

Uptake of Plasma Fibrinogen into the Alpha Granules of Human Megakaryocytes and Platelets

Paul Harrison,* Barry Wilbourn,* Najet Debili,† William Vainchenker,‡ Janine Breton-Gorius,‡ Andrew S. Lawrie,* Jean-Marc Masse,‡ Geoffrey F. Savidge,* and Elisabeth M. Cramer‡

*Coagulation Research, The Rayne Institute, St. Thomas' Hospital, London, United Kingdom; †Institut Nationale de la Santé et de la Recherche Médicale (INSERM) U 91, Hôpital Henri Mondor, Creteil, France; and ‡Département d'Angio-Hématologie and INSERM U 150, Hôpital Lariboisière, 75010, Paris, France

Abstract

The origin of platelet α -granule fibrinogen (Fg), whether from endogenous synthesis or exogenous derivation, remains unknown. Although Fg biosynthesis by megakaryocytes (MK) has been suggested, recent studies have demonstrated that certain α -granular proteins originate primarily from plasma. To study the origin of α -granule Fg, platelet-associated Fg was measured by ELISA and Western blotting, and localized by immunofluorescence and immunoelectron microscopy in a patient with symptomatic congenital afibrinogenemia before and after replacement therapy with cryoprecipitate. α -Granule Fg was detected in the majority of platelets as early as 24 h post-infusion, suggesting that direct platelet uptake was occurring. Platelet Fg reached a maximum value of 42.5% of normal values at 3 d postinfusion and was localized in the α -granules, while plasma levels followed a typical half-life profile. Significant α -granule Fg was still detectable at 13 d postinfusion, with plasma Fg virtually absent. Studies on cultured CFU-MKs from the patient also confirmed that MKs can incorporate exogenous Fg into α -granules. These results indicate that platelet α -granule Fg can be derived from the circulating plasma pool and that Fg uptake can occur in both platelets and MKs.

Introduction

Platelet α -granules are known to contain a diversity of proteins including coagulation factors, which are of fundamental importance in hemostasis (1). Initially these proteins were assumed to originate from endogenous synthesis during megakaryocyte (MK)¹ development (2). Recent evidence, however, has demonstrated that certain plasma proteins such as albumin, Ig, and the tracer protein horseradish peroxidase (HRP) can be incorporated into α -granules (3–5). Although fibrinogen (Fg) synthesis by MKs has been suggested (6–8), recent *in vitro* studies have demonstrated that Fg is packaged in the α -granules of cultured MK at a later stage than other synthe-

sized proteins and only occurs when an exogenous source of the protein is present (9). Despite earlier *in vivo* and *in vitro* studies that failed to demonstrate Fg exchange between α -granules and plasma (10, 11), recent data involving *in vivo* defibrination of rats and the infusion of human Fg into guinea pigs suggest that endocytosis of plasma Fg occurs in the MK with subsequent expression of the protein in circulating platelets (12, 13).

To elucidate more conclusively the origin of platelet Fg, quantitative and qualitative measurements of platelet and plasma Fg were performed for up to 24 d in a symptomatic patient with congenital afibrinogenemia after replacement therapy with cryoprecipitate. In this manner, the kinetics of a putative Fg uptake mechanism into the MKs and platelets could be studied. *In vitro* experiments were also performed on cultured MKs from the same patient before therapy. The experimental data provide the first clear *in vivo* evidence for the endocytic uptake of plasma Fg into human platelet α -granules.

Methods

Patient. A 27-yr-old woman with a known family history of autosomal recessive afibrinogenemia and a life-long history of bleeding symptoms was admitted with menorrhagia. Representative basal plasma Fg levels were 1.8 μ g/ml Fg (antigen by ELISA), < 9 mg/dl clottable Fg (Claus technique) with a prolonged thrombin time ratio > 10.0, international normalized ratio > 11.0, and a bleeding time > 30 min (Ivy method). The patient was not receiving oral contraceptives or any other prescribed medication.

