Characterization of Autoantigenic Epitopes on Platelet Glycoprotein IIb/IIIa Using Random Peptide Libraries

By R.D. Bowditch, P. Tani, K.C. Fong, and R. McMillan

Most patients with chronic immune thrombocytopenic purpura (ITP) have autoantibodies directed against the glycoprotein (GP) IIb/IIIa complex. We have used a filamentous phage library that displays random linear hexapeptides to identify peptide sequences recognized by these autoantibodies. Plasma antibody eluates from two patients were used to select for phage displaying autoantibody-reactive peptides. From patient ITP-1 (known to have two distinct autoantibodies), we identified anti-GPIIb/IIIa antibody-specific phage encoding the peptide sequences Arg-Glu-Lys-Ala-Lys-Trp (REKAKW) and Pro-Val-Val-Trp-Lys-Asn (PVVWKN). Patient ITP-2 bound phage encoding the hexapeptide sequence Arg-Glu-Leu-Leu-Lys-Met. Each phage showed saturable dose-dependent binding to immobilized autoantibody, and binding could be blocked with purified GPIIIa. Patient ITP-1 autoantibody recognition of phage encoding REKAKW could be blocked with a synthetic peptide derived from the GPIIIa cytoplasmic tail; however, the PVVWKN was not. Using sequential overlapping peptides from the GPIIIa cytoplasmic region, an epitope for ITP-1 was localized to the sequence Arg-Ala-Arg-Ala-Lys-Trp (GPIIb/IIIa 734-739). Inhibition studies using synthetic peptides showed that phage REKAKW and PVVWKN were recognized by distinct autoantibodies from patient ITP-1. To determine whether individual patients with ITP possessed autoantibodies that recognize similar antigenic determinants on GPIIb/IIIa, the three phage were tested for binding to five other ITP patient autoantibodies. The phage encoding the peptide PVVWKN was found to bind ITP-1 and one other patient autoantibody. This result suggests that ITP patients recognize a limited number of shared epitopes.

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MATERIALS AND METHODS

Patients

We studied plasma anti-GPIIb/IIIa antibody from two chronic ITP patients (ITP-1 and ITP-2). All chronic ITP patients were diagnosed using standard criteria. Each patient had severe disease, as shown by their failure to attain a complete remission with either corticoste-

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AfJinity Purijication of Phage Mimitopes Recognized by TTCTCACTCGGCCGACGGGGT (NNK)6 GGGGCCGCTGGG-

Monoclonal Antibodies

The aminoterminus by Ala-Asp-Gly-Ala and on the carboxyterminus codes for each of the 20 residues and an amber stop codon. The library was prepared by ligating the synthetic 33-bp containing a core degenerate coding sequence (NNK)6, which enter into fUSE5 and transforming into E coli MC1061 by electroporation. Each individual phage contains one of these random oligonucleotide sequences and will display the appropriate hexapeptide, flanked on the amino- and carboxyterminus by the remainder of the gene III product. This library contains greater than 2 × 10^11 independent clones.

Block of phage binding to autoantibody with synthetic peptides

Peptides were synthesized on a Gilson AMS422 by the Protein/DNA Core Facility, The Scripps Research Institute. The synthetic peptides were Gly-Ala-Arg-Glu-Lys-Ala-Lys-Trp-Gly-Ala (GAREKAKWG) and Gly-Ala-Pro-Val-Val-Trp-Lys-Asp-Gly-Ala (GAPVVWKNGA). Microtiter wells coated with murine monoclonal anti-IgG (HB43) and blocked with 3% BLOTTO were incubated for 1 hour with 100 μg/mL of each peptide or irrelevant phage. After blocking with BSA-PBS-Tween containing a known quantity of affinity-purified antibody, 50 μL of the following were added: patient autoantibody eluate (100 μL) to test for binding of the selected phage and specificity of the affinity purified antibody, 50 μL of affinity purified antibody or control eluate to test for the ability of the selected phage to compete with antibody binding to autoantibody. After 2 hours of incubation at room temperature and washing, 50 μL of substrate (0.7 μL/mL HzO2 and 0.7 mg/mL o-phenylenediamine dihydrochloride in 0.02 mol/L citric acid) was added to each well. After 15 minutes of incubation at room temperature, 50 μL of a 1/2 dilution of the substrate was added to each well. Binding of horseradish peroxidase (ABC System; Vector Laboratories, Burlingame, CA), and 50 μL of substrate (0.7 μL/mL HzO2 and 0.7 mg/mL o-phenylenediamine dihydrochloride is 0.02 mol/L citric acid, 0.05 mol/L Na2HPO4 [pH, 5]). Net optical density after subtraction of control eluate values was determined. In selected studies, bound rabbit anti-human IgG antibody was detected with 125I-affinity-purified goat antirabbit antibody (Sigma Chemical Co, St Louis, MO). In blocking studies, varying concentrations of purified GPIIb/IIIa (affinity-purified using monoclonal anti-GPIIb antibody [2A9] linked to sepharose) was added for 60 minutes before the addition of the phage. Otherwise, the assay was identical.

