The differential uptake of tritium-labeled immunoglobulin G (IgG) cross-linked with bisdiazonium-benzidine (BDB) (\(^{3}H\)-BDB-IgG) by washed, pooled human platelets to sites inaccessible to pronase digestion was tested. Up to 52% of the \(^{3}H\)-BDB-IgG associated with platelets at 37 °C resisted pronase treatment, whereas only 23% of the cross-linked IgG associated with platelets at 4 °C or at 37 °C but in the presence of deoxyglucose/antimycin A, remained refractory to pronase. This effect was not due to platelet agglutination. Pronase resistance reached a maximum after a 60-minute incubation period at 37 °C. With increasing \(^{3}H\)-BDB-IgG input, both the total cross-linked IgG associated with platelets and the fraction resistant to pronase digestion approached saturation at 4 °C, but not at 37 °C. The proportion of \(^{3}H\)-BDB-IgG bound to platelets at 4 °C that was resistant to pronase digestion increased by 13% within five minutes of warming the platelets to 37 °C. Pretreatment of platelets with 10 mmol/L acetylsaliclic acid (or 10 μmol/L prostaglandin E1) prior to the addition of \(^{3}H\)-BDB-IgG led to a 74% (95%) inhibition of the \(^{3}H\)-BDB-IgG-induced \(^{14}C\)-serotonin release, but to only a 44% (49%) inhibition of pronase-digestible bound ligand. In contrast, pretreatment with 10 μmol/L cytochalasin B led to a mere 17% reduction of \(^{14}C\)-serotonin release, whereas acquisition of resistance to pronase digestion by the bound \(^{3}H\)-BDB-IgG was inhibited by 90%. Incubation of platelets at 37°C with \(^{3}H\)-BDB-IgG and removal of unbound material prior to the addition of prostaglandin E1, or deoxyglucose/antimycin A had little effect on the susceptibility of platelet-associated \(^{3}H\)-BDB-IgG to pronase. Thus, after binding, \(^{3}H\)-BDB-IgG becomes transferred in an energy-dependent process to pronase-resistant cellular sites, most likely to the open canalicular system.

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MATERIALS AND METHODS

**Buffers.** Buffer A consisted of 90 mmol/L Tris- (hydroxymethyl) aminomethane (Tris), 30 mmol/L NaCl, 10 mmol/L EDTA, and 6.25 mg/mL bovine serum albumin (BSA), pH 7.6. Buffer B consisted of 46 mmol/L Tris, 7.5 mmol/L N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES), 15.5 mmol/L KCl, 78 mmol/L NaCl, 5 mmol/L EDTA, and 3.13 mg/mL BSA, pH 7.4.

**Platelet preparations.** Platelet-rich plasma was prepared from citrated blood as previously described. Two parts of platelet-rich plasma were mixed with one part of 30 mmol/L citric acid, 3 mmol/L KCl, 102 mmol/L NaCl, and 4.8 mmol/L glucose, pH 6.5, after adjustment of the pH to 6.5. Thereafter, platelet-rich plasma was incubated at 37 °C for 30 minutes, and platelets were washed three times by repeated centrifugation for ten minutes at 1,200 g at room temperature and resuspension in washing buffer containing 3 mmol/L Tris(2-N-morpholino) ethane sulfonic acid (MES), 3 mmol/L KCl, 147 mmol/L NaCl, and 4.8 mmol/L glucose, pH 6.5. Finally, they were resuspended in 28 mmol/L TES, 3 mmol/L KCl, 108 mmol/L NaCl, and 25 mmol/L glucose, pH 6.8, at a concentration of 5.1 x 10^10/mL and incubated for one hour at room temperature. In experiments with deoxyglucose- and antimycin A–treated platelets, glucose was omitted in the third washing step, and 25 mmol/L deoxyglucose and 1 μmol/L antimycin A instead of 25 mmol/L glucose were included in the final resuspension buffer.
Plaquelet Immunoglobulin Internalization

