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Peptide Ligands That Bind IgM Antibodies and Block Interaction with Antigen

Pati M. Glee, Seth H. Pincus, Deirdre K. McNamer, Michael J. Smith, James B. Burritt, and Jim E. Cutler

We have selected a peptide-display phage library on IgM Abs and identified a panel of phage-expressing peptides that bind to IgM Abs in general, but not to Abs of other classes. A synthetic peptide corresponding to one of the displayed peptide sequences also binds to IgM Abs. The peptides bind to both soluble pentameric Abs and to monomeric cell-surface IgM. The phage-displayed and synthetic peptides inhibit the binding of IgM Abs to Ag. These peptides may create confounding artifacts when IgM Abs are used for epitope mapping studies. Nonetheless, the peptides may have both experimental and therapeutic utility. The Journal of Immunology, 1999, 163: 826-833.

The use of bacteriophage display peptide libraries (PDPL), termed epitope or “mimotope” libraries, greatly facilitates identifying peptide ligands for proteins of interest (for reviews, see Refs. 1 and 2). Inspired by advances in peptide synthesis (3), Smith and colleagues pioneered PDPL construction in which vectors were designed for expression of random peptides fused to coat proteins, usually pH11 or pVIII, of filamentous phage (2). Thus, a multitude of different oligopeptides are surface displayed by the viral particles and available for interaction and affinity selection with a ligate of choice. DNA sequence information from the selected clones reveals the sequence of the displayed peptide. Most applications of PDPL technology have focused on protein-to-protein interactions. However, recent data from various labs have indicated the feasibility of finding peptide structures that mimic nonproteinaceous ligands such as biotin (4) or oligosaccharide epitopes (5-8).

We applied phage-display technology to search for structural equivalents of microbial polysaccharide epitopes. We had previously identified carbohydrate epitopes from Candida albicans and group B streptococci (GBS) that elicit protective Ab responses, as well as other carbohydrate epitopes that elicit nonprotective Abs. The Abs used to select the phage were of the IgM class, which are typical of T-independent, anti-carbohydrate responses. While using these IgM Abs to select for carbohydrate-mimetic phage (8), we identified a population of phage that bind to all IgM Abs regardless of antigenic specificity, presumably recognizing determinants in the constant regions. We characterized these phage and demonstrated, surprisingly, that the phage and peptides derived from them can inhibit the interaction between Ag and IgM Ab.

Materials and Methods

J404 nonapeptide PDPL

The J404 PDPL we used in our studies was constructed by one of the authors (J.B.B.) and is described elsewhere (1, 9, 10). The library displays random nonamer peptides from the N-terminal portion of the pIII capsid protein of kanamycin-resistant, filamentous bacteriophage M13K8. The J404 library contains an estimated 5 × 10^10 different nonapeptides at high titer (1 × 10^13 pfu/ml).

Abs

Anti-Candida IgM mAbs B6.1 and B6 were isolated as previously described (11) and produced in serum-free medium (BG 101 Liquid Kit; Irvine Scientific, Santa Ana, CA). The Abs were concentrated by ammonium sulfate precipitation and exhaustively diaлизed against Dulbecco’s PBS (DPBS) (Sigma, St. Louis, MO). Anti-GBS IgM mAbs S7, S9, and S10 (12) were prepared as mouse ascites, and the IgM fraction was isolated by distilled water dialysis. S7 and S9 are directed against carbohydrate Ags, while S10 is specific for the GBS Ibc protein. Monoclonal anti-GBS mAbs 1B1 and 2A6 are specific for the GBS type III capsular polysaccharide (8), as is the IgM S9 (12). Additional mAbs and polyclonal Abs were used to assess peptide binding and Ig class specificity. Murine IgM mAbs used included: H9 and C6 (13); mAb 9A, mAb 5A, mAb 6F, P3 × 63 (14); 2B3.1, A4.1, C3.4, JD3 (M. Riesselman and J.E.C., unpublished observations), and T17 directed against the synthetic peptide (Y,E)-A-K– (15). Murine IgG mAbs included 6C5-H4 (a gift from Kevin C. Hazen, University of Virginia, Charlottesville, VA), 44.1 (9), and 924 (16). Polyclonal preparations included murine ascites and rabbit and human sera.

