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Divalent cation effects on the binding of human anti-phospholipid antibodies

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(1) Anti-phospholipid antibodies from sera of subjects with documented syphilis were measured as a result of their individual interactions with cardiolipin, phosphatidylserine and phosphatidic acid, each impregnated in nitrocellulose paper from chloroform solution, followed by reaction with a labelled second antibody. Binding curves generated by increasing the cardiolipin concentration over a standard area of nitrocellulose paper showed saturation. (2) The presence of Ca^{2+} or Mg^{2+} during the antibody binding step resulted in a complex pattern of binding as a function of the cation concentration. When the extent of binding was normalized to percent of maximum bound, virtually superimposible patterns were seen with different sera. These patterns were distinctive for both the phospholipid and the cation. (3) The speculation is presented, albeit without any direct evidence, that the extent of antibody binding is sensitive to a variety of intermediate phospholipid phase structures which may be initiated by the presence of the specific divalent cation at a particular concentration.

Introduction

The influences of individual phospholipids on membrane structure/function, and membrane-bound enzyme activity are fundamental biological characteristics about which much has yet to be learned [1]. Cardiolipin, 1,3-diphosphatidylglycerol [2], is curious in that it is present in substantial concentration in mammals only in the inner leaf of the mitochondrial membrane [3] where it has an important role in cytochrome *c* oxidase [4] and cytochrome $P\text{-}450_{scc}$ activities [5]. Antibodies to cardiolipin have been well described in patients with syphilis [6] and more recently in patients with systemic lupus erythematosus [7] where there could be important clinical correlations [8]. The biological significance of these antibodies is not clear [9] and the phospholipid-antibody interactions have not been well characterized in molecular terms.

The traditional method of measuring anti-cardiolipin antibodies by flocculation tests appears to require other phospholipids and cholesterol [10] which may not be central to the antigen-antibody interaction. The requirement of the test for secondary interactions may be one of the reasons for conflicting results of measurements based on flocculation to answer questions regarding the antibody interactions with caridolipin, phosphatidylcholine, and cholesterol [9]. There is no doubt, however, that the active phospholipid is diphosphatidylglycerol and that the synthesized phospholipid reacts well in flocculation tests [11].

We have previously demonstrated that syphilitic sera contain antibodies to cardiolipin, phosphatidic acid, and phosphatidyl serine [12]. In the

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present studies we measured specific and reproducible effects of Ca^{2+} and Mg^{2+} on antibody binding to phospholipids impregnated in nitrocellulose paper. The observed binding patterns in the presence of the cations showed striking similarity using different sources of donor serum and were specific to the phospholipid.

Materials and Methods

Normal human sera were obtained from the Red Cross (Buffalo, NY), and sera from syphilitic patients were obtained through the courtesy of the Public Health Division of the Erie County Laboratories. The latter were defined as sera exhibiting positive flocculation tests in routine screening procedures for syphilis, and positivity in two specific tests for spirochetal antigens. Affinity purified goat anti-human IgG was obtained from Bio-Rad Laboratories (Rockville Center, NY) and rabbit anti-human IgG from Jackson ImmunoResearch Laboratories (Avondale, PA). [1-^{14}C]Acetic anhydride (30–40 mCi/mmol) was obtained from New England Nuclear (Boston, MA). The purified antibody was labelled with [^{14}C]acetic anhydride as previously described [13]. The labelled second antibodies from goat and rabbit sources could be used interchangeably. Measurement of the specific activity of the labelled antibody protein permitted the conversion from disintegrations per minute to moles of second antibody. The phospholipids were obtained from Sigma Chemical Company (St. Louis, MO) and from Avanti Polar Lipids (Birmingham, AL). These were studied for purity in two-dimensional thin-layer chromatography as previously described [14]. Lipid phosphorus was measured with the Barlett technique [15].