Protein Preparations and Labeling With Tritium. The IgG was obtained by cold ethanol fractionation and contained IgG: IgM:IgA in a ratio of 600:8:1. It was cross-linked with BDB according to Pfueiler et al.15 To remove IgG not cross-linked and smaller aggregates, BDB-IgG was gel filtered on a Biogel A-5m column (Bio-Rad Laboratories; Richmond, Va), and only protein eluted in the void volume consisting of aggregates with ten to 30 IgG molecules was used. BDB-IgG, monomeric IgG (IgG obtained from the upper half of the contents in a test tube after centrifugation at 100,000 g for one hour at 4 °C), and human serum albumin were labeled with 3H by reductive methylation as described.19 In brief, 3 to 4 mg of protein in 500 μL 10 mmol/L Na-phosphate and 0.14 mol/L NaCl, pH 7.0, were mixed at 4 °C with 800 μL of 0.5 mol/L Na-diethylborate, pH 9.0, and 155 μL of fresh 20 mmol/L formaldehyde, and 30 seconds later, 100 μL of 8.8 Ci/mmol/L NaBH4 (approximately equal to 5 mCi in 10 mmol/L NaOH; 8.8 Ci/mmol/L; New England Nuclear, Boston) were added. After 20 minutes at 4 °C, 20 μL of 0.1 mol/L NaBH4 was added, and after a further 20 minutes, 20 μL of glycine was added and the pH adjusted to about 7 with 20% acetic acid. After exhaustive dialysis against 10 mmol/L Tris-HCl and 0.14 mol/L NaCl, pH 8.0, labeled protein was stored at −70 °C in aliquots.

Measurement of labeled protein associated with washed platelets and pronase treatment. Before use, 3H-BDB-IgG and BDB-IgG were centrifuged for ten minutes at 4 °C and 40,000 g to remove insoluble material, and monomeric IgG preparations were centrifuged at 100,000 g at 4 °C for one hour, whereupon only the upper half of the supernatant was used to eliminate self-aggregated IgG as far as possible. Four parts buffer A were incubated at 37 °C or 4 °C with two parts washed platelets (5.1 ± 10^7/mL) and one part 0.15 mol/L NaCl with slow stirring. After 5 minutes, one part labeled protein was added and incubation continued with slow stirring for 30 minutes at the temperature indicated. Then all tubes were cooled to 4 °C and centrifuged at 11,000 g for 3 minutes. The supernatants were discarded and the sediments resuspended with eight parts cold buffer B. Duplicate 600-μL aliquots of each sample were incubated either with 200 μL BSA (20 mg/mL in 0.15 mol/L NaCl) or with 200 μL pronase (20 mg/mL in 0.15 mol/L NaCl, 77 PUK [enzyme activity liberating folin positive amino acids] per mg; Sigma Chemical Co, St Louis). Digestion of surface-bound ligand was allowed to proceed usually for four hours at 4 °C, a time lag considered to be optimal because incubation for a further five hours led only to a 4% decrease in remaining platelet-associated radioactivity and because incubation of platelets with pronase for longer time periods (16 hours at 4 °C) resulted in some platelet damage as determined by the release of 3H-serotonin and 3H-adenine. After incubation of the platelets with pronase and BSA, 150 μL of a mixture of deoxynylphosphatidylcholine and butylphosphatidylcholine (1:2.56) were added, and the tubes were centrifuged at 11,000 g for 30 seconds, separating the platelets from the water phase through the phthalate mixture. Aliquots of the supernatants were counted and the tubes inverted. After removing traces of supernatant using absorbent paper, 150 μL 0.5% Triton X-100 (Bio-Rad, Richmond, Calif) and two drops of xylol were added to the sediments before sonication with a Branson sonifier (Branson Sonic Co, Danbury, Conn). Solubilized platelets were transferred to a counting vial and radioactivity measured in a Packard Tri Carb 2450 Liquid Scintillation Spectrometer (Downers Grove, Ill). Inhibitors of platelet activation by 3H-BDB-IgG were added to the incubation mixture instead of the one part 0.15 mol/L NaCl. Experiments were always done in duplicate, which usually did not differ from one another by more than 5%.