Preparation of selection matrices

Ab affinity matrices for interaction with the J404 PDPL were mAb B6.1 adsorbed to polystyrene dishes, mAb B6.1 conjugated to Sepharose 4B (CL-4B-200; Sigma), or mAb S10 conjugated to Sepharose beads. Polystyrene dishes (Falcon 35 mm; Becton Dickinson, San Diego, CA) were coated for 2 h at room temperature with 1 mg mAb B6.1/ml DPBS and washed five times with cold DPBS. For mAb-conjugated Sepharose matrices, the Sepharose 4B was activated with cyanogen bromide as previously described (17) and coated with mAb B6.1 or S10 (3 mg per ml packed beads, 16 h at 4°C), washed, and blocked in 1% BSA (ICN Biomedicals, Aurora, OH) in DPBS before incubation with phage.

Interaction of the J404 library with IgM mAb matrices

All Ab-coated dishes and beads were preblocked for 1 h in DPBS plus 1% BSA before incubation with the library. Three independent selections (mAb B6.1-dish, MAb B6.1-Sepharose, and mAb S10-Sepharose) were performed with aliquots from the J404 PDPL. Manipulation of the nonapeptide PDPL and appropriate Escherichia coli K91 host cells were essentially as described (9). Briefly, aliquots of the nonapeptide PDPL...
Wells were washed, and an HRP-conjugated goat anti-mouse polyvalent Ig was added and incubated 2–4 h. Substrate containing O-phenylenediamine, H₂O₂ in 0.1 M sodium citrate, pH 5.0, was added, and the color was developed for 10–30 min. Reactions were stopped by the addition of 10% H₂SO₄, and A₄05 was measured.

GBS were coated onto microtiter wells using poly-lysine and glutaraldehyde as described elsewhere (8). Plates were blocked with 1% BSA. IgM anti-GBS Abs were mixed with phage or with KLH-conjugated peptides derived from the phage and incubated in microtiter wells at 4°C for 16 h. The plates were washed and incubated with AP-conjugated anti-Ig for 6 h, followed by washing and the addition of p-nitrophenyl phosphate. A₄05 was then determined.

**Latex agglutination for measurement of rheumatoid factor (RF)**

Latex beads coated with aggregated IgG were obtained from REFSSCAN Kit (Becton Dickinson, Cokkesville, MD). Serum containing high titered RF activity were either contained within the kit or were obtained from the Division of Rheumatology, University of Utah School of Medicine (Salt Lake City, UT). Pretitered dilutions of serum were premixed with phage and then added to the latex beads. The mixture was stirred and allowed to incubate with slow shaking for 20 min before photography.

**Flow cytometry for cell surface IgM**

Murine B lymphoma cells expressing cell surface IgM (designated SFS) or differentiated to express IgA (4F10) (20, 21) were incubated with phage in PBS/BSA/0.1% sodium azide, washed, incubated with rabbit anti-M13 antisera, washed, and then stained with FITC-conjugated anti-rabbit IgG (Cappell Laboratories, Durham, NC). Alternatively, cells were directly stained with FITC-conjugated anti-mouse IgM (Cappell).

**Preparation of synthetic peptide–carrier protein conjugate**

The nonapeptide displayed by phage clone ed1 was chosen for conjugation to KLH with a heterobifunctional cross-linker, m-maleimidobenzoyl-N-hydroxy-succinimide ester (MBS) (Pierce, Rockford, IL). A synthetic peptide containing a 4-aa extension including cysteine for cross-linking (YWIPS-SAWGFC) was made (Bio-Synthesis, Lewisville, TX). Briefly, 10 mg KLH (Cappell KLH; Pierce) in degassed, nitrogen-sparged 0.05 M citrate-phosphate buffer, pH 5.0, was stirred gently with 2 mg cross-linker for 1 h at room temperature under a N₂ cap, then passed over a Sephadex G-25 column to separate the carrier-protein-linker product from unreacted cross-linker. The MBS-KLH was placed into a fresh glass tube and stirred 6 h (N₂ cap, room temperature) with 5 mg of the synthetic ed1 peptide, which was dissolved in 50 μl dimethyl formamide. The sample was dialyzed against 3 L of the citrate-phosphate buffer, pH 5.0, to remove free peptide and then dialyzed against three changes of DPBS. Aliquots of the ed1-KLH-conjugate preparation were stored at −20°C before use.

