Increased Fibrinogen Binding to Platelets from Patients with Familial Hypercholesterolemia

Giovanni DiMinno, Melvin J. Silver, Anna M. Cerbone, Annibale Rainone, Alfredo Postiglione, and Mario Mancini

Familial hypercholesterolemia (FH) is a disease marked by a high incidence of thrombotic episodes and hypersensitivity of the patients' platelets to naturally occurring aggregating agents. Prostaglandin/thromboxane (PG/Tx) formation, adenosine 5'-diphosphate (ADP) secretion, and fibrinogen binding to platelets are all believed to be involved in the mechanisms of platelet aggregation. Therefore, we studied the interrelated roles of these processes in the platelets of nine FH patients and 10 controls. In response to ADP, collagen, or thrombin, FH platelets bound about twice as much 12S l-fibrinogen as controls. This ratio did not change after suppression of PG/Tx formation by aspirin. With or without aspirin, FH platelets always aggregated in response to significantly lower concentrations of these agents than did platelets from normal controls. After stimulation with thrombin or collagen, the hyperaggregable platelets from FH patients were shown to bind significantly more fibrinogen than control platelets even when PG/Tx formation was suppressed (aspirin) and secreted ADP was scavenged (apyrase). To determine whether the increased fibrinogen binding observed in FH platelets is due to a qualitative or quantitative abnormality of the platelet receptor, we used a monoclonal antibody (B79.7) that is specific for the receptor. The amount of B79.7 that bound to platelets from control and FH subjects was similar. In addition (as in normal individuals), the antibody inhibited aggregation and fibrinogen binding of FH platelets. (Arteriosclerosis 6:203–211, March/April 1986)

Several mechanisms have been suggested by which alterations in plasma lipids may promote atherosclerosis.7, 10–13 Hypercholesterolemia may favor endothelial injury13 and thus initiate and promote the progress of the atherosclerotic plaque. In addition, high levels of cholesterol can enhance platelet aggregation and prostaglandin (PG) production. In vitro experiments show that the enrichment of normal platelets with cholesterol is associated with increased sensitivity to aggregating agents, increased formation of thromboxane A₄ (TXA₄) in response to thrombin, and increased activity of platelet phospholipases.14–18 These findings correlate with the observations that platelets from patients with familial hypercholesterolemia have high levels of free cholesterol19 associated with in vitro hypersensitivity to aggregating agents;19–22 increased secretion of nucleotides,21 β-thromboglobulin, and platelet Factor 4;23 and increased formation of malondialdehyde and thromboxane.22–25

Evidence is now accumulating that platelet aggregation involves exposure of receptors for fibrinogen on the platelet surface and binding of fibrinogen to platelets;26–29 that adenosine 5'-diphosphate (ADP)30, 34, 35 as well as PG endoperoxides and thromboxane36–39 secreted by platelets may play a direct role in the exposure of fibrinogen receptors on normal platelets; and that the glycoprotein IIb-IIIa (GPIIb-IIIa) complex is the receptor for fibrinogen on the platelet surface.36, 38, 39 In this paper we examine the roles of ADP secretion and PG endoperoxide/thromboxane formation in the exposure of fibrinogen receptors and aggregation of platelets from nine FH patients.
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A 100 mM stock solution of EDTA (Fisher Scientific Company, Fairlawn, New Jersey) was made up in distilled water (pH adjusted to 7.4 with NaOH solution). Bovine serum albumin (Pentex, Fraction V) was purchased from Miles Laboratories Incorporated, Elkhart, Indiana. Apyrase (grade 1), creatine phosphate (CP), creatine kinase (CPK), hirudin (grade IV), ADP, and aspirin (crystalline) were purchased from Sigma Chemical Company, St. Louis, Missouri. Apyrase, hirudin, CP, and ADP were dissolved in distilled water and stored at −20°C in small aliquots. Aspirin was dissolved in 0.3 M sodium acetate immediately before each series of experiments. CPK solutions (1 mg/ml in distilled water) were prepared fresh daily.

Arachidonic acid (AA) (more than 99% pure) was provided by Dr. Renato Saggiorato, Menarini, Diagnostici, Firenze, Italy; it was dissolved in 100 mM sodium carbonate. Thrombin was purchased from Parke Davis & Company, Detroit, Michigan, was dissolved in distilled water, and was stored in small aliquots at −70°C. A stable analogue of prostaglandin endoperoxide/Tx A2, U-46619, was kindly provided by Dr. John Pike (the Upjohn Company, Kalamazoo, Michigan) and prepared and stored as previously described.40 Equine tendon collagen was purchased from Hormon Chemie, München, West Germany. Sodium iodide (125I) was from New England Nuclear, Firenze, Italy. Highly specific antibody to Tb±2 (Seragen Incorporated, Boston, Massachusetts) exhibited less than 0.03% cross reactivity with prostaglandin E2, F2α, 6-keto-PGF1α, or D2. Silicon oils (methyl-silicone 1.0, 0 DC 200 and Hi phenylsilicone 125 DC 550) were purchased from W.F. Ney Incorporated, Specialty Lubricants, New Bedford, Massachusetts. Mixtures of DC 200 and DC 550 were prepared as described previously.34 Aspirin tablets (500 mg) were from Bayer Company, West Germany.