Calculations. Normally the amount of platelet-associated ligand remaining after BSA or pronase treatment was calculated first by using the specific activity of the labeled compound. The percentage of pronase-resistant protein was then calculated as 100 times the ratio between the platelet-associated protein in the presence of pronase and in the presence of BSA. In the initial experiments, 200 μg/mL of insulin and 43 μCi of [3H]-hydroxymethylulin (30 μCi/mL, 2.14 μCi/mg; Amersham, England) were included in buffer B (no Pronase) to correct for labeled protein trapped within the platelet sediment but not associated with the platelets themselves. Calculations were made as previously described.14 These corrections were found to be irrelevant, and therefore the insulin preparations were omitted in later experiments.

Measurement of 3H-serotonin release by washed platelets. Platelets were labeled with [3H]-serotonin (5-hydroxy(side chain-2-[3H])-tryptamine creatinine sulfate, 55 mCi/mmol/L [Amersham, England]) by incubation with the labeled compound at a concentration of 125 nCi/mL of platelet suspension for one hour at room temperature. On an average, 92% of the [3H]-serotonin was taken up by the platelets. To measure the release of 3H-serotonin induced by 3H-BDB-IgG, incubations were done in the same way as for measurement of 3H-BDB-IgG association, but imipramine (2 mmol/L, final concentration) was added to prevent reuptake of released 3H-serotonin. After the indicated incubation period at 37 °C or 4 °C, 400 μL of the mixture was centrifuged at 11,000 g for 30 seconds, and 200 μL of supernatant was added to 2 mL of scintillator to be counted in the Packard liquid scintillation spectrometer. The formula for the calculation is as follows: [H-serotonin released (%)] = [(X − B)/(T − B)] × 100, where X is the cpm of the sample after centrifugation, T is the total cpm contained in 200 μL before centrifugation, and B is the control—cpm of 200 μL of supernatant after centrifugation but without 3H-BDB-IgG.

Other methods. (1) Platelets were counted in a TOA platelet counter (TOA, Medical Electronics Co, Kobe, Japan) according to the instructions of the manufacturer. (2) To measure possible degraded products of platelet-associated 3H-BDB-IgG, platelets incubated with 3H-BDB-IgG at 4 °C or 37 °C for 30 minutes were solubilized with 1% sodium dodecyl sulfate (SDS; Bio-Rad, Richmond, Calif), one aliquot was immediately counted, and trichloroacetic acid was added to the other aliquot (10% final concentration). After a four-hour incubation period at 4 °C, the samples were centrifuged, and radioactivity in the supernatants was determined. (3) Slab gel electrophoresis of SDS solubilized and reduced platelets previously incubated with 3H-BDB-IgG at 4 °C or 37 °C in a 5% to 15% gel was done according to Laemmli,16 and fluorography of the gels was performed as previously described.14 (4) Prostaglandin E and cytochalasin B were obtained from Sigma Chemical Co, St Louis. They were dissolved in dimethylsulfoxide to a concentration of 10 mmol/L and stored at −20 °C. Dilutions were made with 0.15 mmol/L NaCl. All other substances were of reagent grade. (5) Protein concentrations were determined with a modified Lowry method as described15 using BSA as a standard.

RESULTS

Pronase susceptibility of 3H-BDB-IgG or 3H-monomeric IgG (3H-mIgG) associated with washed human platelets at 4 °C or 37 °C. In a first step, the capacity of platelets to take up 3H-BDB-IgG was evaluated under three different conditions: at 37 °C, at 4 °C, and at 37 °C after treatment with deoxyglucose/antimycin A. Figure 1A shows that platelets were able to take up more 3H-BDB-IgG at 37 °C than at 4 °C (0.60 μg compared to 0.38 μg), and in the presence of deoxyglucose/antimycin A, association at 37 °C was reduced to 0.20 μg.