**Results**

Independent IgM selections enrich for phage expressing similar peptide sequences

Phage were selected in four distinct selections on three different mAbs, immobilized either on Sepharose beads or on polystyrene dishes. In each case, the final selection pools contained phage clones that bound to multiple IgM Abs in ELISA or immunoblot analysis (see below). Nonapeptide sequences from individual IgM-binding phage clones are shown in Table I. Phage displaying some sequences, especially those designated ed1, ed4, and ps1, were isolated considerably more frequently than the others. All sequences, especially those designated ed1, ed4, and ps1, were isolated considerably more frequently than the others. All sequences contain a tryptophan residue in the C-terminal or penultimate position, and the sequences shown in Table I are aligned to that residue. While there is variability in the sequences capable of binding to IgM, certain motifs are strongly represented, such as aspartate on the amino-terminal side of the conserved tryptophan and W I/S/P/S/Q-X D W in many clones. The absolute or minimal sequence requirements necessary for IgM binding activity have not been established.

**Binding of phage and peptide to IgM Abs**

We have used immunoblotting and ELISA to demonstrate binding to IgM by phage and peptides derived from the phage. Phage clones were initially identified as binding to IgM by plaque lift immunoblots (data not shown).
TABLE I. Peptide ligands of IgM Abs

<table>
<thead>
<tr>
<th>Phage Clone</th>
<th>Sequences</th>
</tr>
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<tbody>
<tr>
<td>ed4, es87, ed8, ed36</td>
<td>SWISSKDWT</td>
</tr>
<tr>
<td>ps1</td>
<td>SWISSKDWT</td>
</tr>
<tr>
<td>ps58</td>
<td>TWISSNDDT</td>
</tr>
<tr>
<td>S10-3</td>
<td>KGWISANDW</td>
</tr>
<tr>
<td>S10-4, S10-8</td>
<td>QRWISSADW</td>
</tr>
<tr>
<td>es92</td>
<td>DWIDMQYDN</td>
</tr>
<tr>
<td>es19</td>
<td>QRWISSANDW</td>
</tr>
<tr>
<td>ed1, es63</td>
<td>YDWIPSSAW</td>
</tr>
<tr>
<td>es81, es83, es86, ed38</td>
<td>DWIPAQASWE</td>
</tr>
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<td>ed42</td>
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</tr>
<tr>
<td>ed46, es84, es85</td>
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<td>ed2, ed23</td>
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<tr>
<td>es50, es107</td>
<td>EAAILSDDNA</td>
</tr>
<tr>
<td>ed9</td>
<td>ENVYSKDWL</td>
</tr>
<tr>
<td>jb</td>
<td>FYGVTGDN</td>
</tr>
<tr>
<td>jb</td>
<td>NRVVQWQDW</td>
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<tr>
<td>jb</td>
<td>NKWISQGDN</td>
</tr>
<tr>
<td>jb</td>
<td>PSWISQVDN</td>
</tr>
<tr>
<td>jb</td>
<td>NTVIWNDN</td>
</tr>
<tr>
<td>jb</td>
<td>LSWISGEDW</td>
</tr>
</tbody>
</table>

