Deoxyribonucleic Acid-Binding Properties of Human Monoclonal Anti-Platelet Antibodies Obtained from Patients with Idiopathic Thrombocytopenic Purpura

Mitsuru MATSUMOTO, Kenichi MOCHIZUKI and Yuzuru KOBAYASHI

Recent studies of human monoclonal lupus autoantibodies have revealed that some of them are cross-reactive with platelets. To clarify further the bivalencies in anti-DNA and anti-platelet autoantibodies, we established nine human B cell lines producing monoclonal anti-platelet antibodies from patients with idiopathic thrombocytopenic purpura by Epstein-Barr virus transformation and studied the DNA-binding properties of these antibodies. Three of the antibodies showed cross-reactivity with single-stranded DNA (ssDNA), as determined by enzyme-linked immunosorbent assay and one of them was also found to bind to the synthetic polynucleotides; poly (dT) and poly (l), as well as to ssDNA. These findings suggest that cross-reaction between anti-DNA and anti-platelet antibodies is based on overlaps in specificity existing as common part of both autoantibodies.

Key words: Human monoclonal anti-platelet antibody, EBV transformation, Anti-DNA antibody, Cross-reaction

Human monoclonal lupus autoantibodies, screened and selected on the basis of binding to denatured DNA (dDNA), are known to react with platelets (1, 2). The platelet-binding properties of monoclonal anti-DNA autoantibodies are considered to be clinically significant, because thrombocytopenia is often seen in patients with systemic lupus erythematosus (SLE) (3, 4). For adequate evaluation of the bivalencies in anti-DNA and anti-platelet autoantibodies, however, studies on DNA-binding properties of monoclonal anti-platelet antibodies are also required.

Therefore, we established human B cell lines producing monoclonal anti-platelet antibodies from patients with idiopathic thrombocytopenic purpura (ITP) by Epstein-Barr virus (EBV) transformation and then tested the DNA-binding properties of these antibodies for this study.

In the present paper, we report human monoclonal anti-platelet antibodies cross-reactive with DNA in order to reach a better understanding of the overlapping specificity of these autoantibodies.

MATERIALS AND METHODS

1. Isolation of lymphocytes

Lymphocytes were obtained from three anti-DNA seronegative patients with ITP with their consent. Mononuclear cells from heparinized peripheral blood from one patient were purified by gradient centrifugation over Ficoll-Conray. Spleen cells from two patients were obtained at the time of splenectomy. The spleens were cut into small pieces followed by passage through a screen in RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 10 mM Hepes, 100 μg/ml streptomycin and 100 U/ml penicillin, and the purification of mononuclear cells was carried out as described above.
2. Preparation of EBV

A marmoset cell line B95-8 producing EBV (5) was kindly provided by Dr. K Yamamoto, Tokyo Medical and Dental University, Japan. The cells were grown in RPMI 1640 medium supplemented with 20% heat-inactivated fetal calf serum (FCS) (Gibco), referred to hereafter as the culture medium, at a density of $7 \times 10^5$ cells per ml for seven days at 37°C. The culture supernatant was then harvested, passed through a 0.45-$\mu$m membrane filter (Millipore Corp., Bedford, MA) and stored at −70°C until use.

3. EBV transformation

Twenty million lymphocytes from each patient were suspended in 10 ml of culture medium, mixed with an equal volume of the B95-8 culture supernatant and then the cells were plated at a density of $2 \times 10^5$ cells per 0.2 ml in flat-bottomed microculture plates (Falcon 3072, Oxnard, CA). Cultures were incubated in a humidified 5% CO2 incubator at 37°C and half of the medium was replaced every four days. After two weeks, the supernatants of EBV-transformed B-lymphocyte cultures were screened for antibody to platelet by the following method and cells from positive wells were cloned by limiting dilution or soft agar cloning using mitomycin C-treated (16 $\mu$g/ml, at 37°C for 30 min) Raji cells as the feeder layer.