The capacity of pronase to remove platelet-associated 3H-BDB-IgG was also assessed: 52% of the 3H-BDB-IgG...
associated with platelets at 37 °C resisted subsequent pronase treatment, whereas only 23% of the 3H-BDB-IgG associated to platelets at 4 °C or to deoxyglucose/antimycin A–treated platelets at 37 °C was refractory to pronase digestion (Fig 1A). Thus, at 37 °C, a 29% increase of pronase-resistant protein was found. When platelets were incubated with 3H-BDB-IgG for 30 minutes at 37 °C, washed, treated with deoxyglucose/antimycin A at room temperature for one hour, and then further incubated at 37 °C for 30 minutes, they still carried 70% of the initial pronase-resistant 3H-BDB-IgG. Thus, the deoxyglucose/antimycin A treatment was unable to make substantial amounts of pronase resistant 3H-BDB-IgG available to pronase digestion. When experiments were carried out with 3H-monomeric IgG (3H-mIgG) instead of 3H-BDB-IgG, they revealed that incubation at either 37 °C or 4 °C allowed similar amounts of 3H-mIgG to become associated with platelets in a way that was highly susceptible to pronase treatment at both temperatures (Fig 1B). To exclude self-aggregated IgG in the mIgG preparation as much as possible, the IgG preparations were always centrifuged immediately before use for one hour at 100,000 g and 4 °C, and only the upper half of the supernatants was used.

Although all experiments were done in the presence of 5 mmol/L EDTA, which is known to prevent platelet aggregation, we investigated whether the decreased pronase susceptibility of 3H-BDB-IgG associated to platelets at 37 °C was possibly due to the entrapment of protein within microscopic aggregates or agglutinates. In fact, pronase resistance of labeled, platelet-associated mIgG was as low as indicated in Fig 1B whether or not 20 μg/mL BDB-IgG was included in the samples during the 37 °C incubation period to activate the platelets, and the same result was obtained using labeled human serum albumin instead of 3H-mIgG (data not shown).

This is incompatible with platelet clumping as the major reason for the increased pronase resistance of platelet-associated 3H-BDB-IgG. Nevertheless, platelet activation produced by incremental amounts of 3H-BDB-IgG resulted in some microscopic aggregation or agglutination even in the presence of EDTA as seen from a decrease in platelet numbers determined by electronic counting (Fig 2). However, comparison of aggregation to pronase susceptibility of platelet-associated 3H-BDB-IgG revealed no correlation: 50% pronase-resistant platelet-associated 3H-BDB-IgG was observed with 5 μg/mL of 3H-BDB-IgG, whereas the reduction of platelet count at this concentration was only 2% (Fig 2). In addition, pronase resistance was readily saturable, whereas the reduction in platelet count progressively increased with increasing 3H-BDB-IgG inputs.

The time course of the acquisition of resistance to pronase by 3H-BDB-IgG associated with platelets at 37 °C and at 4 °C is shown in Fig 3. It appears that pronase resistance becomes detectable after two minutes and reaches a plateau after one hour, whereas at 4 °C, pronase was always able to remove the bulk of platelet-associated radioactivity. The same saturation characteristics were obtained when the time course of the 3H-BDB-IgG association with platelets was examined after BSA or pronase treatment (data not shown).

Concentration dependence of 3H-BDB-IgG binding to platelets and its susceptibility to pronase digestion. Increasing amounts of 3H-BDB-IgG were incubated with washed platelets at 37 °C or at 4 °C for 30 minutes in the presence or absence of 5 mg/mL unlabeled mIgG. Then the amount of 3H-BDB-IgG associated with the platelets after the four-hour incubation with pronase or BSA at 4 °C was determined. Specific binding is referred to as the difference of the amounts of 3H-BDB-IgG found associated to platelets with and without unlabeled mIgG in the system. In parallel tubes, release of 14C-serotonin was measured at 37 °C (Fig 4). As can be seen, specific 3H-BDB-IgG binding approached saturation at 4 °C but not at 37 °C with or without pronase treatment. Significant binding had already occurred at a very low 3H-BDB-IgG concentration, which did not result in serotonin release at 37 °C. Nonspecific binding was substan-
were taken and kept on ice. After the last aliquot had been taken, 3H-BDB-lgG. 3H-BDB-lgG (10 μg/mL) was incubated with washed platelets at 37 °C or at 4 °C (from platelets prelabeled with 14C-serotonin) was measured for 30 minutes at 37 °C or 4 °C in the presence or absence of 5 mg/mL BSA to the other, and further processing was done as described in Materials and Methods.