Infusion studies. We studied this patient on four separate occasions when she required treatment for severe menorrhagia. Platelet Fg was assessed qualitatively by immunoelectron microscopy (IEM) and quantitatively by ELISA and Western blotting of platelet lysates. In these studies regular samples were taken up to 24 d postinfusion of cryoprecipitate (3.4–5.3 g Fg).

Platelet preparation procedures. Venous blood was obtained by clean venipuncture using the two-syringe technique, and anticoagulated in 1:10 vol of 3.2% trisodium citrate and EDTA (10 mM). Platelet-rich plasma was prepared by centrifugation at 250 *g* for 20 min. Platelets and washed buffy coats were applied to a discontinuous arabinogalactan gradient using a modification of the method of Gralnick et al. (14). The differentiation of platelet membrane associated from internalized Fg was ensured by enzymatic digestion of surface Fg on intact platelets with 1 mg/ml pronase (4 U/mg; Sigma Chemical Co., St. Louis, MO) using a BSA control sample for 4 h at 4°C before washing and lysis. After adjustment of the platelet count to 10⁶/ μ l, the cells were lysed with 1:40 vol of 20% Triton at 37°C for 1 h and subsequently stored at –70°C. Centrifugation at 10,000 *g* was necessary to remove platelet membranes before analysis. The efficiency of removal of plasma proteins was assessed in control samples by the addition of ¹²⁵I-Fg (Amersham Corp., Arlington Heights, IL) to whole blood before platelet lysis. Quantitation of Fg in normal platelet lysates from 20 healthy donors was performed to establish a reference range.

IEM. 1 vol blood was taken into tubes containing 9 vol of fixative (3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2) according to

Address correspondence and reprint requests to Dr. Elisabeth Cramer, Département d'Angio-Hématologie, Hôpital Lariboisière, 75010, Paris, France.

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1. Abbreviations used in this paper: Fg, fibrinogen; HRP, horseradish peroxidase; IEM, immunoelectron microscopy; MK, megakaryocyte.

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Stenberg et al. (15). Cells were fixed for 40 min at 20°C, washed three times in phosphate buffer (0.1 M, pH 7.4), and embedded in glycol-methacrylate (16, 17). Cultured MKs were fixed and treated the same way. Immunocytochemistry was performed on thin sections according to the method of De Mey (18). Sections were incubated for 2 h in the presence of rabbit anti-human vWf (DAKOPATTS, Copenhagen, Denmark) or Fg (Cappel laboratories, Cochranville, PA). Goat anti-rabbit IgG coupled to 5-nm gold particles (GARG5; Janssen Pharmaceutica, Beerse, Belgium) was used as the second antibody. Samples were assessed without knowledge of their time of collection. 50 platelet sections were observed in each sample. Platelets were considered positive for Fg when at least two of five or more α -granules in platelets were labeled.

Culture and immunofluorescence procedures. PBMC from the patient (preinfusion) were separated by Ficoll-Hypaque centrifugation and cultured in Iscove's medium, 1% BSA, 5% PHA-lymphocyte-conditioned medium, and either 10% aplastic plasma or 10% aplastic serum. After 13 d culture cells were recovered, cytocentrifuged, and fixed for 1 min with methanol. Platelet samples were assessed in the same manner. They were subsequently double-labeled using rabbit anti-human Fg (Cappel Laboratories) and a pool of anti-vWf MAbs (a generous gift of Dr. D. Meyer, INSERM U 143, Hôpital de Bicêtre, Paris, France). Goat anti-rabbit IgG (rhodamine conjugated; Nordic Immunology, Tillbury, The Netherlands) and sheep anti-mouse F(ab')₂ fragments (fluorescein conjugated; Selenius, Hawthorn, Australia) were used as the secondary antibodies.

