Antigenic Specificity of the "Anticardiolipin" Antibodies

To the Editor:

We read with great interest the thorough review by Roubey\(^1\) on the antiphospholipid syndrome. However, we would like to comment on one point. To explain why autoantibodies to pure \(\beta_2\)-glycoprotein I (\(\beta_2\)-GPI) may be detected by enzyme-linked immunosorbent assay (ELISA) only when \(\beta_2\)-GPI is coated on irradiated plates, Roubey supports the first possibility, ie, that the antibodies require a relatively high density of immobilized antigen that cannot be achieved on untreated polystyrene. We present data that support the second possibility, ie, that the antibodies are specific for a conformational epitope of \(\beta_2\)-GPI formed when the protein is bound to an anionic surface, such as irradiated polystyrene or cardiolipin.

Two monoclonal antibodies were used. Cof23 is a mouse monoclonal antihuman \(\beta_2\)-GPI antibody developed by immunizing mouse with human \(\beta_2\)-GPI emulsified with Freund’s complete adjuvant. WB-CAL-1 is a monoclonal "anticardiolipin" antibody derived from (NZWXBXS)F\(_1\) (WBF\(_1\)) male mice, an animal model of antiphospholipid syndrome.\(^2\) We compared the binding of these antibodies to \(\beta_2\)-GPI by ELISA systems using various polystyrene plates. The plates used were plain polystyrene plates (Sumilon plates; Sumitomo Bakelite Ltd, Tokyo, Japan), plain plates coated with cardiolipin, irradiated plates, and commercially oxygenated plates (Sumilon C plates). Plates were incubated with 40 \(\mu\)g/mL \(\beta_2\)-GPI overnight at 4°C, followed by incubation with either WB-CAL-1 or horseradish peroxidase (HRP)-labeled Cof23. WB-CAL-1 bound to solid-phase \(\beta_2\)-GPI was detected by HRP-labeled antimouse IgG.

Binding of "anticardiolipin" WB-CAL-1 to \(\beta_2\)-GPI was detectable on cardiolipin-coated plates, irradiated plates, and commercially oxygenated plates (Table 1). WB-CAL-1 binding to \(\beta_2\)-GPI was not detected when \(\beta_2\)-GPI was coated on plain plates. In contrast, binding of Cof23 to \(\beta_2\)-GPI was easily detectable with any of the plates used, showing that comparable amounts of \(\beta_2\)-GPI were present on the surface of these plates regardless of irradiation or commercial oxygenation. Analysis of surface oxygen on the plate surface\(^3\) showed high oxygen introduction on irradiated plates and commercially oxygenated plates.

Therefore, we believe that WB-CAL-1 binds to an epitope on \(\beta_2\)-GPI that is normally cryptic but is newly expressed when the protein is bound to an anionic surface, such as irradiated polystyrene or cardiolipin.

Table 1. Binding of Anti-\(\beta_2\)-GPI Antibody (Cof23) or \(\beta_2\)-GPI-Dependent Anticardiolipin Antibody (WB-CAL-1) to Various Polystyrene Plates

<table>
<thead>
<tr>
<th>Plates</th>
<th>mol % of Oxygen Atoms on Surface</th>
<th>(\beta_2)-GPI Binding (OD at 450 nm)</th>
<th>aCL Binding (OD at 450 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain</td>
<td>1.3</td>
<td>1.062</td>
<td>0.062</td>
</tr>
<tr>
<td>Cardiolipin-coated</td>
<td>NT</td>
<td>0.629</td>
<td>1.179</td>
</tr>
<tr>
<td>Irradiated ((\gamma)-ray 100 kGy)</td>
<td>9.3</td>
<td>1.156</td>
<td>1.012</td>
</tr>
<tr>
<td>Commercially oxygenated</td>
<td>19.8</td>
<td>0.782</td>
<td>0.892</td>
</tr>
</tbody>
</table>

Binding of anti-\(\beta_2\)-GPI antibody (Cof23) or \(\beta_2\)-GPI-dependent anticardiolipin antibody (WB-CAL-1) to various polystyrene plates was compared by enzyme immunoassays. Mol % of oxygen atoms on surface indicates the atomic mol % of oxygen molecules on the polystyrene surfaces calculated by x-ray photoelectron spectroscopy.\(^3\) Abbreviations: \(\beta_2\)-GPI, anti-\(\beta_2\)-GPI antibody; aCL, anticardiolipin antibody; NT, not tested.
Response to Harris et al and Koike et al

Although certain immunologists in the past may have been biased against the possible existence of antibodies to phospholipids, this is not true of those investigators whose recent work was summarized in my review.1 By the late 1980s, most researchers studying the "antiphospholipid" antibody syndrome were working under the prevailing notion that the autoantibodies associated with the syndrome were directed against anionic phospholipids. For example, both Galli et al23 and McNeil et al44 the groups that first reported the key role of β2-glycoprotein I (β2-GPI), had previously published a number of reports directly involving or assuming phospholipid specificity. Their subsequent discovery arose from experiments in which putative affinity-purified "anticardiolipin" antibodies did not behave as expected; ie, the antibodies did not bind to cardiolipin in the absence of serum or plasma.7 8 To suggest that these findings arose, even in part, from an a priori bias against the possible existence of antibodies to phospholipids is inaccurate. Furthermore, as discussed in my review, a number of laboratories have reported that authentic antiphospholipid antibodies do occur in patients with syphilis and other infectious diseases, but do not appear to be associated with the "antiphospholipid" syndrome.9,11

