of sodium bicarbonate per liter. After 2 weeks, when stromal cells were established, fetal calf serum was reduced to 10% and hydrocortisone was reduced to 0.1 μM. Every week 5 ml of culture was replaced with fresh medium. Colony-forming cells were cultured in medium containing methylcellulose as described by Messner and Fauser (1).

RESULTS

Effect of Fibrinogen on Growth in Minimal Media. T and B lymphoid cell lines were studied for their response to fibrinogen in minimal media.

JM cell line. This cell line was derived from a human T lymphoma (16). When JM cells grown in medium containing 10%

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Abbreviations: CFU-GM, granulocyte–macrophage colony-forming unit; IMD medium, Iscove's modified Dulbecco's medium.

fetal calf serum were washed twice and resuspended in IMD medium at a concentration of 2×10^5 cells per ml, no residual growth was observed. However, when transferrin was added and cell concentration was carefully maintained between 2×10^5 and 1×10^6 cells per ml, a doubling time of 31 hr was observed for at least 30 days. By adding 1–10 μg of pure fibrinogen per ml (3–30 nM), growth was strongly stimulated, and a doubling time of 18 hr was obtained (Fig. 1).

When cells were seeded in IMD medium supplemented with transferrin at low density (i.e., 2×10^3 instead of 2×10^5 cells per ml), no growth occurred. However, if fibrinogen was added at 10 μg/ml, all of the single cells started to grow and formed clones, which could be isolated and expanded for continuous growth in this medium. Therefore IMD medium supplemented with transferrin and fibrinogen constitutes a minimal medium for growth of the JM cell line at low cell density.

In low cell density cultures, no stimulating cell contacts occur (20) and diffusible factors, such as T-cell growth factor (21), produced by the cells are diluted. Such conditions have been used for the proliferation of JM cells. A maximal effect was obtained with 10 μg of fibrinogen per ml—a concentration 1/300th that normally found in plasma (Fig. 2).

The mitogenic effect of fibrinogen was tested in a situation where fibrin could not be formed. Plasmin treatment progressively degrades the fibrinogen molecule producing two D fragments and one E fragment per molecule. Fragment D not only cannot form fibrin but even inhibits its formation (22). Purified fragment D, but not fragment E, had a proliferative effect similar to that of the intact fibrinogen molecule (Fig. 2). In the culture conditions described in Fig. 2, an optimal response was obtained for fragment D at 10 μg/ml (0.1 μM). These results suggest that fibrin formation is not a prerequisite for the proliferative effect of fibrinogen.

Raji cell line. The Raji cell line contains mainly pre-B-cells but early B cells are also present (23). The minimal medium used for JM did not sustain continuous growth of the Raji cells; however, it allowed growth for at least 10 divisions, whether cells were grown at low or at high cell densities. At low cell densities no residual growth was observed in IMD medium alone. Addition of transferrin allowed DNA synthesis and two karyokineses to occur. However, no cytokinesis took place because polykaryons were formed (Fig. 3) and died after 48 hr.

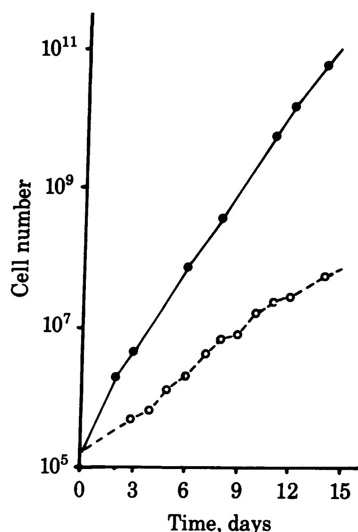

FIG. 1. Growth of JM in defined media with and without fibrinogen: cumulative cell number of cultures replated every 2 or 3 days. ---, IMD medium with 10 nM transferrin; ——, IMD medium with 10 nM transferrin and 30 nM fibrinogen.