Immunological methods. Plasma and platelet vWf and Fg were assayed by standard ELISA techniques using polyclonal monospecific antibodies (Dako, High Wycombe, UK). Discontinuous electrophoresis in 2% agarose gels was performed on plasma and platelet lysates by a modification of the method of Ruggeri and Zimmerman (19). After electrophoresis the proteins were rapidly transferred to nitrocellulose by electroblotting (20). Fg and vWf-Ag were visualized using rabbit anti-human Fg or vWf-Ag as first antibody (Dako) and HRP-conjugated swine anti-rabbit IgG (Dako) as the second antibody, and developed with 4-chloro-naphthol and H₂O₂.

Results

To study the time course of the appearance of intracellular Fg, a combination of immunocytochemical techniques and biochemical studies of washed platelets, platelet lysates, and plasma were performed at various time intervals up to 24 d postinfusion on four separate occasions.

Biochemical measurements. ELISA measurements and Western blotting demonstrated the absence of Fg in the patient's platelets before therapy, with trace concentrations of Fg in the plasma (0.08% normal). Plasma Fg reached almost 50% of normal levels immediately after infusion and followed a typical half-life decay. In contrast, platelet Fg levels remained low immediately after infusion and slowly increased, reaching a maximum of ~40% normal between 2 and 4 d postinfusion while plasma levels were falling. After 5 d the platelet Fg level began to decrease, reaching 18.7% of normal levels at 7 d postinfusion. Fig. 1 shows a representative profile of plasma and platelet Fg after infusion of ~5.2 g of Fg in cryoprecipitate. At 13 d postinfusion the platelets were still found to contain significant intracellular Fg as assessed by ELISA (11.8% normal), whereas plasma Fg returned to near basal levels (1.25%). Even at 24 d postinfusion trace amounts of platelet Fg were still detectable (2.0%). The degree of Fg staining by Western blotting mirrored the antigen levels (results not shown). Comparison of platelet vWf-Ag levels by ELISA and Western blotting revealed that there were no significant differences in lysate samples, and platelet counts remained constant throughout the study ($293\text{--}302 \times 10^9/\text{liter}$).

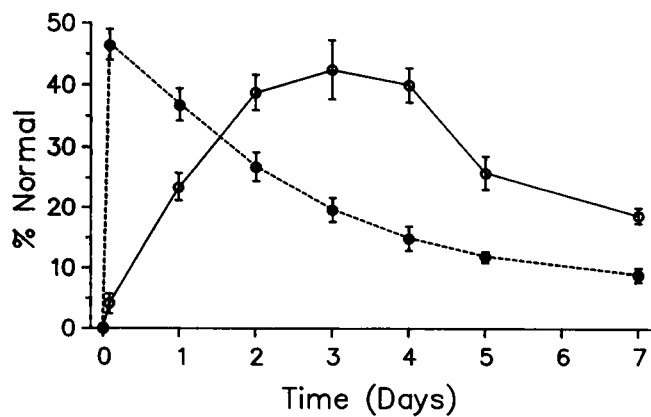


Figure 1. Time course of plasma and platelet lysate Fg levels (mean \pm SD) following infusion of cryoprecipitate (5.2 g of Fg) into a patient with symptomatic congenital afibrinogenemia. Plasma Fg (\bullet) was expressed as a percentage of normal pooled plasma (2.1 mg/ml Fg). Platelet Fg (\circ) was expressed as a percentage of an established normal range from 20 healthy donors ($74.55 \pm 20.7 \mu\text{g}/10^9$ platelets).

Immunocytochemistry. Before treatment the patient's platelets demonstrated no detectable Fg by immunofluorescence (results not shown) and IEM (Fig. 2 a). However, in the same sample vWf-Ag was detectable in the platelet α -granules according to its characteristic eccentric localization (Fig. 2 a, inset). At 2 h postinfusion Fg labeling was still negative in the α -granules by IEM. Immunofluorescence staining could detect a typical granular pattern of Fg labeling in the platelets at 24 h postinfusion, with 80% of platelet sections positive for Fg by IEM. After 48 h positively labeled α -granules were found in 92% of platelets by IEM (Fig. 2 b). At 3 and 4 d postinfusion 100% of platelets were positive with the number of gold particles per platelet greater than in earlier samples. The percentage of labeled platelets started to decrease after 5 d. At 13 d postinfusion 12% of platelet sections were still positive for Fg in the α -granules by IEM, whereas at 24 d postinfusion all platelets were negative for Fg.