Monoclonal Antibodies B79.7 (IgG1) and B59.2 (IgG2a) were kindly provided by Dr. Perumal Thiagarajan, Thomas Jefferson University, Philadelphia, Pennsylvania. B79.7 was purified from ascites fluid using a protein A-sepharose column and appeared to be similar to Antibody B59.2 which had been previously shown to be specific for the GPIb-IIIa complex.34 Indeed, like B59.2, Antibody B79.7 inhibited aggregation and fibrinogen binding to platelets in suspension (see results) and bound only negligibly to platelets from three patients with Glanzmann’s thrombasthenia. Platelets from these patients lack GPIb-IIIa.33–39 Moreover like unlabeled B59.2, Antibody B79.7 cross-competes more than 98.7% with labeled Antibody B59.2 for binding on stimulated or unstimulated platelets from controls or FH subjects. For binding studies, the antibodies were labeled with 125I by the iodine monochloride method34 and had a specific activity of 470 µCi/mg

### Methods

#### Subjects

Nine FH patients (five females, four males, 7 to 36 years old) and 10 normal, control subjects matched for sex and age were studied. None of the controls had a history of any disease known to alter platelet aggregation. Neither FH patients nor controls had taken any medication for at least 10 days before donating blood. There was no difference between FH subjects (109.9 ± 10.6 mg/dl, mean ± SEM) and controls (112.6 ± 12.5 mg/dl) in total triglyceride levels. FH subjects had fasting plasma glucose concentrations 14 hours at the time of blood collection. Plasma cholesterol and triglycerides were determined on the day of the study. Both control and FH subjects had been fasting for 12 to 14 hours at the time of blood collection. Plasma cholesterol and triglycerides were determined on the day of the study.

#### Materials

Table 1. Plasma Cholesterol and Apoprotein Profile of Nine Patients with Familial Hypercholesterolemia

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (yrs), Sex</th>
<th>Total (mg/dl)</th>
<th>VLDL (mg/dl)</th>
<th>LDL (mg/dl)</th>
<th>HDL (mg/dl)</th>
<th>Apoproteins (mg/dl)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>24 M</td>
<td>506</td>
<td>10</td>
<td>452</td>
<td>31</td>
<td>272</td>
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<tr>
<td>2</td>
<td>18 F</td>
<td>682</td>
<td>13</td>
<td>590</td>
<td>36</td>
<td>314</td>
</tr>
<tr>
<td>3</td>
<td>14 F</td>
<td>785</td>
<td>9</td>
<td>680</td>
<td>15</td>
<td>350</td>
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<tr>
<td>4</td>
<td>12 F</td>
<td>944</td>
<td>13</td>
<td>770</td>
<td>18</td>
<td>430</td>
</tr>
<tr>
<td>5</td>
<td>7 M</td>
<td>850</td>
<td>9</td>
<td>715</td>
<td>23</td>
<td>410</td>
</tr>
<tr>
<td>6</td>
<td>36 F</td>
<td>652</td>
<td>25</td>
<td>610</td>
<td>18</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>19 M</td>
<td>690</td>
<td>38</td>
<td>618</td>
<td>17</td>
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<td>8</td>
<td>23 M</td>
<td>550</td>
<td>3</td>
<td>510</td>
<td>42</td>
<td>276</td>
</tr>
<tr>
<td>9</td>
<td>11 F</td>
<td>710</td>
<td>7</td>
<td>651</td>
<td>30</td>
<td>310</td>
</tr>
</tbody>
</table>

Controls (n = 10)

(n = 5) 8-30 M 160-220 <35 <144 <40 80-130 160-180

(n = 5) 13-38 F 160-220 <35 <144 <40 80-130 160-180
protein. For both aggregation and binding studies, mouse immunoglobulins were used as controls. F(ab')2 fragments of both antibodies were prepared by peptic digestion of the gammaglobulin and isolation of the fragments on a Protein A-sepharose column.³⁴