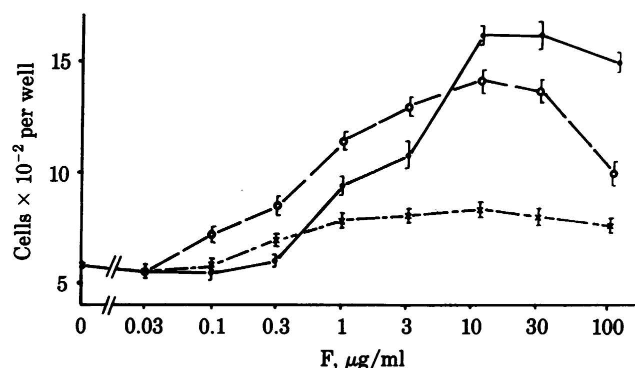

FIG. 2. Proliferative dose–response curves of JM cells. Response to various concentrations of fibrinogen (——), its fragment D (— —), or its fragment E (— - —). Cells were counted on day 3. Values are expressed as the mean of triplicate samples (±SEM). F, fibrinogen or its fragments.

When 10 μg of fibrinogen per ml was added to IMD medium containing transferrin, cytokinesis occurred normally, and <1% polykaryons were found. Fig. 3 shows groups of two cells after 24 hr, four cells after 30 hr, and eight cells after 48 hr of culture. Successive observations of cultures seeded as sparse, well-separated, single cells showed that the progressive doublings in cell number per group was not due to random agglutinations but to clonal growth. Indeed, Fig. 3 shows that the number of groups remained constant during growth. Cell division became relatively synchronized after the addition of fibrinogen, and the duration of the cell cycle was estimated to be 16–18 hr under these conditions. This value was similar to the doubling time of cultures seeded at high cell densities.

As was observed with JM, fragment D could replace fibrinogen in the minimal medium. The proliferative responses of Raji cells to various concentrations of fragments D and E are shown in Fig. 4; they are quite similar to those obtained with JM (Fig. 2).

Absence of Soluble Growth Factors in Fibrinogen Preparations. To eliminate the possibility that soluble low molecular weight growth factors might be contaminating the fibrinogen preparation, the supernatants obtained after fibrinogen clotting were tested for their ability to stimulate Raji cell proliferation. No stimulation was observed, regardless of the concentrations used (data not shown). Thrombin, at the concentration used, was neither inhibitory nor stimulatory for the growth of Raji cells. To ensure that fibrin did not trap possible growth factors during clotting, we mixed radiolabeled insulin with the fibrinogen solutions. After clotting and fibrin extraction, 81% of the radiolabeled insulin but only 2.7% of the fibrinogen proteins were found in the supernatant. The fibrinogen proteins in the supernatant approximated the amounts of peptides A and B released on clotting.

Effect of Fibrinogen on Growth in Nonminimal Defined Media. In addition to lymphoid cell lines, myeloid cell lines were studied for their proliferative responses to fibrinogen. Table 1 compares JM and Raji cells with U 937, K 562, and HL 60 cells for their respective abilities to grow in various defined media with or without fibrinogen. The effect of fibrinogen was tested on cells growing at high density (2×10^5 to 1×10^6 cells per ml) and at low cell density (<2×10^3 cells per ml).

Cultures at high cell densities. The shortest doubling times obtained with defined media (Table 1) were similar to those found with 10% fetal calf serum. When the shortest doubling times were obtained without fibrinogen in serum-free media, fibrinogen addition did not improve growth. For all cell lines other than JM, the lipid fraction *L* induced a marked growth