4. Measurement of anti-platelet antibodies

Antibodies to platelet or DNA were measured using a modified enzyme-linked immunosorbent assay (ELISA) (1).

Blood was collected from donors of blood group O using 3.8% sodium citrate. Platelet-rich plasma was obtained by centrifugation at $110 \times g$ for 15 minutes, followed by further centrifugation at $110 \times g$ for 10 minutes to remove red and white cell contamination. After three washings with phosphate-buffered saline, pH 7.2 (PBS) containing ACD-A, platelets were resuspended in this buffer and adjusted to $5 \times 10^8/\mu$l. A 50-$\mu$l aliquot of this platelet suspension was added to each well of several poly-L-lysine (10 $\mu$g/ml)-treated polystyrene microtiter plates (Flow Laboratories, Irvine, Scotland, UK) and the platelets were sedimented by centrifugation at $300 \times g$ for 5 minutes. Immediately after addition of 50 $\mu$l of 0.25% glutaraldehyde solution in PBS, the plates were again centrifuged at $300 \times g$ for 5 minutes and incubated at 22°C for 10 minutes. The wells were washed three times with PBS-Tween 20, and then 200 $\mu$l of PBS containing 1% egg albumin (Difco Laboratories, Detroit, MI) was added to each well. After a 2-hours incubation at 37°C, the plates were washed three times with PBS-Tween 20 and kept at 4°C until assay. One hundred microliters of culture supernatant was added to each well and the plates were incubated at 37°C for 1 hour. After three washings with PBS-Tween 20, 100 $\mu$l of horseradish peroxidase-conjugated goat anti-human immunoglobulin (IgG + IgM) (Cappel Laboratories, Cochranville, PA) was added. The plates were then incubated at 37°C for 1 hour and again washed three times. Two hundred microliters of substrate solution (o-phenylenediamine, 1 mg/ml in 0.1 mole citrate buffer, pH 4.5) was added to each well and the plates were incubated at 22°C for 20 minutes before determining the optical density at 492 nm for each well in a Titertek Multiskan MC (Flow).

5. Measurement of anti-DNA antibodies

For the preparation of single-stranded DNA (ssDNA), calf thymus DNA (Sigma Chemical Co., St. Louis, MO) was dissolved in PBS and denatured by heating at 100°C for 10 minutes followed by rapid cooling in ice-water. Double-stranded DNA (dsDNA) was isolated from peripheral white blood cells according to the method originally described by Blin and Stafford with slight modification (6). A 50-$\mu$l aliquot of each of these DNA solutions (2.5 $\mu$g/ml) was added to the wells of poly-L-lysine-treated polystyrene microtiter plates and the plates were dried at 37°C for 18 hours. The plates were washed three times with PBS-Tween 20 and then blocked with egg albumin buffer as the platelet-coated plates. One hundred microliters of culture supernatant was added and each plate was incubated at 37°C for 1 hour. The remainder of the assay was carried out as for the measurement of anti-platelet antibodies. In all assays, plates without antigens served as controls.

6. Competitive inhibition assay

Equal volumes of culture supernatant containing monoclonal antibody and various concentrations of the inhibitor were mixed, incubated at 37°C for 1 hour, washed three times with PBS-Tween 20 and then incubated at 37°C for 1 hour. After three washings with PBS-Tween 20, 100 $\mu$l of horseradish peroxidase-conjugated goat anti-human immunoglobulin (IgG + IgM) (Cappel Laboratories, Cochranville, PA) was added. The plates were then incubated at 37°C for 1 hour and again washed three times. Two hundred microliters of substrate solution (o-phenylenediamine, 1 mg/ml in 0.1 mole citrate buffer, pH 4.5) was added to each well and the plates were incubated at 22°C for 20 minutes before determining the optical density at 492 nm for each well in a Titertek Multiskan MC (Flow).
hours and then tested for residual binding to the antigen-coated plate by ELISA. The synthetic polynucleotides poly(dA), poly(dT), poly(C), poly(G), poly(I) and poly(U) were purchased from PL Biochemicals (Milwaukee, WI) and yeast RNA and cardiolipin were from Sigma Chemical, ssDNA and dsDNA were prepared as described above.