Evaluation of Autoantibody Specificity for the Panned Phage

Specificity was evaluated using 2 methods, (1) phage binding to autoantibody and lack of phage binding either to autoantibody that had been adsorbed with platelets or to control eluates and (2) blocking of phage binding to autoantibody by purified GPIIb/IIIa.

Phage expressing autoantibody mimotopes were affinity-purified by sequential adsorptions to immobilized autoantibody. Plasma autoantibody eluate (100 μL) was added to an aliquot of phage library (phage concentration was adjusted to contain at least 100 copies of each random peptide in 0.1% bovine serum albumin [BSA]/PBS-Tween), and the mixture was incubated overnight at 4°C. Then, 100 μL of a 1/2 dilution of staph-A agarose in either BSA/PBS-Tween or 3% BLOTTO/PBS-Tween were added to each tube and incubated with constant mixing for 60 minutes at room temp. The BSA and BLOTTO were alternated to prevent purification of phage, which may bind to these proteins. The mixture was transferred to a 15-mL capped tube, and the sepharose (containing bound autoantibody and any bound phage) was washed 9 times with 10 mL of PBS-Tween and once with 10 mL of normal saline (to remove any buffering capacity). To the pellet was added 600 μL of elution buffer (0.1 N HCl, pH 2.2, with glycine) and, after 15 minutes of incubation, the mixture was centrifuged for 2 minutes at 3000g. The supernatant fluid was aspirated, neutralized with 36 μL of 2 mol/L Tris-base (pH, 9.0), and added to 400 μL of E coli K91-Kan. After incubation for 15 minutes at 37°C, 9 mL of Superbroth was added, and the mixture was incubated at 37°C for 30 minutes with aeration. Next, 10 μL of tetracycline (200 μg/mL) was added, and the mixture was incubated for 60 minutes at 37°C. Dilutions (10^-4 to 10^-6) were plated on LB-tetracycline plates, and the percent recovery was determined.

To amplify the selected phage, each 10-mL aliquot of infected bacterial suspension was added to a 500-mL flask containing 90 mL of Superbroth with tetracycline (12.5 μg/mL) and incubated overnight at 37°C with aeration. The bacteria were removed by centrifugation at 10,000g for 5 min at 4°C, and the phage were precipitated from the supernatant fluid by adding 4 g of polyethylene glycol-8000 and 3 g NaCl, incubating on ice for 3 hours, and then centrifuging at 10,000g for 30 minutes at 4°C. The phage were resuspended in 2.5 mL of PBS/azide and titered. A total of four sequential affinity purifications were performed. Phage produced by individual bacterial colonies, harvested after the fourth purification, were tested for specificity as described below. Single-stranded DNA was isolated from purified phage and sequenced to determine the encoded peptide sequence.
incubated with patient autoantibody eluate for 2 hours. After washing, wells were incubated for 60 minutes with 40 µL of either synthetic peptide (800 µg/mL) or BSA (1 mg/mL). Next, 10 µL of purified phage was added, and the mixture was incubated for an additional 60 minutes. Bound phage were detected with rabbit anti-M13 phage antibody and 125I-affinity-purified goat antirabbit antibody as described above.