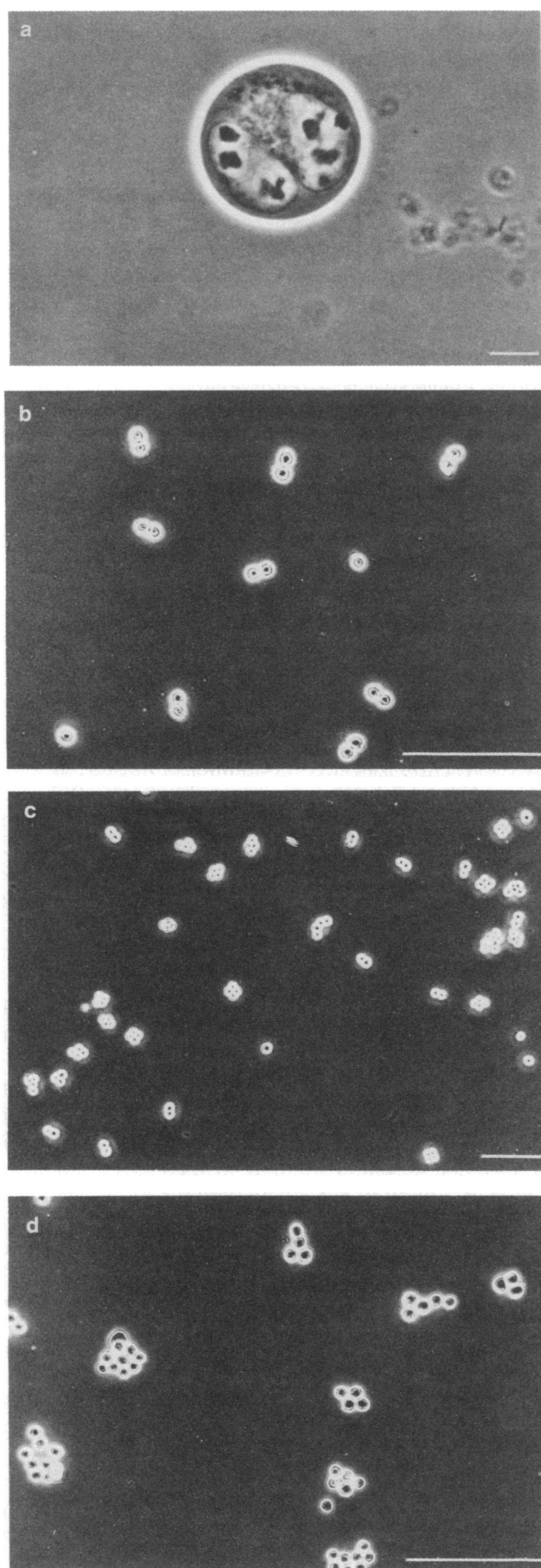

FIG. 3. Effect of fibrinogen on the proliferation of the Raji cell line. Raji cells were seeded at 5×10^3 cells per ml in 33-mm Petri dishes in a total volume of 2 ml of medium. (a) Control culture with IMD medium and transferrin: polykaryons are present after 18 hr. (Bar = 10 μm.) (b–d) Cultures in IMD medium with transferrin and 30 nM fibrinogen: 24 hr, two-cell clones (b); 30 hr, four-cell clones (c); and 48 hr, eight-cell clones (d). (Bars = 100 μm.)

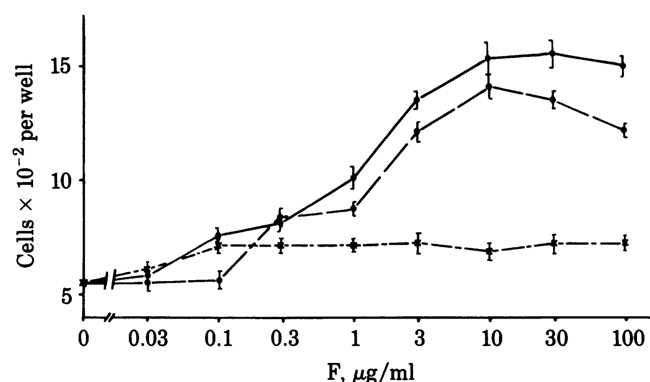

FIG. 4. Proliferative dose–response curves of Raji cells. Response to various concentrations of fibrinogen (——), its fragment D (— —) or its fragment E (— - —). Cells were counted on day 3. Values are expressed as the mean of triplicate samples (±SEM). F, fibrinogen or its fragments.

enhancement, which was not further improved by addition of fibrinogen. However, with JM, which responded poorly to the lipid fraction L, addition of fibrinogen strongly reduced the

Table 1. Effect of fibrinogen on the growth of lymphoid and myeloid cell lines in various defined media