7. Treatment of antigen-coated plates with DNAase I

A 50-μl aliquot of DNAase I (Boehringer Mannheim, Mannheim, W. Germany) was added to ssDNA- or platelet-coated wells of microtiter plates in the presence of 10 mM Mg^{2+} (pH 7.2) and the plates were incubated at 22°C for 30 min. After five washings with PBS-Tween 20, measurement of antibodies on these plates was carried out as described above.

8. Determination of antibody class

Culture supernatants from each cell line were concentrated by the 50% saturated ammonium sulfate precipitation method and then analyzed by the micro-Ouchterlony method.

RESULTS

1. Binding characteristics of human monoclonal anti-platelet antibodies

Nine human B cell lines producing monoclonal anti-platelet antibodies were established. All of them produced IgM-class immunoglobulin and the light chains were of the kappa type in all except No. 7. Table 1 shows the reactivities of these monoclonal antibodies with platelet and ssDNA, as determined by ELISA. They reacted with platelet to different degrees and three of them, Nos. 3, 5 and 8, also reacted with ssDNA. None of these monoclonal antibodies bound to plates without antigens, nor did culture medium show any reactivity with these antigen-coated plates.

2. Cross-reactive specificities of human monoclonal anti-platelet antibodies

The specificities of three monoclonal anti-platelet antibodies cross-reactive with ssDNA were determined using direct-binding ELISA (Table 2). These monoclonal antibodies bound to ssDNA, but not to dsDNA.

Moreover, the binding specificity of antibody No. 8, that most strongly reactive with ssDNA, was determined by competitive inhibition assay (Fig. 1). This monoclonal antibody bound to the synthetic polynucleotides; poly(dT) and poly(I), as well as to ssDNA, but not to dsDNA, RNA, poly(dA), poly(C), poly(G), poly(U) or cardiolipin. The binding of antibody No. 8 to platelet was also inhibited by ssDNA, poly(dT) and poly(I) with the same pattern as that shown in Fig. 1 (data not shown). Conversely, the reactivity of this monoclonal antibody with ssDNA was also inhibited by glutaraldehyde-fixed platelets (Fig. 2), indicating that antibody No. 8 was polyspecific, including cross-reactivity with ssDNA.

To exclude the possibility that this cross-reactivity could have been due to the location of DNA itself on the surface of platelets, we treated platelet- or ssDNA-coated plates with DNAase I and then tested antibody No. 8 for its binding to these DNAase I-treated plates, respectively. The treatment of DNA-coated plates with DNAase I at a concentration of

<table>
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<th>Patients</th>
<th>No. of antibodies</th>
<th>Platelet</th>
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<th>No antigen</th>
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<tbody>
<tr>
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<td>0.000</td>
<td>0.006</td>
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<tr>
<td>2</td>
<td>2</td>
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<td>3</td>
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<tr>
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<td>0.014</td>
</tr>
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<td>0.154</td>
<td>0.003</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
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</tr>
<tr>
<td>8</td>
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<td>0.004</td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>0.322</td>
<td>0.048</td>
<td>0.035</td>
</tr>
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</table>

ssDNA: single-stranded DNA
* Values represent optical densities read at 492 nm in ELISA.