Immunoblots of SDS-PAGE-separated phage proteins were used to demonstrate that IgM binding by phage involves the chimeric pIII-displayed peptide and also to show the specificity of binding. IgM-binding or nonbinding irrelevant phage were subjected to SDS-PAGE, blotted onto nitrocellulose, and were incubated with either one of two different IgM mAbs or a murine IgG mAb. The results of the immunoblot with the IgM mAb B6 is shown in Fig. 1. Signal occurred at the appropriate mobility for the pIII-nonapeptide chimeric protein in the lanes containing the IgM-binding phage (lane 4, es81; lane 6, ed9; lane 7, ed23; lane 8, ed36; lane 9, ed42; and lane 17, ed1), but not with any of the irrelevant phage clones (lanes 2, 3, 5, and 10–16) or with the parental phage M13KBst (lane 18). Duplicate blots were prepared and tested against a second IgM mAb, B6.1, or an IgG mAb, 6C5-H4. The identical pattern was seen with the IgM, and no reactivity was seen with the IgG (data not shown).
Phage clone ed1 was chosen for further analysis. A preparative blot of the phage proteins was evaluated for reactivity with additional IgM Abs, including murine mAbs and polyclonal Abs from various species, as well as to IgG Abs (Fig. 2). All IgM mAbs bound the ed1 pIII-displayed nonapeptide (Fig. 2A, lanes 1–13). Because the mAbs in Fig. 2A were primarily directed against carbohydrate Ags, two mAbs against protein Ags, T17 and S10, were tested for binding to ed1. Fig. 2, B and C, lanes 1 and 2, demonstrate that these IgMs also bind to ed1, but not to an irrelevant phage. In lanes 3 and 4, we showed that two IgG mAbs are not bound by ed1 (Fig. 2B), but that one of the IgGs will bind to phage displaying the epitope recognized by that IgG (Fig. 2C). IgM Abs in a murine polyclonal ascites (Fig. 2A, lane 14) are bound by ed1. IgM Abs from human and rabbit serum samples also bound the pIII band (Fig. 2A, lane 15, and Fig. 2B, lane 3). The ed1 pIII did not bind IgG Abs, neither mAbs as described above, nor IgGs found in the affinity-purified secondary Abs used for the control strips (Fig. 2A, lanes 16 and 17, Fig. 2B, lanes 5 and 6, and Fig. 2D, lanes 1, 2, and 4).

Table II. Binding of phage to IgM, but not to IgG

<table>
<thead>
<tr>
<th>Phage</th>
<th>Ab-Coating Wells*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>ps55</td>
<td>0.24*</td>
</tr>
<tr>
<td>S9-24</td>
<td>0.03</td>
</tr>
<tr>
<td>S7-B</td>
<td>0.05</td>
</tr>
<tr>
<td>S10-4</td>
<td>0.07</td>
</tr>
<tr>
<td>S10-8</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Wells were coated with IgM (S7, S9, B6.1), IgG (924) at 5 μg/ml, or no Ab.

ELISA was performed to demonstrate the specificity and avidity of phage binding. Specificity of binding of the phage to IgM Abs is shown in Table II. Two different IgM-binding phage (S10-4 and S10-8) were compared with three different Ab-specific phage (8) in an ELISA in which microtiter wells were coated with different Abs. S10-4 and S10-8 bound to wells coated with each of the IgM, IgM Abs in a murine polyclonal ascites (Fig. 2A, lane 14) are bound by ed1. IgM Abs from human and rabbit serum samples also bound the pIII band (Fig. 2A, lane 15, and Fig. 2B, lane 3). The ed1 pIII did not bind IgG Abs, neither mAbs as described above, nor IgGs found in the affinity-purified secondary Abs used for the control strips (Fig. 2A, lanes 16 and 17, Fig. 2B, lanes 5 and 6, and Fig. 2D, lanes 1, 2, and 4).

FIGURE 4. Phage and peptide conjugates bind to anti-GBS mAb S9. A, Binding of phage to S9. ELISA plates were coated with mAb S9. Varying amounts of phage were added to the wells, incubated, and then washed. Phage binding was detected with rabbit anti-M13 antiserum followed by AP-conjugated anti-rabbit Ig. The results are reported as A₄₀₅. B, Binding of peptide to S9. ELISA plates were coated with mAb S9. Varying concentrations of ed1-KLH conjugate, pep2 (WENWMMGNA)-KLH conjugate, or KLH were added to the wells, incubated, and then washed. Binding was detected with rabbit anti-KLH antiserum followed by AP-conjugated anti-rabbit Ig. The results are reported as A₄₀₅.