Cell line	Cell density*	Supplements†	Doubling time, hr: No fibrinogen	Doubling time, hr: Fibrinogen
JM	>200	none	‡	‡
	>200	S	18	18
	>200	T	31	18
	>200	TL	31	18
	<2	T	‡	18
Raji	>200	none	‡	‡
	>200	S	18	18
	>200	T	§	17 (10)¶
	<2	T	§	17 (10)¶
U 937	>200	none	‡	‡
	>200	S	30	30
	>200	T	51	34
	>200	TL	34	32
	>200	TLI	34	32
	<2	T	‡	34
	1	TL	‡	ND
	3	TL	48	ND
	10	TL	37	ND
HL 60	>200	none	‡	‡
	>200	S	36	36
	>200	T	55 (11)	52
	>200	TI	48 (11)	44
	>200	TL	44	42
	>200	TLI	39	37
	<2	TI	‡	46
K 562	>200	none	‡	‡
	>200	S	24	24
	>200	L	48 (8)	42 (9)
	>200	LI	42 (9)	38 (15)
	>200	TL	28	28
	>200	TLI	30	30

ND, not determined.
* Shown as cells $\times 10^{-3}$ per ml.
† S, fetal calf serum; T, transferrin; I, insulin; L, lipid fraction (see *Materials and Methods*).
‡ No growth.
§ Polykaryons.
¶ Residual growth. The number of doublings is enclosed in parentheses.

Table 2. Effect of fibrinogen on the number and size of granulocyte–macrophage colonies in cultures seeded at various cell densities

Seeding concentration, cells/ml	Proliferation index* of n-cell colonies in the presence of fibrinogen					
	Day 3			Day 14		
	$n = 2$	$n = 4$	$n = 6–8$	$n < 20$	$n = 20–50$	$n > 50$
5×10^4	168 ± 16 (452:269)	178 ± 14 (168:94)	212 ± 18 (32:15)	— (0:0)	— (0:0)	— (0:0)
2×10^5	67 ± 10 (284:425)	150 ± 11 (180:120)	166 ± 19 (64:38)	93 ± 11 (273:293)	425 ± 72 (371:87)	2100 ± 692 (450:22)

As the optimal cell density may vary with cells from one donor to another, three experiments were performed with cells from one normal donor.

* Proliferative index of n-cell colonies in the presence of fibrinogen (10 μg/ml) is calculated as follows:

$$\frac{\text{Number of } n\text{-cell colonies with fibrinogen}}{\text{Number of } n\text{-cell colonies without fibrinogen}} \times 100.$$

The ratio of the average number of colonies in cultures with fibrinogen to that in cultures without fibrinogen is shown in parentheses.

doubling time. The synergistic effect of fibrinogen and transferrin was observed with U 937 as with JM and Raji cells but not with HL 60 cells at high cell densities. In contrast to what has been observed with other cell lines, insulin had little or no effect (11).

Cultures at low cell densities. At low cell densities, cell factors are diluted. Under these conditions, the growth of JM, Raji, U 937, and HL 60 cells, in a defined medium of IMD medium supplemented with transferrin only, was prevented. Addition of fibrinogen was then sufficient to allow growth to start. The doubling time obtained was similar to that obtained at high cell densities. On the contrary, when fibrinogen was replaced by the lipid fraction L, the doubling time was strongly dependent upon cell density as detailed in Table 1 for U 937 cells. This suggests that the effects of fibrinogen were more independent of cell factors than those of the lipid fraction L.

Effect of Fibrinogen on the Proliferation of Human Bone Marrow Colony-Forming Cells. *Colony assay.* The effect of fibrinogen on normal human bone marrow colony-forming cells grown in a medium with serum and methylcellulose was studied. Cultures seeded at 5×10^4 and 2×10^5 cells per ml with or without fibrinogen were compared (Table 2). After 3 days, regardless of the starting cell density, clones with two, four, and eight cells were more numerous in cultures supplemented with fibrinogen. Eleven days later, cultures seeded at 5×10^4 cells per ml were dead. On the other hand, in cultures seeded at the higher concentrations, addition of fibrinogen increased the number of 20–30 cell colonies by a factor of 4 and the number of colonies with more than 50 cells by a factor of 20. In the presence of erythropoietin, fibrinogen was also found to increase the number and size of erythroid colonies (data not shown).