Cardiolipin (bovine heart), phosphatidylserine (bovine brain), and phosphatidic acid (egg yolk) were dried separately under ultrapure nitrogen and dissolved in chloroform in the specified concentrations. 5-μl aliquots were spotted on circles of nitrocellulose paper (Bio-Rad) which had been cut into 1-cm diameter membranes. After drying, these membranes were then blocked for 1 h in a buffer containing 0.05 M Tris (pH 7.4), 0.15 M NaCl, 0.05% Tween 20, 0.02% NaN_3 and 3% gelatin and added cation were indicated. The papers were then incubated overnight at room temperature with the sera in dilutions of 1 : 50 to 1 : 1000 in blocking buffer to a final volume of 4.0 ml. The papers were then washed three times in the same buffer.

The circles were reacted for 1 h with ^{14}C-labelled anti-human IgG, diluted 1 : 200 in 20 mM phosphate (pH 7.4) containing 150 mM NaCl in a final volume of 10 ml. After washing, the circles were placed in suitable scintillation media and the radioactivity was measured in a Packard 460CD scintillation counter.

The cations were tested in the form of $MgCl_2$ and $CaCl_2$, EDTA or EGTA at the concentrations specified, and were added to the blocking buffer during the serum incubation. Membranes with no added phospholipid were used for the assessment of the non-specific binding of IgG from the patients' serum to nitrocellulose paper. Such binding was usually very low, was almost linear in form, and was subtracted for most calculations. There was negligible binding of the diluted labelled second antibody to the nitrocellulose paper itself and to the paper with phospholipid but without human serum.

Results

Fig. 1 shows binding curves of two individual sera from patients with syphilis and two normals. The binding of ^{14}C-anti-IgG was measured as a function of the amount of cardiolipin added. It can be seen that the binding curves for syphilitics rose fairly steeply to plateau levels at approximately 50 nmol of cardiolipin. The measurement at zero cardiolipin concentration gave an indication of the non-specific binding to nitrocellulose paper. Further inspection of these binding curves revealed that at the plateau binding level one antibody molecule was bound per 8000 molecules of antigen.

In Fig. 2 the binding of labelled anti-IgG was measured as a function of the concentration of Mg^{2+} added at plateau levels of other reactants. Data are shown for cardiolipin and for phosphatidylserine. It is apparent in the case of cardiolipin that the binding first decreased slightly, exhibited an apparent peak at 1 mM, then a later rise. The pattern with phosphatidylserine was quite different, showing a broader peak at lower con-

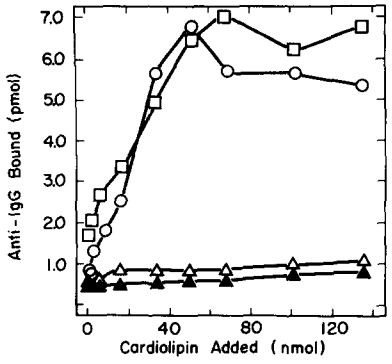

Fig. 1. Binding curves of two sera from syphilitic subjects (open squares, open circles) and two normals (open triangles, closed triangles). Cardiolipin in chloroform in the indicated amounts was spotted on 1 cm diameter nitrocellulose papers, dried, blocked and 50 μl sera added for overnight incubation. After washing, ^{14}C-labelled goat anti-human IgG was added as indicated in Methods.

centrations of Mg^{2+} and clearly no peak at 1 mM. The effects of Mg^{2+} on antibody binding to these phospholipids was reproducible. For cardiolipin, antibody binding peaked at exactly 1.0 mM Mg^{2+} in 11 of 15 experiments. For phosphatidylserine, binding peaked at 0.75–0.80 mM Mg^{2+} in 4 of 4 experiments. Binding patterns were otherwise similar for each phospholipid.