When platelets were treated with 100 μg/mL 3H-BDB-IgG at 4 °C for 60 minutes, washed, and warmed to 37 °C for a further five- or 30-minute incubation, pronase susceptibility after both 37 °C incubation periods was decreased by 13% as compared to such susceptibility measured after the 4 °C time period. At the same time, 42% of the 14C-serotonin was released by the platelets. Lower 3H-BDB-lgG inputs (20 μg/mL or 50 μg/mL) resulted in no significant decrease of pronase susceptibility of associated protein whereas 14C-serotonin release was below 18%.

Inhibition of 3H-BDB-lgG association with platelets by monomeric IgG and by reagents interfering with platelet activity at different levels. Inhibition of the 3H-BDB-lgG association with washed platelets at 37 °C or 4 °C induced by incremental amounts of mlG is shown in Fig 5. For these experiments, mlG was added to the platelets five minutes before 3H-BDB-lgG, and it can be seen that mlG inhibits the uptake of 3H-BDB-lgG by the platelets at both incubation temperatures independently whether or not pronase was added. Pronase resistance of platelet-associated 3H-BDB-lgG was also inhibited by mlG, inhibition of the 3H-BDB-lgG association occurring at lower mlG inputs than inhibition of pronase resistance (data not shown).

The effects of acetylsalicylic acid (which interferes with the prostaglandin pathway by inhibiting cyclooxygenase2'), prostaglandin E1 (which elevates the intracellular cAMP concentration2') and cytochalasin B (which interacts with actin2') on pronase susceptibility of platelet-associated 3H-

Table 1. Comparison of the Percentage of Pronase-Resistant 3H-BDB-lgG Specifically Associated With Washed Platelets and Serotonin Release in Relation to Increasing 3H-BDB-lgG Additions to Washed Platelets at 37 °C and 4 °C

<table>
<thead>
<tr>
<th>3H-BDB-lgG (μg/mL)</th>
<th>37 °C</th>
<th>4 °C</th>
<th>Percentage of Pronase-Resistant 3H-BDB-lgG Specifically Associated With Washed Platelets at 37 °C</th>
<th>4 °C</th>
<th>14C-Serotonin Release at 37 °C</th>
<th>4 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>46</td>
<td>23</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>13.5</td>
<td>54</td>
<td>28</td>
<td>10</td>
<td></td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>77</td>
<td>46</td>
<td>31</td>
<td></td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>72</td>
<td>41</td>
<td>31</td>
<td></td>
<td>49</td>
<td></td>
</tr>
</tbody>
</table>

*Experimental procedures as described in Materials and Methods. Values in brackets represent the difference of values at 37 °C and 4 °C.

Fig 3. Time course of pronase-resistant platelet-associated 3H-BDB-lgG. 3H-BDB-lgG (10 μg/mL) was incubated with washed platelets at 37 °C (●) or 4 °C (○). At the indicated times, aliquots were taken and kept on ice. After the last aliquot had been taken, 5 mg/mL pronase was added to one half of each aliquot and 5 mg/mL BSA to the other, and further processing was done as described in Materials and Methods.

Fig 4. Dose dependence of specific 3H-BDB-lgG association with washed platelets after BSA or pronase treatment (-----) and simultaneous measurement of serotonin release. (---) Washed platelets were incubated with increasing amounts of 3H-BDB-lgG for 30 minutes at 37 °C or 4 °C in the presence or absence of 5 mg/mL mlG, and the amount of 3H-BDB-lgG specifically associated with 7.5 x 10⁷ platelets was determined. Specific association represents the difference of association measured in the presence and absence of mlG. Key: ●, platelets incubated at 37 °C before the addition of BSA; ■, platelets incubated at 37 °C before the addition of pronase; ○, platelets incubated at 4 °C before the addition of BSA; and □, platelets incubated at 4 °C before the addition of pronase. The 3H-BDB-lgG-induced serotonin release at 37 °C (from platelets prelabeled with 14C-serotonin) was measured (△) in parallel tubes.