Megakaryocyte culture studies. CFU-MKs from the patient were grown in liquid culture. MKs identified by their positive immunostaining for vWf (Fig. 3 a) represented ~2% of the total cell population. Cells grown in the presence of plasma were able to express Fg as shown by immunofluorescence (Fig. 3 b) or IEM (Fig. 4). In contrast, MKs grown in the presence of serum remained negative for Fg but with normal labeling for vWf-Ag.

Mathematical modeling. A simplistic model of the MK/platelet incorporation of Fg was calculated from the data in Fig. 1 to estimate the relative importance of MK versus platelet uptake (Fig. 5). The percentage of Fg-positive platelets derived from MK uptake was estimated by assuming that 10% of circulating platelets were replaced with new platelets every 24 h. This model assumed that there was no significant time delay between MK Fg uptake and thrombopoiesis, that the positive platelets were saturated with Fg (i.e., $74.55 \mu\text{g}/10^9$ platelets), and that Fg uptake only occurred for the first 4 d after therapy while plasma Fg levels were high. The platelet uptake curve was calculated by subtracting the MK curve from the observed platelet Fg levels. The resulting curves (Fig. 5) clearly demonstrate that both MK and platelet uptake were occurring during the first 4 d after therapy. Exclusive MK uptake appeared to be operative after 4 d postinfusion.