**Preparation of Platelet Suspensions**

Blood was collected from FH patients and controls on two occasions, before and 2 hours after ingestion of 500 mg aspirin. For each individual subject and on each occasion, 90 ml of blood were collected from the antecubital vein via a 19-gauge scalp vein needle into a plastic tube containing 10 ml of 3.8% trisodium citrate. Platelet-rich plasma (PRP), obtained by centrifugation (200 g, 15 minutes) at room temperature, was cooled on ice for 30 minutes and EDTA was added to a final concentration of 5.6 mM. After centrifugation at 2000 g for 6 minutes at 4°C in a Beckman, Model J-68 centrifuge (Beckman Analytical, Milan, Italy), the platelets were resuspended in a volume of buffer (0.14 M NaCl, 20 mM Tris, 5 mM glucose, 1 mM EDTA, pH 7.4) equal to that of the discarded plasma; they were centrifuged at 900 g for 6 minutes at room temperature and were resuspended at room temperature at counts in the range of 1 to 5 x 10⁸ platelets/ml in a Tyrode's buffer (0.14 M NaCl, 2.7 mM KCl, 1 mM CaCl₂, 12 mM NaHCO₃, 0.4 mM NaH₂PO₄, pH 7.4) containing 5 mM glucose and 3.5 mg/ml bovine serum albumin. Platelet counts were measured in the presence of fibrinogen was 80% to 90% of that seen in autologous PRP and remained unchanged for at least 2 hours. All aggregation and binding studies were conducted within this 2-hour period.

**Purification and Labeling of Fibrinogen**

Fibrinogen was purified from citrated plasma of normal donors by the method of Martinez et al.⁴¹ The purified fibrinogen contained less than 0.8% immunosassayable fibronectin and von Willebrand factor. Fibrinogen, labeled with ¹²⁵I by the iodine monochloride method of McFarlane,⁴¹ had a specific activity of 2 x 10⁷ to 3.8 x 10⁸ cpm/μg protein. Unlabeled and labeled fibrinogen showed the characteristic Aα, Bβ, and γ peaks without any degradation products when electrophoresed on reduced SDS-polyacrylamide gels.⁴³ Further details of the properties of fibrinogen that was prepared and labeled in this manner have been previously reported.³⁴, ⁴³

**Studies on Platelet Aggregation and Thromboxane Formation**

Platelet aggregation tests were performed in plastic cuvettes essentially as described elsewhere, except that the ELVI-80 aggregometer, (ELVI Logos, Milan, Italy) was used. The apparatus was adjusted so that the platelet suspension and the buffer produced 10% and 90% light transmittance, respectively.

When thrombin was the stimulus used, hirudin (0.1 U/ml, added 3 minutes after thrombin and immediately before fibrinogen) was used to prevent clotting. AC₅₀ was defined as the lowest concentration of an aggregating agent that caused a 50% light transmittance within 3 minutes. TxB₂ levels were measured by radioimmunoassay³⁴ in aliquots of the supernatant solution removed 3 minutes after the completion of tests for platelet aggregation. The sensitivity was such that as little as 0.5 pmol TxB₂ could be detected. The effect of aspirin on aggregation and TxB₂ formation was determined on platelet suspensions incubated at 37°C for 30 minutes with 1 to 4 mM aspirin. The effect of apyrase (or CP/CPK) was evaluated by incubating platelet suspensions with 500 to 2000 μg/ml apyrase (or with 6 mM CP plus 15 U/ml CPK) for 5 minutes before adding aggregating agents. When aspirin and apyrase (or CP/CPK) were used in combination, they were added sequentially.

**Studies on Fibrinogen and Antibody Binding to Platelets**

Binding of ¹²⁵I-fibrinogen or B79.7 to platelets was performed as reported previously³⁴ with some modifications. Briefly, the time-dependence of binding was studied by incubating 6 ml of unstimulated platelet suspension (1–5 x 10⁸/ml) with either 10 μM AA, 1 μg/ml collagen, 1 μM U-46619, 10 μM ADP, 10 μM/ml thrombin, or with microliter amounts of buffer for 3 minutes at room temperature. Labelled fibrinogen (600 nM) or B79.7 (100 μg/ml) was then added. At appropriate time intervals, 0.4 ml of the platelet suspension was removed and layered onto 50 ml of silicone oil in a 0.5 ml micro-Eppendorf tube; free and platelet-bound ligand were separated by centrifugation for 2 minutes at 12,000 g in an Eppendorf centrifuge (Beckman, Milano, Italy) and were counted in a Beckman DP 5500 gammacounter. When thrombin was used, hirudin (0.1 U/ml) was used to prevent clotting and was added 3 minutes after thrombin and immediately before fibrinogen.