FIGURE 5. Binding of phage to cell-surface IgM. Murine B cell lymphomas 5F5 (surface IgM⁺) and 4F10 (surface IgM⁻) were incubated with 3 × 10⁶ phage, washed, and incubated with rabbit anti-M13 antisemur followed by FITC-conjugated anti-rabbit Ig (two left sets of panels) or were incubated only with an FITC-conjugated anti-mouse μ-chain (right). Labeled cells were analyzed by flow cytometry. Cell number is on the vertical axis, fluorescence intensity is on the horizontal.
but not IgG Abs, while the Ab-specific phage only bound to the selecting Ab. Fig. 3 shows that phage ed1 and S10-4 bind to IgMs displaying specificity for both carbohydrate (S9) and protein Ags (S10 and T17), but do not bind to IgG Abs. The parental phage M13KBst and an irrelevant phage, J508.22 do not bind to the IgMs. Because J508.22 represents the epitope bound by mAb 44.1 (9), it binds well to the IgG mAb 44.1.

The relative avidity of phage binding to IgM is shown in Fig. 4A. Seven different IgM-binding phage and the parental phage M13KBst were serially diluted, and the IgM binding activity was measured. The results demonstrate at least a 10-fold difference in relative avidity between those phage with the highest avidity (ed1, ed42) and those with the lowest (ed9).

The ability of a peptide derived from the phage to bind to IgM Abs was tested. A synthetic peptide corresponding to the ed1-displayed sequence was conjugated to KLH. The conjugate, but not the peptide, was water soluble. Thus the conjugate was used in these experiments. Microtiter wells were coated with IgM mAb S9 and incubated with either KLH, KLH conjugated to the ed1 peptide, or KLH conjugated to an irrelevant peptide (designated pep2). Following an incubation and washing, binding was detected with rabbit anti-KLH antiserum and AP anti-rabbit Ig. The ELISA results are shown in Fig. 4B and demonstrate that binding occurred with the KLH-ed1 conjugate, but with neither KLH nor KLH-pep2.

Table III. Inhibition of Ab binding to GBS by phage

<table>
<thead>
<tr>
<th>Phage</th>
<th>S9</th>
<th>S7</th>
</tr>
</thead>
<tbody>
<tr>
<td>No phage</td>
<td>2.08</td>
<td>2.25</td>
</tr>
<tr>
<td>M13KBst</td>
<td>2.03</td>
<td>2.44</td>
</tr>
<tr>
<td>S10-4</td>
<td>0.49</td>
<td>0.71</td>
</tr>
<tr>
<td>S9-24</td>
<td>1.56</td>
<td>2.29</td>
</tr>
<tr>
<td>S7-B</td>
<td>2.10</td>
<td>0.48</td>
</tr>
</tbody>
</table>

* Binding of the indicated Ab (0.3 μg/ml) to ELISA wells coated with GBS was detected with AP-conjugated anti-mouse IgM.
* Abs were premixed with 10^10 of the indicated phage prior to addition to ELISA plate.
* A_{0.05}, mean of duplicate samples.

Indirect immunofluorescence and flow cytometry were used to demonstrate that IgM-specific phage bind to the monomeric cell-surface form of IgM (Fig. 5). Two different B cell lymphoma lines were used, one expressing cell-surface IgM (5F5), the other IgA (4F10). FITC-conjugated anti-mouse IgM Abs were used to demonstrate that 5F5 does express IgM and 4F10 does not (right panels). Binding of phage to the cells was detected by first incubating the cells with phage, washing, and then incubating with rabbit anti-M13 antiseraum, followed by FITC-conjugated anti-rabbit Ig. As shown in the left panels of Fig. 5, S10-4 phage bind to the IgM-expressing cells, but not to those with cell-surface IgA. The parental phage, M13KBst, bind to neither (center panels).

Inhibition of Ag binding by phage and phage-derived peptides

We next tested the ability of the IgM-binding phage to inhibit Ag binding by IgM Abs. We have performed these experiments in multiple different antigenic systems.

The ability of phage to block the binding to GBS of two anti-GBS mAbs directed against two different carbohydrate epitopes (12) is shown in Table III. For each mAb, the inhibition caused by the IgM-binding phage S10-4 was compared with the inhibition seen with mAb-specific phage described in a previous publication (8). Phage S10-4 inhibited both mAbs, the parental M13KBst inhibited neither, and each mAb-specific phage inhibited its respective mAb, but not the other. The inhibition obtained with S10-4 was equal or greater than that obtained with the mAb-specific phage, which presumably mimics the antigenic structure and binds within the Ab-combining site. Fig. 6 shows that both ed1 and S10-4 inhibit the binding of two IgM Abs, one specific for carbohydrate Ag (S9) and the other for a protein structure (S10), to GBS. However, they inhibit neither of two IgG Abs (2A6 and 1B1) with the same antigenic specificity as S9. The parental phage, M13KBst, does not inhibit Ag binding at all.