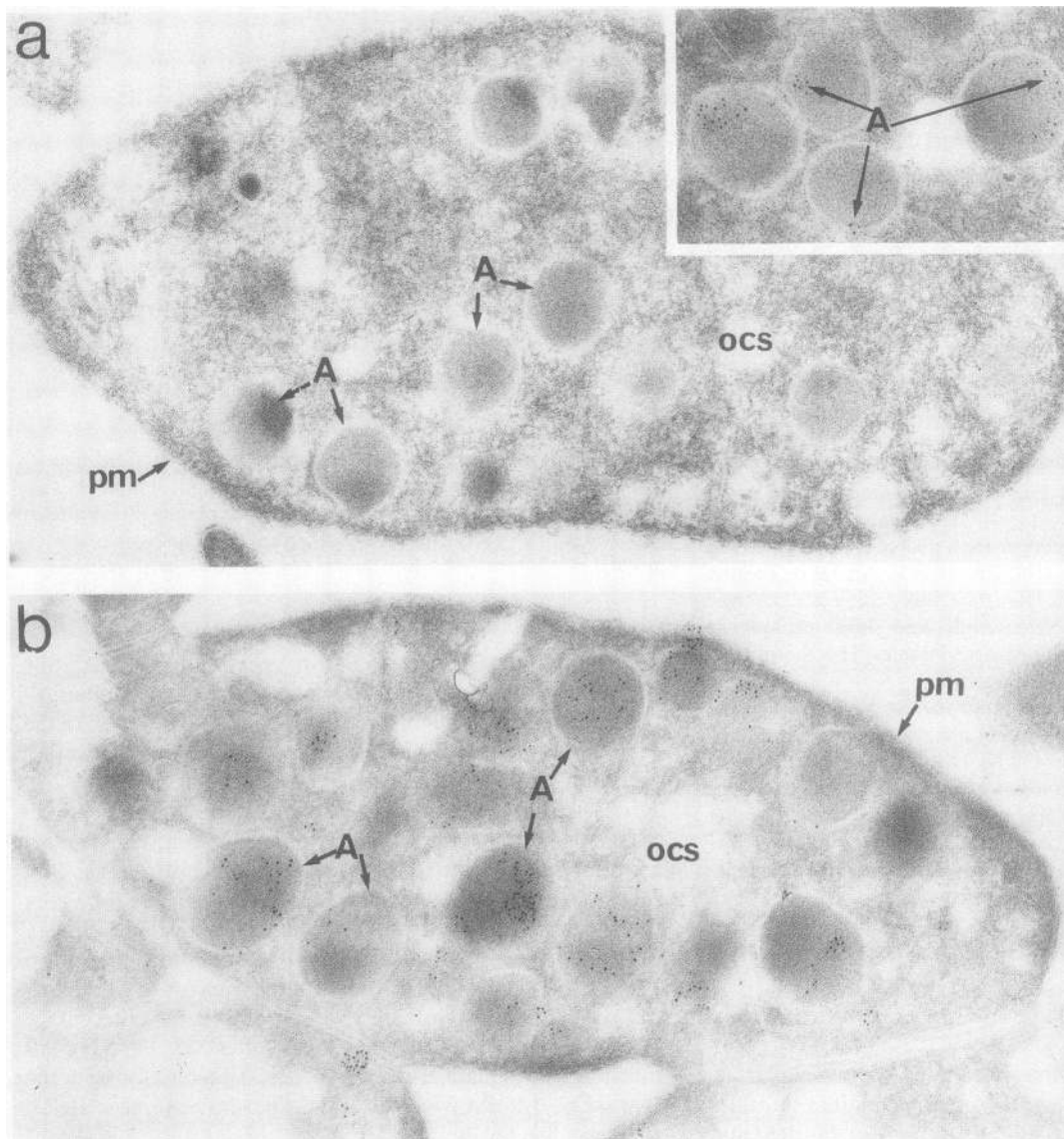


Figure 2. Immunogold labeling for Fg in platelets from an afibrinogenic patient. (a) No Fg was seen in the α -granules (A) before treatment, whereas vWf was detected according to its specific eccentric localization. (b) 48 h after infusion of cryoprecipitate Fg was present within the α -granules (A). The plasma membrane (pm) and open canicular system (ocs) were not labeled. $\times 48,000$.

Discussion

In this study we combined biochemical and immunocytochemical techniques to study whether plasma Fg could be incorporated within platelet and MK α -granules. The experimental data derived from the study of platelets from a patient with congenital afibrinogenemia demonstrate that the incorporation of plasma Fg into α -granules occurs as early as 24 h postinfusion of cryoprecipitate. Platelet levels continued to rise and reached a plateau of $\sim 40\%$ normal with 100% of positive platelets after 3–4 d. The early high proportion (80%) of positive platelets at 24 h is much greater than would be expected if Fg uptake were occurring exclusively in the MK, as only a maximum of 10–14% of circulating platelets would be positive. However, the persistence of detectable Fg within the platelets and the return of plasma levels to basal levels at 13 d posttreatment also support the possibility of Fg uptake occurring in the MKs. Perhaps both mechanisms are occurring *in vivo* since mature α -granules of normal bone marrow MKs were shown to contain less stainable Fg by IEM than in circulating platelets (9). The relative importance of MK versus platelet uptake was estimated by the application of a simple mathematical model based on normal platelet kinetics (Fig. 5).

The resulting curves suggested that a combination of both MK and platelet uptake of Fg was operative until 4 d postinfusion, after which only exclusive MK uptake of Fg occurred. However, the model is only an approximate estimate of MK/platelet uptake, particularly as the platelet kinetics were likely to be abnormal in the patient studied because of the severe menorrhagia before therapy. Previous studies may have failed to

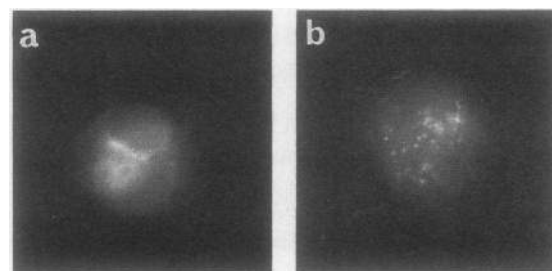


Figure 3. Double immunofluorescent labeling for vWf and Fg in 13-d cultured afibrinogenic MKs. In the presence of plasma, cells identified as MKs by their vWf content (a) were strongly positive for Fg (b).