Fig 5. Inhibition by mlG of 3H-BDB-lgG association with washed platelets. Washed platelets were incubated with increasing amounts of mlG for five minutes at 37 °C or 4 °C before the addition of 3H-BDB-lgG (16 μg/mL). After a further incubation for 30 minutes at the corresponding temperature, platelet-associated protein was determined as described in Materials and Methods. For symbols see Fig 4.
BDB-IgG were studied. In a first set of experiments, washed platelets were incubated with the inhibitor for five minutes at 37 °C before the addition of 3H-BDB-IgG and a further incubation for 30 minutes at 37 °C. Then the percentage of pronase-resistant, platelet-associated 3H-BDB-IgG was determined. The same experiment was also conducted at 4 °C. At this temperature, at which platelets are not activated by 3H-BDB-IgG, the percentage of pronase-resistant protein did not change significantly and was within a range of 18% to 23% whether or not inhibitors were present. Table 2, column 2, shows the inhibition of the increased pronase resistance of platelet-associated 3H-BDB-IgG induced during the activation of the platelets at 37 °C. In parallel tubes, the 3H-BDB-IgG-induced release of 14C-serotonin at 37 °C was also measured (Table 2, column 3). Although acetylsalicylic acid and prostaglandin E1 had a marked inhibitory effect on 3H-BDB-IgG-induced serotonin release, their effect on preventing pronase from removing 3H-BDB-IgG associated to platelets was less distinct. In contrast, addition of cytochalasin B led to an increased protease susceptibility of platelet-associated 3H-BDB-IgG but had little or no inhibitory effect on 3H-BDB-IgG-induced serotonin release. In a second set of experiments, platelets were incubated with 3H-BDB-IgG for 30 minutes, one sample at 37 °C, the other at 4 °C; both samples were washed at 4 °C and incubated thereafter with inhibitor at the corresponding temperature for 30 minutes (Table 2, column 3). It was found that 10 μmol/L prostaglandin E1 had no effect on the pronase resistance of platelet-associated 3H-BDB-IgG, whereas increasing cytochalasin B input led to an inhibition of the increased pronase resistance of platelet-associated 3H-BDB-IgG obtained at 37 °C, the extent being not so marked as the decrease observed upon simultaneous incubation of cytochalasin B and 3H-BDB-IgG with platelets. Again, both inhibitors had no effect on the pronase resistance of 3H-BDB-IgG associated with platelets at 4 °C.

Finally, we were interested to know whether platelet-associated 3H-BDB-IgG is processed further by intrinsic platelet enzymes. For this purpose, 3H-BDB-IgG was incubated with platelets at 37 °C and 4 °C for 30 minutes or 120 minutes, respectively; then the platelets were solubilized with SDS, and trichloroacetic acid–precipitable radioactivity was measured: no decrease in precipitable radioactivity was found. In addition, gel electrophoresis of 3H-BDB-IgG obtained from the solubilized platelets and subsequent fluorography of the gel showed no degradation of the aggregated IgG into smaller components.

**Table 2. Effect of Inhibitors of Platelet Functions on the Resistance to Pronase of Platelet-Associated 3H-BDB-IgG and the Induction of Serotonin Release by 3H-BDB-IgG**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition of Pronase Resistance (%)*</th>
<th>Inhibition of 14C-Serotonin Release Induced by 3H-BDB-IgG at 37 °C (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylsalicylic acid</td>
<td>By Simultaneous Incubation of Inhibitor and 3H-BDB-IgG With Platelets†</td>
<td>By Incubation of Inhibitors With Platelets Pretreated With 3H-BDB-IgG‡</td>
</tr>
<tr>
<td>1.0 mmol/L</td>
<td>19</td>
<td>ND</td>
</tr>
<tr>
<td>10.0 mmol/L</td>
<td>44</td>
<td>ND</td>
</tr>
<tr>
<td>Prostaglandin E1</td>
<td>0.2 μmol/L</td>
<td>15</td>
</tr>
<tr>
<td>1.0 μmol/L</td>
<td>24</td>
<td>ND</td>
</tr>
<tr>
<td>10.0 μmol/L</td>
<td>49</td>
<td>0</td>
</tr>
<tr>
<td>Cytochalasin B</td>
<td>0.2 μmol/L</td>
<td>11</td>
</tr>
<tr>
<td>0.5 μmol/L</td>
<td>40</td>
<td>16</td>
</tr>
<tr>
<td>1.0 μmol/L</td>
<td>60</td>
<td>31</td>
</tr>
<tr>
<td>10.0 μmol/L</td>
<td>90</td>
<td>77</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

*Numbers represent the percent inhibition of the increased pronase resistance of 3H-BDB-IgG associated with platelets at 37 °C. This is calculated as 100 times the difference between the pronase resistant 3H-BDB-IgG from samples incubated at 37 °C and 4 °C in presence of the inhibitor and the same difference without the inhibitor.