The effects of increasing concentrations of aggregating agents on the extent of fibrinogen binding to platelets were assessed by incubating unstimulated platelet suspensions with ADP (2.5 to 100 μM), collagen (0.25 to 10 μg/ml), AA (2.5 to 50 μM), thrombin (5 to 40 μg/ml), or U-46619 (0.25 to 10 μM). After 3 minutes, labeled fibrinogen (600 nM) was added, and the extent of binding was determined 5 minutes later. For concentration-response studies, 500 μl aliquots of unstimulated platelets were incubated with each aggregating agent (or equal amounts of buffer) for 3 minutes, after which labeled fibrinogen or B79.7 was added at respective final concentrations of 1.5 to 1200 nM or 25 nM to 2 μM. Fibrinogen binding was measured after 5 minutes and antibody binding was measured after 1 minute. Specific binding was calculated by subtracting the binding measured in the presence of a 20-fold excess of unlabelled fibrinogen from the total bound fibrinogen. This latter "non-specific" binding represented 13.8% ± 2.7% SEM of the total bound. Specific fibrinogen binding was not corrected for the amount of this protein bound in the absence of stimulation, which was 4% to 5% of stimulated binding.

For the parallel study of inhibition by antibody B79.7 of aggregation and fibrinogen binding to platelets from FH or control subjects, microliter amounts of B79.7 (from 25 nM to 2 μM, final concentration) were incubated for 1 minute with 0.25 ml of platelet suspensions which had been stirring at 37°C for 1 minute at 1000 rpm. Collagen (1 μg/ml) was then added and followed by unlabelled fibrinogen (600 nM, final), and platelet aggregation was measured. In parallel, aliquots of 0.5 ml of unstimulated platelet suspensions (1 to 5 x 10⁹/ml) were incubated at room temperature with 1 μg/ml collagen. After 3 minutes, increasing concentrations (see above) of B79.7 were added, and 1 minute later, ¹²⁵I-
fibrinogen (600 nM) was added. After 5 minutes, free and platelet-bound fibrinogen were separated on silicone oil and counted.

The in vitro effect of aspirin and/or apyrase (or CP/CPK) on fibrinogen binding to platelets was determined on platelet suspensions processed for platelet aggregation as described above.

**Protein Concentration**

Protein concentration was calculated from the absorbance at 280 nm by using a 1% molar extinction coefficient of 15.1 for fibrinogen and 14.3 for antibody.34

**Statistical Analysis and Analysis of the Binding Data**

Student's t test for paired or grouped data were used as appropriate for statistical analysis. The binding to platelets of \( ^{125} \text{I-fibrinogen} \) was analyzed according to Scatchard as reported elsewhere.34 46

Informed consent was obtained from all the volunteers in accordance with accepted institutional practice. The studies were carried out according to the principles of the Helsinki declaration.

**Results**

**Platelet Aggregation: Familial Hypercholesterolemia Platelets Compared to Control Platelets**

In the absence of added fibrinogen, washed platelet suspensions (1 to 3 x 10^9/ml) from control or FH subjects did not aggregate in response to ADP (100 \( \mu \text{M} \)), collagen (0.2 to 1 \( \mu \text{g/ml} \)), U-46619 (0.5 \( \mu \text{M} \)), or AA (10 to 40 \( \mu \text{g/ml} \)). They aggregated partially (i.e., about 20% light transmittance) in response to thrombin (10 \( \mu \text{M} \)). When fibrinogen (600 nM) was added, full aggregation occurred, and the minimal concentrations of ADP, collagen, and thrombin required to cause 50% light transmittance in the absence of aspirin were lower (\( p < 0.01 \)) in suspensions from FH patients than in those from control subjects (Table 2, compare controls to FH without aspirin for each aggregating agent). The AC\(_{50}\) for U-46619 was similar in control and FH platelet suspensions even in the presence of added aspirin. Aspirin suppressed aggregation in response to AA and caused elevation of the AC\(_{50}\) for ADP, collagen, or thrombin both in control and FH platelets. It is to be noted that, whether or not aspirin was present in vitro, the AC\(_{50}\) for each of these three agents with FH platelets was less than with control platelets (\( p < 0.01 \)). Similar results were found using platelets obtained from control or FH subjects 2 hours after the ingestion of 500 mg aspirin.