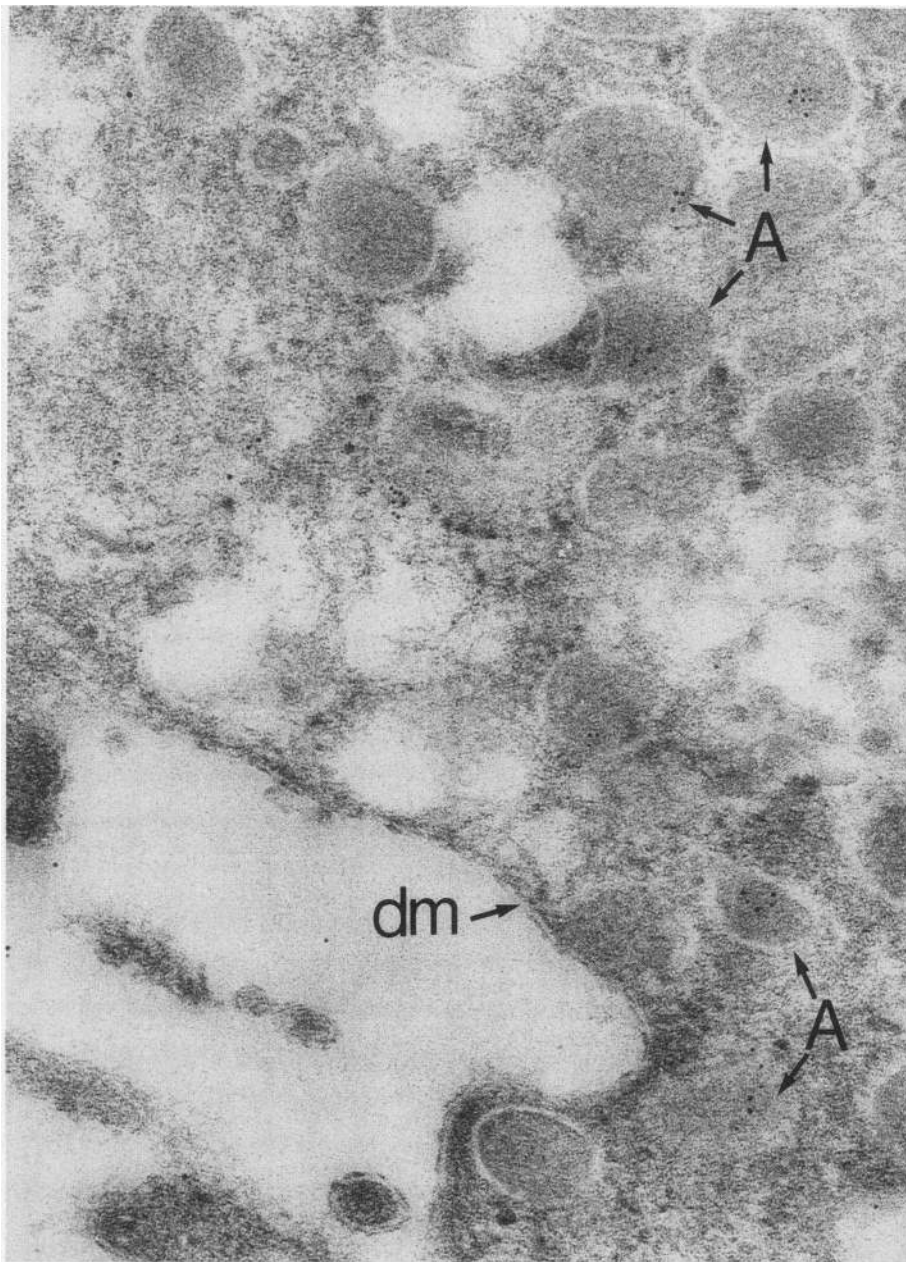


Figure 4. Part of a 12-d cultured MK from the afibrinogenemic patient grown in the presence of normal plasma and immunolabeled for Fg. Labeling is present in some of the α -granules (A) of this large MK containing a prominent demarcation membrane system (dm). $\times 48,000$.