†Washed platelets were incubated with the inhibitor for five minutes before the addition of 3H-BDB-IgG (30 μg/mL) and a further 30-minute incubation. Two samples were run, one at 37 °C, the other at 4 °C. Release of 14C-serotonin induced by 30 μg/mL 3H-BDB-IgG at 37 °C was assessed in parallel tubes. At 4 °C there was no release of 14C-serotonin.

‡Washed platelets were incubated with 3H-BDB-IgG at either 37 °C or 4 °C for 30 minutes, washed at 4 °C, and resuspended in buffer B before the addition of inhibitors and a further 30-minute incubation at the corresponding temperature.

**DISCUSSION**

We demonstrate that part of aggregated immunoglobulin used for the activation of platelets becomes inaccessible to prolonged pronase digestion probably because of displacement to intracellular sites. Our findings may explain the observation that saturable and reversible association of aggregated IgG with washed human platelets can be obtained at 4 °C but can only be demonstrated at 37 °C in the presence of metabolic inhibitors deoxyglucose and antimycin A. Thus, evidence is provided that the engulfment of aggregated IgG taking place at 37 °C is energy dependent and does not result from either passive diffusion to pronase-inaccessible sites or entrapment within platelet aggregates or agglutinates. This is based on the following arguments: (1) EDTA (5 mmol/L), which is known to prevent platelet aggregation, was always included in the reaction mixtures. (2) Although with the addition of incremental amounts of 3H-BDB-IgG to constant amounts of platelets some decrease in the platelet count occurred in the reaction tubes (Fig 2), a simultaneous evaluation of pronase resistance revealed an increase of engulfed material that already occurred at much lower 3H-BDB-IgG inputs and then plateaued. In addition, the day-to-day variation in the platelet count decrease was substantial during these experiments, whereas pronase resistance remained constant within small limits. (3) There was no change in the proportion of platelet-associated labeled mlgG or human serum albumin that was resistant to pronase digestion after the addition of 20 μg/mL BDB-IgG, a concentration of BDB-IgG known to activate the platelets even in the presence of small amounts of 3H-mlgG or human serum albumin. (4) The omission of stirring during the incubation of 3H-BDB-IgG with the platelets did not lead to
a decreased pronase resistance of platelet-associated $^3$H-BDB-IgG (data not shown).

If such a reduction of pronase-accessible surface area by aggregation or agglutination of platelets is not the reason why part of the $^3$H-BDB-IgG associated at 37°C escapes pronase digestion, then the assumption appears justified that the binding of $^3$H-BDB-IgG to the platelet plasma membrane is followed by redistribution to an area not accessible to pronase digestion. There is a difference in the extent of the $^3$H-BDB-IgG associated with platelets and its pronase resistance depending on whether the platelets were incubated with the IgG aggregates at 37°C or preincubated with the aggregates at 4°C and then warmed to 37°C after the removal of unbound material. When incubation was at 4°C prior to warming to 37°C, the fraction of $^3$H-BDB-IgG that was not susceptible to pronase digestion was only increased by 13% even at the highest input of IgG aggregates (100 μg/mL), with no significant increase at lower inputs of $^3$H-BDB-IgG. In contrast, incubation of the platelets at 37°C with low amounts of $^3$H-BDB-IgG (20 μg/mL) resulted in a 29% increase of pronase-resistant platelet-associated $^3$H-BDB-IgG. Simultaneous measurement of the $^1$C-serotonin release by platelets treated with $^3$H-BDB-IgG at 4°C and then warmed up revealed a reduced release compared to that measured by the incubation of $^3$H-BDB-IgG with washed platelets at 37°C, although the extent of reduction was not so pronounced as compared to the reduction of pronase resistance. It appears that during centrifugation after the 4°C incubation period a considerable part of the $^3$H-BDB-IgG is lost from its binding sites, consistent with earlier findings in which it was shown that most of reversibly bound IgG aggregates dissociate from the membrane within minutes,14,16,17 although binding at 4°C to platelets is essentially complete after a 60-minute incubation period (data not shown).17 In addition, it cannot be excluded that the binding of aggregates to sites from where they could redistribute at 37°C is temperature dependent and could be diminished at 4°C. Nevertheless, both serotonin release and the limited increase of the pronase resistance of associated $^3$H-BDB-IgG occurred immediately, within five minutes after warming to 37°C, consistent with a receptor-mediated process in which the binding of the ligand is the rate-limiting step.52 This obviously was the case in our experiments because incubation of $^3$H-BDB-IgG with platelets at 37°C resulted in a maximal increase of pronase-resistant platelet-associated $^3$H-BDB-IgG at much later stages of the incubation period (Fig 3). Moreover, high amounts of mIgG inhibited both the total association of $^3$H-BDB-IgG to washed platelets and the pronase-resistant part of it (Fig 5).