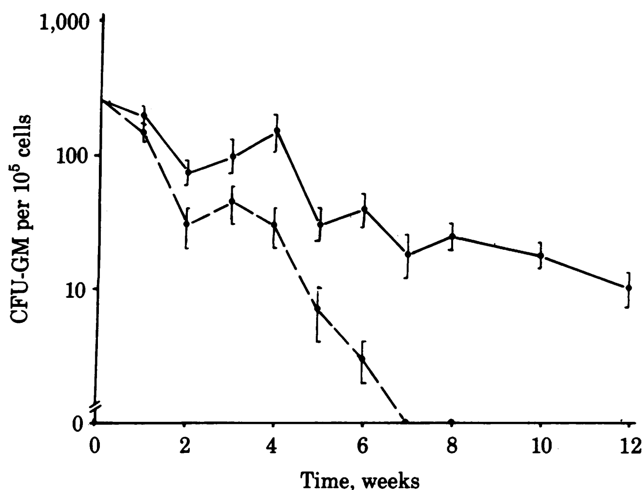

FIG. 5. CFU-GM production in long-term human bone marrow suspension cultures with (——) or without (— – —) fibrinogen. The results represent the means (±SEM) of at least three colony assays in four different flasks.

Long-term bone marrow cultures. The addition of fibrinogen to a long-term serum-containing culture greatly reduced the time-associated decrease in the production of colony-forming cells. Fig. 5 compares the number of granulocyte–macrophage colony forming units (CFU-GM) at different times during the long-term propagation of a bone marrow culture with or without addition of fibrinogen at each medium renewal. After 6 weeks, cultures with fibrinogen produced 50 CFU-GM per 10^5 cells, whereas control cultures without fibrinogen no longer produced any. After 3 months, cultures with fibrinogen still produced 10 CFU-GM per 10^5 cells. In similar experiments (data not shown), the addition of fibrinogen-free medium, after 3 weeks of culture in the presence of fibrinogen, resulted in a decrease in the number of colony-forming cells down to the level found in the control cultures to which fibrinogen was not added from the outset.

DISCUSSION

Fibrinogen at 3–30 nM (1–10 μg/ml) stimulated the proliferation of various cell types derived from the human hemopoietic system. The growth effect of fibrinogen was particularly obvious at low cell densities where cell-to-cell contact did not occur and cell factors were diluted. With JM, Raji, U 937, and HL 60 cells, fibrinogen showed a strong synergism with transferrin, allowing growth at low density to start.

We observed that purified fragment D had a mitogenic effect similar to that seen with the complete fibrinogen molecule. This indicates that the capacity of fibrinogen to stimulate proliferation does not require fibrin formation.

It is not yet clear how fibrinogen controls proliferation. In the case of Raji cells, fibrinogen stimulated cytokinesis because without it, in defined media, polykaryons were formed. This recalls the proliferative effect of fibronectin observed by Orly and Sato (24) with RF-1 fibroblasts. In this case, "fibronectin-mediated adhesion" stimulates cytokinesis of cells that are anchorage-dependent for growth. However, a different mechanism may be involved with fibrinogen because all of the cell lines described here grow in suspension culture. Fibronectin is a common contaminant of fibrinogen preparation: whether it is removed or not on a gelatin agarose column does not change

the mitogenic effect of fibrinogen.

We observed that fibrinogen was active *in vitro* at concentrations ≈1/300th that of the concentration found in plasma, and fragment D was less effective at 100 than 10 μg/ml. One possible explanation for these observations could be that the affinity of these molecules for certain cells are different *in vitro* and *in vivo*. *In vivo*, physiological mechanisms such as hemostatic controls could modify the interaction between cells and fibrinogen. For example, it has been shown that fibrinogen binds to platelets only when they are activated (14).

The effect of fibrinogen also could be observed in the presence of a serum complement in primary human bone marrow cultures. Cell heterogeneity made the analysis of fibrinogen growth-stimulating activity on these cultures difficult. However, the fact that fibrinogen increased the number of larger colonies more than it did the number of smaller ones suggests that fibrinogen is more active on the most immature colony-forming cells. Another possibility would be that fibrinogen slows down differentiation processes, allowing more cell divisions before terminal differentiation. Recently Messner *et al.* (25) have obtained improved results in mixed colony assays by replacing part of the serum with plasma. The presence of fibrinogen in plasma could explain the positive response obtained.

Further analysis is required to determine whether fibrinogen binds specifically to certain cells as has been reported for activated platelets (14). Fibrinogen also could have a binding site for a growth factor. These molecules, present in serum or produced by certain cells, could be active only after binding to fibrinogen or its fragment D which could serve as a carrier protein.

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