Because the phage inhibited the binding of IgM Ab to Ag, we sought to determine whether the phage bound within the combining site by performing a competitive inhibition, attempting to block the binding of phage to Ab S9 with purified GBS capsular polysaccharide. No inhibition was seen (data not shown).

The comparative ability of different IgM-binding phage to inhibit binding to GBS by mAb S9 is shown in Fig. 7A. The results
demonstrate that there are marked differences in the degree of inhibition by phage with different displayed sequences, and, not surprisingly, the relative inhibition correlates with the avidity of phage binding to the IgM Ab (Fig. 4A). We next tested whether Ab binding to Ag could be inhibited with a peptide corresponding to the sequence displayed by the ed1 phage (Fig. 7B). The results showed that the KLH-ed1 conjugate inhibited binding of Ab S9 to GBS, but neither KLH alone nor KLH conjugated to an irrelevant peptide had that effect.

The ability of IgM-binding phage to inhibit the interaction between IgM anti-C. albicans Abs (19) to cell wall extract from C. albicans is shown in Fig. 8. As with the GBS, two different Abs with different epitope specificity were tested. Inhibition of both Abs, mAb B6.1 (Fig. 8A) and mAb B6 (Fig. 8B) was observed with phage ed1, but not with M13KBst. Much greater inhibition was seen when the phage and Ab were premixed and then added to Ag than when the phage were added after the initial Ag-Ab interaction had occurred.

Human RF is an anti-IgG autoantibody, primarily of the IgM class. Fig. 9 shows that ed1 phage, but not M13KBst, inhibited the RF-induced agglutination of IgG-coated latex beads.

**IgM-binding phage do not inhibit complement activation**

By use of ELISA, we tested the ability of phage to inhibit complement activation (22). IgM mAb and Ag were allowed to interact, then phage and human complement-containing serum was added. The binding of C1q and C3b to the immune complexes was assessed with specific antisera. There was no difference in complement binding in the presence of IgM-binding phage, control phage, or no phage (data not shown).

**Discussion**

In this report, we describe a set of peptides with IgM-binding activity. These peptides were inadvertently identified when we used a PDPL to select for peptides that mimic the Ags recognized by IgM Abs. These peptides bound to IgM Abs from mice, rabbits, and humans, but not to IgG Abs from those species. Binding activity was seen when the peptides were displayed as chimeras on the terminus of the pIII protein on intact phage or when a synthetic peptide was conjugated to a carrier protein. IgM Abs specific for both carbohydrate and for protein Ags were bound. Unconjugated peptides were not tested because of insolubility in water at physiological pH. Peptides bound to multimeric IgM and to the monomeric cell-surface form of IgM. Most surprisingly, the peptides inhibited the binding of Ag to IgM Abs. Inhibition of Ag binding...
The inhibition of Ag binding was seen at the site of the Ag combining site on the IgM, because virtually complete sterically hindered. Finally, the stoichiometry of the interaction has not been absolutely ruled out, for several reasons we do not block Ag binding. While the phage displaying the peptides are not used to epitope map IgM Abs, because such peptides appear to bind to an IgM Ab in a manner analogous to Ag. It is only when the specificity of binding and Ag inhibition are tested on irrelevant IgM Abs that the true nature of the peptide binding is revealed.

The peptides described in this publication may have both experimental and therapeutic utility. If the affinity of interaction between peptide and IgM molecule is sufficiently high, the peptides may be used to purify IgM Abs on affinity matrices. Because the peptides bind to cell-surface IgM, they may function as agents that activate B cells. The ability of the peptides to block the interaction between IgM Abs and Ag may be used to prevent deleterious Ag-Ab interactions or dissociate IgM-containing immune complexes. Such pathologic IgM Ab interactions can occur in hemolytic transfusion reactions, autoimmune diseases such as rheumatoid arthritis, and in paraneoplastic syndromes associated with Waldenström's macroglobulinemia.

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**References**