**Binding of Autoantibody to GPIIIa Peptides**

Thirty-nine overlapping biotinylated synthetic 10-residue peptides corresponding to amino acids 721-762 of GPIIIa (Chiron Mimotopes, Victoria, Australia) were dissolved in dimethyl sulfoxide/40% acetonitrile per the manufacturer’s instructions and used as a 1/1000 final dilution in 0.1% BSA in PBS containing 0.1% azide. Separate microtiter wells were coated with 50 µL of streptavidin (5 µg/mL in water) and incubated overnight. After 6 washes with 0.05% Tween-20 in PBS (PBS-Tween), the wells were blocked for 60 minutes with 2% BSA-PBS-Tween. After 6 washes, 50 µL of each peptide was added to separate wells, incubated for 2 hours, and washed 6 times. Next, 50 µL of patient antibody eluate was added and incubated for 2 hours. After washing, bound antibody was detected with 125I-antihuman IgG (~200,000 cpm/well).

**RESULTS**

We studied plasma antibody eluates from two patients with chronic ITP (ITP-1 and -2). The two plasma antibodies were chosen for study, because it was known that a portion of the antibodies bound to the cytoplasmic fragment of GPIIIa and that the REKAKW was on the cytoplasmic fragment of GPIIIa. This suggested that these phage reflected different epitopes. It is known from previously published studies that ITP-1 plasma contains autoantibodies to at least two distinct epitopes. One antibody binds to the cytoplasmic portion of GPIIIa, and the remaining antibody or antibodies bind to other regions of the GPIIib/IIIa heterodimer (patient ITP-3 in Fujisawa et al). To further evaluate the epitopes represented by the phage REKAKW and PVVWKN, we preadsorbed the phage with platelets and a dose-dependent decrease in the binding of both REKAKW and PVVWKN phage when the autoantibody eluate was preincubated with purified GPIIib/IIIa (Fig 2).

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Additional studies were performed to confirm that phage REKAKW and phage PVVWKN bind to distinct anti-GPIIb/IIIa autoantibodies. Phage binding studies were performed in which the patient’s autoantibody eluate was preadsorbed with synthetic peptides containing either the REKAKW or PVVWKN sequence, before addition of the phage. As shown in Fig 3, the synthetic peptides blocked only phage containing the same sequence. Thus, these phage must reflect two distinct epitopes.

The REKAKW sequence was found to be homologous to a region of the GPIIIa cytoplasmic tail (Fig 4). To ascertain if this homologous sequence was indeed an epitope for one of the autoantibodies from ITP-1, we evaluated the binding of this autoantibody to sequential 10-residue peptides from the GPIIIa cytoplasmic fragment. These studies show binding of ITP-1 antibody to five sequential overlapping peptides, all of which contained the sequence RARAKW (Fig 5). This
sequence is similar to the sequence REKAKW, displayed by the phage (Fig 4). It seems likely that the first 3 amino acids are important for binding because, in previous studies, the antibody bound to the synthetic peptide (amino acids 721-744) containing the entire RARAKW sequence (GPIIIa 734-739) but not to one beginning with AKW (amino acids 721-734). In summary, phage expressing the peptides REKAKW or PVVWKN bind to ITP-1 autoantibody eluate. The REKAKW appears to reflect a binding site on the cytoplasmic region of GPIIIa, whereas PVVWKN mimics an epitope in another portion of GPIIIa.

Patient ITP-2. Similar studies were performed using plasma autoantibody eluate from another patient, ITP-2. This autoantibody was known to bind exclusively to the GPIIIa cytoplasmic region as shown by studies using GPIIb/IIIa recombinants expressed on Chinese hamster ovary cells. This patient’s antibody bound strongly to the GPIIb/IIIa recombinant containing intact GPIIIa but did not bind to the recombinant that lacked the cytoplasmic portion of GPIIIa (patient ITP-6 in Fujisawa et al). We isolated phage from the library, expressing the peptide Arg-Glu-Leu-Leu-Lys-Met (RELLKM), which bound strongly in a dose-dependent manner to the patient’s plasma autoantibody eluate when compared with control eluates (Fig 1). Tests for specificity showed (1) minimal reactivity of the phage with eluate that had been adsorbed with platelets to remove antibody reactivity and (2) marked inhibition of phage binding by purified GPIIb/IIIa and by the synthetic GPIIIa cytoplasmic peptide (residues 721-762; Fig 6). The peptide sequence RELLKM is not represented in the cytoplasmic fragment, suggesting that this sequence represents a mimotope of the native epitope. This epitope may require a specific conformation or be made up from nonlinear sequences because this antibody did not bind to an isolated region when studied using sequential GPIIIa 10-residue peptides spanning the cytoplasmic portion of GPIIIa (data not shown). However, this autoantibody does bind to and is inhibited by the synthetic peptide corresponding to the amino acid sequence of the entire cytoplasmic region (amino acids 721-762).