<table>
<thead>
<tr>
<th>No. of antibodies</th>
<th>ssDNA</th>
<th>dsDNA</th>
<th>No antigen</th>
</tr>
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<tbody>
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<td>0.001</td>
<td>0.003</td>
</tr>
<tr>
<td>8</td>
<td>0.585</td>
<td>0.018</td>
<td>0.004</td>
</tr>
</tbody>
</table>

dsDNA: double-stranded DNA
* Values represent optical densities read at 492 nm in ELISA.
50 μg/ml reduced the binding ability of antibody No. 8 to them by almost 90%. On the other hand, the treatment of platelet-coated plates with DNAase I even at a concentration of 5 mg/ml had no effect on the binding of this monoclonal antibody (Fig. 3). We therefore concluded that this cross-reactivity was not due to the presence of DNA itself bound to the surface of platelets.

**DISCUSSION**

In the present study, we showed that some of the monoclonal anti-platelet antibodies obtained from patients with ITP had DNA-binding properties. Recent studies of monoclonal anti-DNA auto-antibodies have revealed that some of them are cross-reactive with platelets (1, 2). The platelet-binding properties of monoclonal lupus autoantibodies are considered to be clinically significant, because thrombocytopenia is often seen in patients with SLE (3, 4). Moreover, Rauch et al found that anti-platelet activity was strongly correlated with anti-dDNA reactivity (2). These reported clones have been screened and selected for anti-dDNA activity and then tested for binding to platelets. In order to clarify further the bivalencies in anti-DNA and anti-platelet autoantibodies, we conversely studied the DNA-binding properties of the EBV-transformed human B cell clones screened and selected for anti-platelet activity. Our results indicated that some of these anti-platelet antibodies were cross-reactive with DNA and that moreover, one of them, No. 8, was also able to react with multiple synthetic polynucleotides of different base composition; some of the monoclonal anti-DNA autoantibodies produced by human-human hybridomas (7) or EBV transformation (8) also have multiple binding reactions of this type. Taken together, these findings suggest that cross-reaction between anti-DNA and anti-platelet antibodies is based on overlaps in specificity existing as common part of both autoantibodies.
We showed that the cross-reactive antigen recognized by antibody No. 8 was not DNA itself bound to the surface of platelets, though DNA receptors have been described on human platelets (9). Moreover, as this antibody showed base-specific preference in the competitive inhibition assay, we believe that the common structural properties of negatively charged arrays in the phosphodiester ribose backbone of DNA do not play a major role in this cross-reaction with platelets. Accordingly, there must be particular epitopes shared by both platelets and DNA, even though they appear to be structurally distinct (10). A monoclonal anti-platelet antibody produced by a hybridoma derived from a mouse immunized with platelets has also been shown to bind to H1 histones of cell nuclei (11). Thus, it is suggested that the structures for antigen recognition by anti-platelet autoantibodies are widely distributed and the immunogenic stimulus for their production are more complex.

At present, we are unable to comment on whether our monoclonal anti-platelet antibodies obtained from patients with ITP are responsible for the pathogenesis of thrombocytopenia in these patients. Studies on idiotypic analysis of these monoclonal anti-platelet antibodies and serum autoantibodies in ITP would provide useful information for this problem. In fact, using the same EBV transformation method, Sasaki et al. have succeeded in establishing the monoclonal anti-DNA producing cell lines expressing the cross-reactive idiotypes of anti-DNA autoantibodies in SLE sera (12, 13). Furthermore, it is now accepted that anti-platelet autoantibodies of the IgM class, like our present monoclonal antibodies, are as significant as those of the IgG class when considering the pathogenesis of platelet destruction in ITP (14, 15). Therefore we think that studies on autoimmune disease using monoclonal antibodies is useful for simplifying the complex autoantibody-antigen interactions occurring in this type of condition.

We showed that overlapping specificity existed as common part of anti-DNA and anti-platelet antibodies. However, the precise role of bivalencies in anti-DNA and anti-platelet autoantibodies in creating the common clinical manifestations seen in SLE and ITP (16, 17) remains to be determined. For the solution of this problem, it will be important to know whether or not the platelet antigens responsible for anti-platelet autoantibodies in ITP are similar to those in SLE.

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REFERENCES