In Fig. 3 the data for Ca^{2+} and the same two phospholipids are illustrated. There was no increased binding over the amount measured with no added cation and no peak at 1 mM, but a small

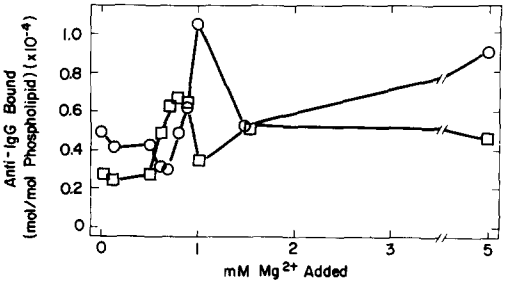

Fig. 2. Effect of Mg^{2+} on anti-phospholipid antibody binding. Extent of anti-human IgG bound using constant serum concentrations (50 μl) from a patient with syphilis, and constant phospholipid (100 μmol). Cardiolipin (open circles), phosphatidylserine (open squares), and increasing concentrations of added Mg^{2+} during the blocking and human serum incubation stages. Otherwise as in Fig. 1.

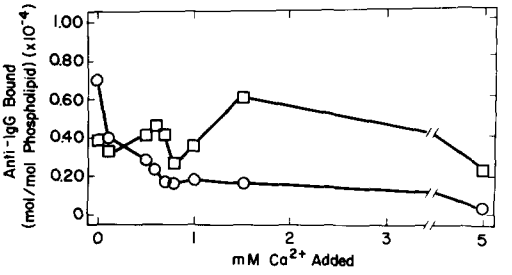

Fig. 3. Effect of Ca^{2+} on anti-phospholipid antibody binding. Cardiolipin (open squares), phosphatidylserine (open circles).

one at 1.5 mM. A total of six experiments with cardiolipin and Ca^{2+} was done. In each case a decrease in binding was evident in the 0.8–0.9 mM Ca^{2+} range. The small peak at 1.5 mM Ca^{2+} was less consistently seen (2 of 6 experiments). For phosphatidylserine the curve was less dramatic showing an irregular decrease in the amount of binding with increased Ca^{2+}. The same pattern of antibody binding was seen in three additional experiments with Ca^{2+} and phosphatidylserine.

In Fig. 4, data have been normalized by referring the extent of binding to the percent of maximum achieved. In these experiments, anti-cardiolipin antibody binding was measured in three additional individual sera from syphilis patients as a function of the concentration of Mg^{2+} added. Antibody binding was decreased in the

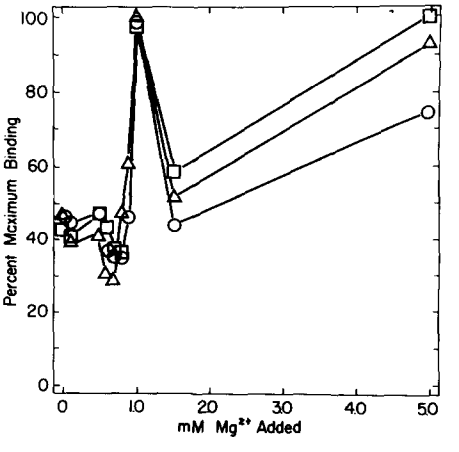

Fig. 4. Effect of Mg^{2+} on human antibody binding to cardiolipin. Binding data for sera from three additional syphilitic subjects are presented, normalized to percent maximum bound.

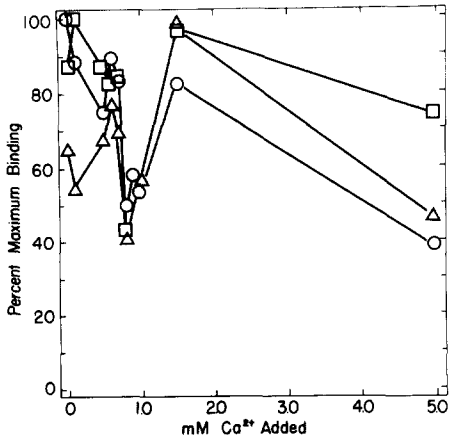

Fig. 5. Effect of Ca^{2+} on human antibody binding to cardiolipin, normalized to maximum percent bound. Otherwise as in Fig. 4.