Binding of Affinity-Purified Phage to Other ITP Autoantibodies

One of the goals of this project is to ultimately develop a panel of affinity-purified phage expressing multiple different epitopes (or mimotopes). This panel will be used to screen a large number of ITP autoantibodies to see if there are common epitopes. We have tested a small number of platelet-associated autoantibodies from ITP patients other than those described above, with the three affinity-purified phage. As shown in Table 1, phage PVWVKN and REKAKW bound to ITP-1 autoantibody and phage RELLKM bound to ITP-2 autoantibody as expected. However, PVWVKN also showed significant binding to autoantibody from patient ITP-4. Subsequent studies showed significant binding (>3 SD) of PVWVKN phage to ITP-4 autoantibody when compared with binding to eluates from 10 different control subjects (ITP-4, 27,695 cpm; mean binding to controls, 14,085 ± 1,559 cpm). In addition, binding of PVWVKN phage to ITP-4 autoantibody could be blocked by preincubation of
autoantibody with purified GPIIb/IIIa (100 μg/mL 57% blocking; 200 μg/mL, complete blocking). These results suggest that there may be a limited number of epitopes among ITP patients.

DISCUSSION

Most autoantibodies in patients with chronic ITP bind to antigens on either GPIIb/IIIa or GPIb/IX, although there are few data defining exact epitope localizations. As noted earlier, platelet-associated anti-GPIIb/IIIa autoantibodies bind to extracellular antigens, most of which are divergent cation-dependent. Attempts to further localize antigen sites on GPIIb/IIIa using large recombinant GPIIa peptides were largely unsuccessful, suggesting that the epitopes were either localized on GPIIb or were dependent on an intact complex. Some plasma anti-GPIIb/IIIa autoantibodies also bind to extracellular antigens, but others bind to antigens on the GPIIIa cytoplasmic region. Studies on anti-GPIIb autoantibodies also show serum antibodies that bind to both extracellular and cytoplasmic portions of GPIIb. Recent studies, using recombinant GPIIb peptides, have localized the binding region of 6 of 16 anti-GPIIb serum antibodies to a specific region on GPIIb (amino acids 389 to 412). Platelet eluates from two of these patients also bound to this peptide. The successful use of GPIIb recombinant peptides when compared with that of the negative studies with GPIIb/IIIa recombinant peptides may be in part due to the calcium-dependent heterodimeric structure of GPIIb/IIIa.

In this study, we describe the successful use of phage display to identify mimotopes of platelet autoantigens. Patient ITP-1 was known to have at least two distinct plasma autoantibodies, one reactive with the GPIIIa c-terminus and the other(s) to a separate, as yet undetermined, region of GPIIb/IIIa. As shown by previous studies, the anti-GPIIIa c-terminus antibody binds to a synthetic peptide corresponding to amino acids 724-744 of the GPIIIa cytoplasmic region but not to two others in this area (amino acids 737-751 and 742-762). Using the linear phage library, we identified two separate phage expressing different peptide sequences that bound strongly to the plasma autoantibody from ITP-1; one expressed the sequence REKAKW, and the other expresses PVVWKN. The binding of both phage was anti-GPIIb/IIIa-specific, because binding could be inhibited by purified GPIIb/IIIa and there was reduced binding to eluate that had been adsorbed with platelets.

Figure 6. Specificity of isolated hexapeptide display phage for ITP-2 anti-GPIIb/IIIa autoantibody. Isolated FUSE5 phage (1 × 10⁶ 10⁶ CFU/mL) expressing the hexapeptide sequence RELLKM were added to microtiter wells containing immobilized autoantibodies from patient ITP-2 alone (None), in the presence of 150 μg/mL purified GPIIb/IIIa (GPIIb/IIIa), or in the presence of 800 μg/mL GPIIIa cytoplasmic tail peptide (residues 721-762). Also shown, is FUSE5 phage binding to microtiter wells containing ITP-2 antibody eluates that were preadsorbed with normal platelets (Pit Adsorbed). Bound phage were detected and quantitated as described in Materials and Methods. Results are expressed as the percentage of maximal binding to ITP-2 autoantibody in the absence any added inhibitor.