In both control and FH platelet suspensions, a concentration of aspirin (500 \( \mu \text{g/ml} \)) that suppressed aggregation in response to ADP (10 \( \mu \text{M} \)) inhibited aggregation significantly (\( p < 0.05 \)), but only partially (27% to 33% of that seen in control, buffered samples) in response to collagen (1 \( \mu \text{g/ml} \)), AA (10 \( \mu \text{M} \)), thrombin (10 \( \mu \text{M} \)), or U-46619 (1 \( \mu \text{M} \)). The combination of apyrase (500 \( \mu \text{g/ml} \)) plus aspirin (1 mM) suppressed ADP- and AA-induced aggregation of control or FH platelets, and inhibited aggregation of control platelets exposed to thrombin (10 \( \mu \text{M} \)) or collagen (1 \( \mu \text{g/ml} \)) by about 71% and 77%, respectively, whereas inhibition of aggregation of FH platelets was about 31% and 37%, respectively. This difference in response of FH platelets and control platelets is evident in the example shown in Figure 1. The eight other FH platelet suspensions gave similar results. Both for control and FH platelets, no further inhibition of aggregation was observed when fourfold higher amounts of aspirin (4 mM) and apy-

| Table 2. The AC\(_{50}\) of Platelet Aggregating Agents on Platelet Suspensions from Control and FH Subjects in the Presence or Absence of Aspirin (1 mM) |
|---------------------------------|---------------------|---------------------|
| Aggregating agent               | Control (n = 10)     | Familial hypercholesterolemia (n = 9) |
|                                 | No aspirin          | Plus aspirin        |
|                                 | 2.15 ± 0.38         | 5.10 ± 0.48†       |
| ADP (\( \mu \text{M} \))        | 0.46 ± 0.09         | 2.30 ± 0.42‡       |
| Collagen (\( \mu \text{g/ml} \))| 11.10 ± 1.14        | >1000              |
| AA (\( \mu \text{M} \))         | 11.06 ± 1.22        | 23.5 ± 2.40‡       |
| Thrombin (mU/ml)                | 0.54 ± 0.11         | 0.44 ± 0.11        |
| U-46619 (\( \mu \text{g/ml} \)) | 0.46 ± 0.11         | 0.44 ± 0.11        |

Values are means ± SEM. AC\(_{50}\) is defined as the lowest concentration of an aggregating agent that causes 50% light transmittance within 3 minutes. ADP = adenosine 5'-diphosphate; AA = arachidonic acid.

* \( p < 0.01 \), controls vs FH (both no aspirin). Student's t test for grouped data.

† \( p < 0.01 \), controls vs FH (both with aspirin). Student's t test for grouped data.

‡ \( p < 0.01 \) (no aspirin vs plus aspirin). Student's t test for data.
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TxB2 Formation in Platelet Suspensions from Normal Subjects or Familial Hypercholesterolemia Patients

At the concentrations of collagen, thrombin, or AA (but not of ADP) studied, there was significantly greater TxB2 formation in platelet suspensions from FH patients than in those from controls (Figure 2). After aspirin, TxB2 formation could not be detected (< 0.05 pmol/3 x 10^9 platelets) in response to any of the stimuli used either in platelets from normal subjects or in those from FH patients (data not shown).

Binding of 125I-Fibrinogen to Control or Familial Hypercholesterolemia Platelets

Exposure of control or FH platelets to ADP, collagen, thrombin, AA, or U-46619 induced time-dependent binding of fibrinogen that was complete within 3 to 5 minutes (the time period within which platelet aggregation is complete). For all the agents tested, fibrinogen binding was concentration-related and reached a maximum at a final concentration of about 600 nM. Between 5 and 10 minutes after the addition of labeled fibrinogen, more than 85% of this could be displaced by a 20-fold excess of unlabeled fibrinogen. Similar results were obtained if the labeled and unlabeled fibrinogen were added together at the start of the binding reaction. EDTA (10 mM) inhibited 85% to 90% of the fibrinogen binding to stimulated platelets. Maximum fibrinogen binding was induced in both normal and FH platelets by 10 /uM ADP, 1 /uM collagen, 10 mU/ml thrombin, 10 /uM AA (Figure 3), or by 1 /uM of U-46619 (not shown).

Apparent dissociation constants (Kd) for normal and FH platelets were similar when determined by Scatchard or Klotz analysis (Tables 3 and 4) and were comparable to those previously reported.34, 40 At saturating levels of add-

Table 3. Scatchard Analysis of 125I-Fibrinogen Binding to Control and Familial Hypercholesterolemia Platelets

<table>
<thead>
<tr>
<th>Stimulating agent</th>
<th>High affinity sites*</th>
<th>Low affinity sites†</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP (10 /uM)</td>
<td>Normal</td>
<td>29.3 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>FH</td>
<td>28.3 ± 5.7</td>
</tr>
<tr>
<td>Collagen (1 /uM/ml)</td>
<td>Normal</td>
<td>36.4 ± 7.2</td>
</tr>
<tr>
<td></td>
<td>FH</td>
<td>35.7 ± 4.7</td>
</tr>
<tr>
<td>AA (10 /uM)</td>
<td>Normal</td>
<td>37.4 ± 7.3</td>
</tr>
<tr>
<td></td>
<td>FH</td>
<td>38.6 ± 5.9</td>
</tr>
<tr>
<td>Thrombin (10 mU/ml)</td>
<td>Normal</td>
<td>42.6 ± 8.2</td>
</tr>
<tr>
<td></td>
<td>FH</td>
<td>40.5 ± 7.3</td>
</tr>
<tr>
<td>U-46619 (1 /uM)</td>
<td>Normal</td>
<td>33.5 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>FH</td>
<td>38.3 ± 7.5</td>
</tr>
</tbody>
</table>