concentration range of 0.5 to 1.0 mM but a peak at 1mM was noted which was approximately twice the control value. Although the absolute extent of binding in these three sera was substantially different (data not shown) when the data were normalized the results were remarkably consistent with these sera as well as for others not shown. In general, there was a 2–4-fold increase in binding at 1 mM Mg^{2+} compared to control. Normalized data are also shown for Ca^{2+} in Fig. 5. In these sera there was slightly more variability but it is apparent that there was a reduction at just under 1 mM, no peak at 1 mM but a peak at 1.5 mM or higher. There is also either a peak or a shoulder at 0.6 mM. At higher concentrations of both Ca^{2+} and Mg^{2+} (20 mM) there was uniform depression of binding to near baseline levels (data not shown).

Discussion

In the system used for the present studies all syphilitic sera which were examined (more than 50) showed antibodies to cardiolipin, and most also to phosphatidylserine and to phosphatidic acid [12]. The latter were found at a lower level of binding activity and usually did not reach saturation under the conditions employed. With increasing amounts of Ca^{2+} and Mg^{2+}, a polyphasic pattern of binding was found, one which was specific to both the phospholipid and the cation. When normalized to percent of maximum binding, the patterns were highly reproducible from serum to serum. The mechanism of the effect of phospholipid structural polymorphism on antibody binding is not clear. There would seem to be too much reproducibility among different serum samples for the explanation to simply be limitation of antibody access to closed structures. Furthermore, in preliminary experiments the hyperbolic (sometimes sigmoid) binding curves generated as a function of increasing serum concentration and with added Mg^{2+} (1.0 mM) became flattened in the presence of EGTA (1.0 mM). This would indicate that the cation had a significant effect on binding affinity. This in turn might suggest specific effects of phospholipid structural changes on antibody binding rather than simply being a function of limiting antibody excess.

Although the stoichiometry between the second antibody and the antibody to cardiolipin is not known the results are quite similar to what was found with ^{14}C-labelled protein A [12] where a 2 : 1 stoichiometry is known to exist [16]. In any case, one antibody molecule is apparently reacting with 5000 to 25 000 molecules of antigen. This observation itself could suggest a structural requirement, yet marked phospholipid specificity is retained. No reactivity was found, for example, with phosphatidylcholine or phosphatidylethanolamine. Interaction of hydrated cardiolipin might occur with the nitrocellulose paper itself, analogous to the charge mediated binding of phospholipids to glass [17]. Such interaction could have some effect on the apparently large molar ratio of cardiolipin molecules to antibody.

Extensive investigations have been made of the influence of divalent cations on the phase structures of phospholipids, including cardiolipin. X-ray diffraction [18] and ^{31}P-NMR techniques [19] have been employed to demonstrate that Ca^{2+} and Mg^{2+} could induce hexagonal (H_{II}) structures from bilayer phase cardiolipin in model systems. In the freeze-fracture system an intermediate phase which consisted of 'inverted' structures was evident [19]. These phase changes were evident in the 1–3 mM concentration range for Ca^{2+} in the case of bacterial cardiolipin [20]. The structure of the hydrated lipid in the nitrocellulose paper and on its surface is not known. Although the present

studies do not directly address this question, one could hypothesize that cation induced intermediate phase phospholipid structures modulated the antibody binding patterns seen in syphilitic sera. In data not shown, high levels of divalent cation (20 mm) resulted in greatly reduced binding, both in the case of Ca^{2+} and Mg^{2+}. Although without supporting data, this might suggest that the polyphasic binding observed in the region of 1 mM could be due to the formation and possibly disappearence of (different) intermediate structures preceding hexagonal phase formation, and that this polymorphism greatly effects antibody binding.

Perhaps the most fascinating aspect of these studies is repeatibility of the pattern of binding with different concentrations of each cation in different syphilitic sera. That biological molecules in the form of human antibodies to phospholipid antigens are so strikingly sensitive to alterations in the cation environment opens up interesting possibilities to probe the structural/functional role of cardiolipin.

Acknowledgements

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