demonstrate transfer of Fg from plasma to platelets due to insufficient consideration for time-dependent platelet uptake (10, 11). The ability of MKs to incorporate other platelet α -granule proteins such as Ig, albumin (3, 5, 13), and an exogenous tracer protein, HRP, (4) has already been established. Studies performed on animal models also suggested that Fg uptake occurs in the MK (12, 13). Recent in vitro studies with cultured MKs have demonstrated that the appearance of Fg in the α -granules, subsequent to the synthesized proteins thrombospondin and vWf, is dependent on an exogenous source of Fg (9). The in vitro results obtained with the afibrinogenemic MKs that are unable to synthesize Fg confirmed these observations and thus discounted the possibility of upregulation of Fg expression.

It is intriguing to speculate on the mechanisms of MK and platelet endocytosis of Fg, particularly as platelets congenitally deficient in the Fg receptor Gp IIb/IIIa, as in Glanzmann's thrombasthenia, do not contain platelet α -granule Fg (21).

Although platelet activation is a prerequisite site for Fg binding to exposed GpIIb/IIIa (22), perhaps Fg can still interact with partially exposed GpIIb/IIIa on resting circulating platelets in a time-dependent manner (23). Recent evidence demonstrates that resting platelets also contain a dynamic cycling pool of GpIIb/IIIa (24), which could transport plasma Fg into the α -granules. Fg possesses six potential GpIIb/IIIa binding domains, one per γ -chain and two per α -chain (25), of which four probably mediate multivalent attachment to the platelets (26), a possible prerequisite for endocytosis. Normal γ -chains are necessary to support normal ADP-induced platelet aggregation (27), but may also be critical for internalization of Fg, as both normal platelets and those from patients with certain forms of dysfibrinogenemia (such as fibrinogen Paris I) do not express any of the circulating variant γ -chains of Fg (28, 29). However, it is also conceivable that the uptake of Fg is via a non-Gp IIb/IIIa-like receptor, which could also mediate the uptake of albumin, Ig, and HRP, its concentration and reten-

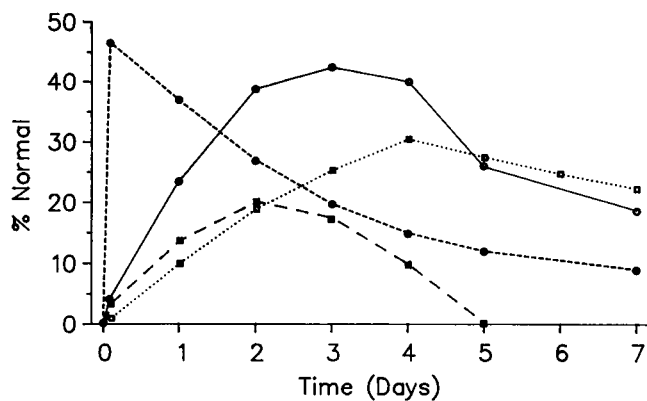


Figure 5. Mathematical model estimating the time course of the relative incorporation of Fg into MKs and circulating platelets after infusion of cryoprecipitate into a patient with congenital afibrinogenemia (derived from data of Fig. 1). The percent of MK-derived platelets was derived by assuming that 10% of circulating platelets were replaced from the bone marrow every 24 h. The percent of Fg uptake into circulating platelets was calculated by subtracting the MK-derived curve from the observed platelet Fg levels. ●, plasma Fg; ○, platelet Fg; □, MK model; ■, platelet model.

tion within the α -granule being under the specific control of GpIIb/IIIa. Finally, although we have demonstrated that platelets and MKs can incorporate exogenous Fg, the study was performed in a symptomatic patient with congenital afibrinogenemia and its relevance to normal physiology remains to be elucidated.

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