**Table 1. Evaluation of Phage-Hexapeptide Binding to Platelet-Associated Autoantibodies**

<table>
<thead>
<tr>
<th>Eluate</th>
<th>REKAKW</th>
<th>PVVWKN</th>
<th>RELKLM</th>
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</thead>
<tbody>
<tr>
<td>ITP-1</td>
<td>15,860</td>
<td>24,632</td>
<td>10,321</td>
</tr>
<tr>
<td>ITP-2</td>
<td>8,732</td>
<td>9,910</td>
<td>30,012</td>
</tr>
<tr>
<td>ITP-3</td>
<td>7,085</td>
<td>7,318</td>
<td>10,650</td>
</tr>
<tr>
<td>ITP-4</td>
<td>7,583</td>
<td>16,265</td>
<td>8,068</td>
</tr>
<tr>
<td>ITP-5</td>
<td>6,751</td>
<td>9,267</td>
<td>9,886</td>
</tr>
<tr>
<td>ITP-6</td>
<td>5,908</td>
<td>7,298</td>
<td>6,623</td>
</tr>
<tr>
<td>ITP-7</td>
<td>9,184</td>
<td>6,965</td>
<td>8,534</td>
</tr>
<tr>
<td>Control</td>
<td>10,536</td>
<td>9,579</td>
<td>10,280</td>
</tr>
</tbody>
</table>

Eluate from ITP Patients (ITP-1 through ITP-7) or control platelets was anchored to microtiter wells that had been coated with murine antihuman IgG (HB-43) followed by blocking with BLOTTO. After washing, phage expressing the hexapeptides REKAKW, PVVWKN, or RELKLM were added. Bound phage were detected with rabbit anti-phage antibody(1:10)-goat antirabbit antibody. Results are the mean bound counts per minute of duplicate studies. Positive values are in boldface.

We determined that these phage represented (or mimicked) two distinct epitopes because the binding of each could be inhibited by an identical synthetic peptide (ie, synthetic peptide REKAKW blocked only the REKAKW phage) but not by a peptide corresponding to the alternative sequence. In addition, binding of the REKAKW phage could be inhibited by a synthetic peptide corresponding to the GPIIIa cytoplasmic region (amino acids 721-762), showing that it reflected an epitope in this area, whereas the PVVWKN phage could not. Using sequential 10-residue synthetic peptides spanning the GPIIIa cytoplasmic region, we could identify ITP-1 autoantibody binding to a 6-amino acid region (amino acids 734-739) containing the amino acid sequence, RARAKW, which has homology to the hexapeptide REKAKW.

Because the PVVWKN sequence was not localized to the GPIIIa cytoplasmic region but was specific for GP IIb/IIIa, it must be located either on the extracellular portion of GPIIb/IIIa or on GP IIb (the sequence PVV is noted on amino acids 472-474 of GP IIb) or must reflect a complex-dependent epitope.

The ITP-2 plasma autoantibody was known from prior studies to bind solely to the GPIIIa C-terminus. Phage binding studies to ITP-2 autoantibody showed GP IIb/IIIa-specific binding of a phage expressing the RELKLM sequence. This phage bound to a site on the GPIIIa cytoplasmic fragment because it was inhibited by a synthetic peptide corresponding to this region (amino acids 721-762). However, unlike the ITP-1 autoantibody, the plasma antibody from ITP-2 did not bind to any of the three smaller synthetic peptides (amino acids 721-744, 737-751, or 742-762) that spanned the cytoplasmic region, suggesting that the epitope might be configurational.

Although one of our reasons for using phage display technology in the study of autoantibodies is to specifically localize autoantigenic epitopes, the major reason is to develop a panel of affinity-purified phage that display peptides that bind to platelet autoantibodies and, therefore, correspond to or mimic the individual epitopes. This panel can then be used to evaluate whether there are several common autoepitopes among chronic ITP patients or a large number of unique epitopes. Although we studied only five platelet-associated autoantibodies from other ITP patients against the three affinity-purified phage (REKAKW, PVVWKN, and RELKLM), one autoantibody (ITP-4) showed significant binding to phage displaying the peptide PVVWKN. This binding could be blocked by purified GPIIb/IIIa. These results suggest that there may be hyperantigenic regions of GPIIb/IIIa that are particularly likely to induce autoantibody formation.

**REFERENCES**


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Characterization of autoantigenic epitopes on platelet glycoprotein IIb/IIIa using random peptide libraries

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