Values are means ± SEM. For definition of Scatchard analysis, see Scatchard G. Ann NY Acad Sci 1949;51:660
ADP = adenosine 5'-diphosphate; AA = arachidonic acid; Kd = dissociation constant.
* p > 0.05 high affinity sites (normal vs FH).
† p < 0.05 low affinity sites (normal vs FH).
ed fibrinogen, however, FH platelets bound 1.5 to two-fold as much ligand as those from normals in response to each aggregating agent tested (Figure 3). Aspirin, either ingested 2 hours before or added in vitro (1 mM) 30 minutes before testing, did not affect binding in control or FH platelets exposed to U-46619, suppressed binding in response to AA, and significantly decreased binding in response to ADP, collagen or thrombin (Table 5). Regardless of aspirin, however, the amount of fibrinogen bound to FH platelets was always significantly greater than the amount of fibrinogen bound to control platelets in response to these three agents. Apyrase (500 μg/ml) almost completely (more than 98%) inhibited binding in response to ADP (10 μM) and reduced by 33% to 39% (p < 0.05 for all the agents tested) binding to control or FH platelets exposed to AA (10 μM), collagen (1 μg/ml), or thrombin (10 μU/ml). Similar results were obtained when 6 mM CP plus 15 U/ml CPK were used instead of apyrase. The combination of aspirin (1 mM) and apyrase (500 μg/ml) inhibited binding of fibrinogen to control platelets in response to thrombin or collagen by about 94% and FH platelets by about 85% (Figure 4). For both control and FH platelets, no further inhibition of binding was observed when four-fold higher concentrations of aspirin (4 mM) and apyrase (2000 μg/ml) were used, or when aspirin was used in combination with 6 mM CP and 15 U/ml CPK. Identical results were obtained when aspirin (500 to 2000 μg/ml) was added to platelets obtained from control or FH subjects 2 hours after ingestion of 500 mg aspirin. Apyrase (500 μg/ml) alone (not shown) or in combination with 1 mM aspirin (Figure 4) decreased fibrinogen binding in response to U-46619 by about 75% in both control and FH platelets. Similar results were found when 6 mM CP and 15 U/ml CPK were used in combination with 1 to 4 mM aspirin.

### Table 4. Klotz Analysis of 125I Fibrinogen Binding to Control and Familial Hypercholesterolemia Platelets

<table>
<thead>
<tr>
<th>Stimulating agent</th>
<th>Control</th>
<th>FH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kd (μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP (10 μM)</td>
<td>0.25 ± 0.05</td>
<td>0.27 ± 0.06</td>
</tr>
<tr>
<td>Collagen (1 μg/ml)</td>
<td>0.21 ± 0.06</td>
<td>0.23 ± 0.08</td>
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<tr>
<td>AA (10 μM)</td>
<td>0.29 ± 0.09</td>
<td>0.21 ± 0.05</td>
</tr>
<tr>
<td>Thrombin (10 U/ml)</td>
<td>0.34 ± 0.07</td>
<td>0.20 ± 0.10</td>
</tr>
<tr>
<td>U-46619 (1 μM)</td>
<td>0.30 ± 0.08</td>
<td>0.26 ± 0.09</td>
</tr>
</tbody>
</table>

Values are means ± SEM. For explanation of Klotz analysis, see Klotz IM. Science (Wash) 1982;217:1274. Kd = dissociation constant. p > 0.05 for all the comparisons.

### Table 5. Specific Fibrinogen Binding (pmol/10⁸ Platelets) to Control and FH Platelets in Response to Aggregating Agents In the Presence or Absence of Aspirin (1 mM) and at Saturating Levels of Added Fibrinogen (600 nM)

<table>
<thead>
<tr>
<th>Aggregating agent</th>
<th>Control (n = 10)</th>
<th>FH (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before aspirin</td>
<td>After aspirin</td>
</tr>
<tr>
<td>ADP (10 μM)</td>
<td>5.80 ± 0.82</td>
<td>2.48 ± 0.40†</td>
</tr>
<tr>
<td>Collagen (1 μg/ml)</td>
<td>8.25 ± 0.41</td>
<td>4.83 ± 0.33†</td>
</tr>
<tr>
<td>AA (10 μM)</td>
<td>8.51 ± 0.29</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Thrombin (10 U/ml)</td>
<td>13.19 ± 1.10</td>
<td>6.73 ± 0.57</td>
</tr>
<tr>
<td>U-46619 (1 μM)</td>
<td>8.40 ± 1.35</td>
<td>7.50 ± 1.40</td>
</tr>
</tbody>
</table>

Values are means ± SEM.