Because pronase resistance of $^3$H-mIgG was not influenced by the presence of BDB-IgG, eg, on activated platelets, receptor clustering through the simultaneous binding of one BDB-IgG molecule to at least two receptors must be envisaged as the triggering mechanism for escape from pronase action. Receptor clustering has been described for lymphocytes,24,25 whereby two forms are distinguished, the energy-independent patching and the energy-dependent capping that engages the cytoskeleton of the cell. Recently, patching and capping of concanavalin A receptors have also been described on platelets.26 The capping phenomenon was not dependent on extracellular divalent cations and was inhibited by cytochalasin B, implying that an intact microfilament structure was necessary. Our results obtained with acetylsalicylic acid, prostaglandin E1, and cytochalasin B strongly indicate that the redistribution phenomenon of $^3$H-BDB-IgG on platelets closely resembles a capping phenomenon. Engagement of $^3$H-BDB-IgG was less inhibited by acetylsalicylic acid and prostaglandin E1 than was serotonin release. In contrast to these inhibitors, cytochalasin B, which binds to actin, was able to inhibit engulfment to a greater extent than release of serotonin. We conclude from these results that engulfment of $^3$H-BDB-IgG depends on a functionally intact cytoskeleton.

Although we have no definitive proof of the site in the platelet to which $^3$H-BDB-IgG moves to become resistant to pronase digestion, there are strong indications that it is transferred to the open canalicular system. Incubation of platelets pretreated with $^3$H-BDB-IgG at 37°C with prostaglandin E1 or deoxyglucose/antimycin A had no or only little effect on pronase resistance, whereas cytochalasin B administrations led to a substantial decrease in pronase-resistant $^3$H-BDB-IgG (Table 2). This supports the idea that most of the engulfed $^3$H-BDB-IgG must still be connected to the surface, thus becoming accessible to pronase digestion after disruption of actin filaments. Whether cytochalasin B allows engulfed $^3$H-BDB-IgG to float back to the surface or whether the opening of the open canalicular system is dilated by the drug cannot be decided from our results. Uptake of proteins into the open canalicular system has been shown by electron microscopy with horseradish peroxidase, cationized ferritin, or ferritin-conjugated wheat germ agglutinin. Peroxidase and cationized ferritin were readily taken up at 37°C but not into washed and fixed platelets.28,29 Although hardly any pinocytic vesicles were observed,27 coated pits have been reported.28,29 Therefore, further processing of engulfed $^3$H-BDB-IgG is possible, eg, in lysosomes. As yet, we could not demonstrate detectable digestion of $^3$H-BDB-IgG associated with platelets after two hours at 37°C, a time long enough to reliably detect digested ligand in phagocytic cells.31 Whether small amounts of engulfed $^3$H-BDB-IgG are transferred to storage organelles or whether further engulfment is dependent on extracellular divalent cations needs further investigation.

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REFERENCES

Part of the activating cross-linked immunoglobulin G is internalized by human platelets to sites not accessible for enzymatic digestion

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