Harris et al point out, as stated in the review, that the initial studies describing the specificity of "antiphospholipid" autoantibodies for certain plasma proteins involved relatively small numbers of patients. However, as documented in the review, the fundamental observations regarding β2-GPI and prothrombin specificity have been reproduced by a number of independent laboratories, and the number of patients studied is growing. For example, autoantibody reactivity to β2-GPI has now been reported by at least seven different groups in nearly 100 total patients.7,12-17 Two reports published after the review went to press provide additional supportive data. First, Ichikawa et al18 characterized 5 human monoclonal "anticardiolipin" antibodies derived from 3 patients with the "antiphospholipid" syndrome. All 5 antibodies bound β2-GPI in the absence of phospholipid, and binding to anionic phospholipids occurred only in the presence of β2-GPI. Secondly, Permpikul et al19 studied lupus anticoagulants from 10 patient samples and found that in at least 9 the anticoagulant activity was caused by antibodies specific for prothrombin. In the remaining patient, the anticoagulant activity could have been caused by antibodies reactive with phospholipid, although this was not directly studied.

Data obtained using purified Igs and/or plasma-free assays that support the phospholipid specificity of lupus anticoagulants are limited to two studies.20,21 Whether the discordance between these reports and those from other groups of investigators,20,22-25 reflects true heterogeneity among patients or methodological differences remains to be clarified.

Several of the arguments proffered by Harris et al warrant further discussion. First, the phospholipid dependence of lupus anticoagulant activity is compatible with nonphospholipid antibody specificities. The fact that antibodies to both β2-GPI and prothrombin bind predominantly, or exclusively, to phospholipid-bound antigens would account for this effect. For example, the anticoagulant activities of several murine monoclonal antibodies to β2-GPI exhibit the same phospholipid dependency (Fig 1).26,27 Secondly, the data regarding annexin V are adequately explained by the very high affinity of annexin V for anionic phospholipids. The strength of binding of annexin V to anionic phospholipid vesicles28 is nearly 1,000-fold greater than that of prothrombin, factor Xa, and, based on unpublished data from our laboratory, β2-GPI. Accordingly, annexin V would be expected to displace these proteins from a phospholipid surface, thereby inhibiting antibody binding. Thirdly, although the animal studies involving passive antibody transfer or immuniza-
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tion29-31 cited by Harris et al support a direct pathogenic role for autoantibodies in the "antiphospholipid" syndrome, these data do not address the issue of phospholipid specificity. All three studies used patient Ig fractions rather than affinity-purified antibodies, and in no instance was phospholipid reactivity shown in the absence of serum or plasma. The murine monoclonal antibody CAM, studied by Blank et al,26 is a 16/6 Id+ antibody that binds both DNA and cardiolipin and lacks lupus anticoagulant activity. Binding of CAM to cardiolipin was demonstrated in the presence of bovine serum.

Lastly, I strongly agree with Harris et al that it would be inappropriate to recommend the clinical use of any new tests for autoantibodies to prothrombin, β3GPI, protein C, or other antigens at this time. However, there are sufficient preliminary data to justify evaluation of such tests on a research basis. For example, we recently conducted a pilot study comparing an anti-β3GPI ELISA and a standard "anticardiolipin" assay in a group of 160 rheumatology patients.27 The data suggest that the sensitivity of both tests for clinical manifestations of the "antiphospholipid" syndrome was comparable, whereas the anti-β3GPI ELISA had significantly greater specificity and positive predictive value. Further research is necessary to determine if such new tests will truly enhance our diagnostic and prognostic capabilities and if certain autoantibodies will, in fact, correlate with clinical subsets of the "antiphospholipid" syndrome. Data and conclusions regarding these issues should be judged solely on their scientific merit, irrespective of whether they lead to a variety of clinically relevant antigens and a change in the consensus opinion.

The data reported by Koike et al demonstrate that the murine monoclonal antibody WB-CAL-1 binds to epitopes on β3GPI that are available when β3GPI is bound to certain surfaces but not to others. This finding supports the argument that β3GPI undergoes certain conformational changes when it is bound to an anionic surface. However, these data do not address the issue of whether the polyclonal autoantibody response to β3GPI that occurs in patients may be directed exclusively against one or a few conformational epitopes. Based on studies of numerous other autoantibodies,28 such a limited response is possible but would be unusual.

Recently published data from our laboratory suggest that autoantibodies to β3GPI are of intrinsically low affinity, requiring bivalent binding to a high density of immobilized antigen for detection in ELISA.29 Briefly, this conclusion is based on the ability of fluid-phase β3GPI to inhibit the binding of patient IgG fractions to β3GPI coated on irradiated ELISA plates. These inhibition studies showed that the affinity of antibody binding to fluid-phase β3GPI was relatively low (Kd values ~10^-11 mol/L). The role of antibody bivalency was determined by evaluating the binding of bivalent and monovalent fragments of patient IgG to immobilized β3GPI. Fab' fragments demonstrated little or no binding in the anti-β3GPI ELISA, as compared with intact IgG and F(ab')2 fragments. If antibody reactivity was solely a result of specificity for neo- or cryptic epitopes, Fab' binding should have been equivalent.

In conclusion, whereas data from Koike et al support the importance of secondary and/or tertiary antigen structure, data from our lab indicate the importance of antigen density or clustering, ie, a quaternary structure. As stated in the review, these two possibilities are not mutually exclusive and both support the hypothesis that phospholipid-bound β3GPI is the in vivo target of "anticardiolipin" autoantibodies.

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Antigenic specificity of the "anticardiolipin" antibodies [letter; comment]

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