* p < 0.05, control vs FH (both no aspirin). Student's t test for grouped data.

†† p < 0.01, control vs FH (both after aspirin). Student's t test for grouped data.

†† p < 0.01, no aspirin vs plus aspirin. Student's t test for paired data.

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**Binding of Antibody B79.7 to Platelets**

Binding of 125I Antibody B79.7 (or of labeled B59.2) to unstimulated platelets was complete in 1 minute. The amount of specific B79.7 binding at saturation was similar in control and FH platelets (3.61 ± 2.13 pmol/10⁸ platelets in normal vs 3.89 ± 1.97 in FH subjects, p > 0.01). Pre-treatment of platelets with aspirin (1 mM) alone or in combination with apyrase (500 to 2000 μg/ml) did not affect the amount of binding observed. The amount of Antibody B79.7 bound was not increased by pretreating platelet suspensions from control or FH subjects with ADP (10 μM), collagen (1 μg/ml), thrombin (10 μU/ml), or U-46619 (1 μM).

**Inhibition of Platelet Aggregation and Fibrinogen Binding by Antibody B79.7**

It has been previously shown that Antibody B59.2 inhibits aggregation, secretion, and binding of fibrinogen to normal platelets stimulated by ADP, collagen, or AA. Similarly, Antibody B79.7 (but not equimolar concentrations of mouse immunoglobulins) inhibited aggregation and fibrinogen binding to FH or control platelets in a concentration-dependent fashion (Figure 5). At a 0.2 μM concentration of B79.7, aggregation in response to collagen was suppressed in both types of platelets, and fibrinogen binding to FH platelets stimulated with ADP, collagen, thrombin, AA, or U-46619 was similar to that seen in suspensions from control platelets (Table 6). Similar results were found when...
binding to FH platelets. At a concentration that completely inhibited fibrinogen binding in response to 10 μM ADP both in platelets from normal subjects and FH patients, aspirin (or CP/CPK) decreased binding in response to AA, collagen, or thrombin by 30% to 40%. Aspirin abolished AA- induced binding and reduced binding to control and FH platelets exposed to ADP, collagen, or thrombin by 50% or more. The combination of aspirin and apyrase suppressed binding of fibrinogen to control or FH platelets exposed to ADP or AA and produced significant (95% and 85%, respectively) but not complete inhibition of binding to normal and FH platelets exposed to collagen or thrombin.

The very low concentrations of thrombin that we used in our study explain the marked inhibition of platelet aggregation and fibrinogen binding in response to the combination of aspirin plus apyrase, since it is known that aggregation by low concentrations of thrombin depends on prostaglandin synthesis and nucleotide secretion.\(^5\)\(^6\)\(^7\)\(^8\)\(^9\)

The AC\(50\) and the amount of fibrinogen binding and aggregation in response to U-46619 were similar and were reduced in similar fashion by aspirin and/or apyrase in both types of platelets. This suggests that in FH platelets, neither qualitative nor quantitative abnormalities of the platelet receptor for fibrinogen are involved in the increased aggregation and binding observed in response to ADP, collagen, AA, or thrombin. This is also suggested by studies in which monoclonal antibodies to the platelet receptor for fibrinogen are used (Table 6). It is currently believed that the GPIIb-IIIa complex is the receptor for fibrinogen on the platelet surface and that normal platelets have about 40,000 to 50,000 of these receptors.\(^15\)\(^16\)\(^17\)\(^18\) Our antibodies only detected 20,000 receptors.\(^28\)\(^29\)\(^30\)\(^31\)\(^32\)\(^33\)\(^34\)\(^35\)\(^36\)\(^37\)\(^38\)\(^39\) Our antibodies only detected 20,000 to 30,000 sites on normal as well as FH platelets. A similar finding has also been reported for another monoclonal antibody to the platelet receptor for fibrinogen.\(^40\) It is possible that divalent antibodies could have bound to different, but adjacent, GPIIb-IIIa resulting in an underestimation of the number of these complexes. On the other hand, as suggested by Melero and Gonzalez-Rodriguez,\(^51\) it is conceivable that the epitope of the GPIIb-IIIa complex that reacts with our antibodies, as well as with their antibody, is not readily available on the platelet surface. This would be consistent with the observation that treatment with proteolytic enzymes almost doubles the number of binding sites for B59.2 on normal platelets (S. Niewiarowski, personal communication).

With 40,000 to 50,000 GPIIb-IIIa complexes on platelets, one would expect to find 40,000 to 50,000 molecules of fibrinogen bound per platelet. This number is an average value and many examples of wide variations are present in the literature. For example, Coller et al.\(^37\) found that their antibody to the GPIIb-IIIa binds to as few as 12,000 and to as many as 83,000 sites on platelets from normal subjects.

### Table 6. Fibrinogen Binding (pmol/10^8 Platelets) to Control and FH Platelets In the Presence of Antibody B79.7

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Control platelets (n = 10)</th>
<th>FH platelets (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer</td>
<td>B79.7</td>
</tr>
<tr>
<td>ADP (10 μM)</td>
<td>5.80 ± 0.82</td>
<td>2.72 ± 0.43*</td>
</tr>
<tr>
<td>Collagen (1 μg/ml)</td>
<td>8.26 ± 0.41</td>
<td>5.61 ± 0.44*</td>
</tr>
<tr>
<td>AA (10 μM)</td>
<td>8.51 ± 0.29</td>
<td>5.91 ± 0.57*</td>
</tr>
<tr>
<td>Thrombin (10 μU/ml)</td>
<td>13.19 ± 1.10</td>
<td>8.07 ± 0.10*</td>
</tr>
<tr>
<td>U-46619 (1 μM)</td>
<td>8.40 ± 1.31</td>
<td>4.63 ± 0.50*</td>
</tr>
</tbody>
</table>

Values are means ± SEM. The final concentration of antibody B79.7 was 0.2 μM.

*p < 0.01, buffer vs B79.7-treated platelets.
Plow and Marguerie reported that at maximal exposure of binding sites for fibrinogen, some normal subjects may have as many as 76,000 sites capable of binding this protein. Niewiarowski et al. reported an average of 50,000 sites for fibrinogen on platelets from normal individuals, and Komacek et al. found 88,000 to 400,000 sites for fibrinogen after treatment of platelets with ADP or chymotrypsin. Since these authors reported affinity constants for fibrinogen comparable to those found in studies in which 40,000 to 50,000 sites were observed, one has to assume that either more than 40,000 to 50,000 GPIIb-IIIa sites are present on normal platelets or that additional binding sites (with an affinity for fibrinogen similar to that of the GPIIb-IIIa complex) come into play. While there is no definite answer to this question, it is worth mentioning that thrombospondin secreted by platelets after exposure to thrombin or collagen can bind to free fibrinogen, as well as to fibrinogen already bound to GPIIb-IIIa, and that this interaction may play a major role in enhancing platelet aggregation. It has previously been shown that the secretion of other granular components is increased in platelets from FH patients. There may be increased secretion of thrombospondin in hypercholesterolemia to favor increased binding of fibrinogen and to reinforce the strength of a hemostatic platelet plug or a thrombus. This might also explain the abnormally high sensitivity of FH platelets to collagen and thrombin.

We did not find a direct correlation between inhibition of fibrinogen binding and inhibition of platelet aggregation by the combination of aspirin plus apyrase. A possible explanation for this may be that fibrinogen is secreted from platelets in response to thrombin or collagen in amounts that may favor aggregation but are not sufficient to detectably compete with exogenous labeled fibrinogen for binding to platelets.

We found that the smallest amount of ADP that induces 50% aggregation in the presence of aspirin (which inhibits secretion and secretion-dependent aggregation) is significantly lower in platelets from FH patients. This suggests that platelets from these patients also have enhanced primary aggregation, since only primary aggregation is measured in the presence of aspirin.

In spite of uncertainties concerning the mechanisms involved in the abnormally high binding of fibrinogen to FH platelets, the observation that a monoclonal antibody to the platelet receptor for fibrinogen inhibits the aggregation of platelets from FH patients suggests that therapy with this kind of antibody or drugs that have similar effects on fibrinogen binding and inhibition of platelet aggregation by fibrinogen already bound to GPIIb-IIIa, and that this interaction may favor increased primary aggregation is increased in platelets from FH patients. There may be increased secretion of thrombospondin secreted by platelets after exposure to thrombin or collagen can bind to free fibrinogen, as well as to fibrinogen already bound to GPIIb-IIIa, and that this interaction may play a major role in enhancing platelet aggregation. It has previously been shown that the secretion of other granular components is increased in platelets from FH patients.

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In spite of uncertainties concerning the mechanisms involved in the abnormally high binding of fibrinogen to FH platelets, the observation that a monoclonal antibody to the platelet receptor for fibrinogen inhibits the aggregation of platelets from FH patients suggests that therapy with this kind of antibody or drugs that have similar effects on platelets might reduce the number of thrombotic episodes in FH patients.

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