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Cholesterol effects on the interaction of cardiolipin with anti-cardiolipin antibody

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(1) Human antibodies to cardiolipin, phosphatidic acid and phosphatidylserine were assessed by binding to nitrocellulose paper and subsequent reaction with an enzyme-linked or radioactively labelled second antibody to human IgG. (2) The addition of cholesterol to constant amounts of cardiolipin impregnated in the nitrocellulose paper resulted in a profound fall in antibody binding beginning at a 0.5 to 1 molar ratio of cholesterol to cardiolipin and stabilizing at about 15% of the original level. (3) Antibody binding to phosphatidic acid and phosphatidylserine also showed extensive cholesterol-induced inhibition beginning at a slightly lower molar ratio of cholesterol to phospholipid. (4) The structural array of neither the cardiolipin alone impregnated in nitrocellulose nor the phospholipid together with cholesterol is known. It is possible that the specific cardiolipin phase structure required for human antibody recognition was disrupted by cholesterol.

Introduction

The structural role of cholesterol in the phospholipid bilayers of cell membranes is complex and affects membrane characteristics in diametrically opposed ways in different model systems [1–4]. A functional role for cholesterol in membrane adhesion has been recently proposed [5]. The physical effects of the interaction of cholesterol and cardiolipin in model membranes could not be assessed in one spin-label study in which such attempts were made [6]. In the case of cytochrome $P\text{-}450_{scc}$ cardiolipin appeared to exert its specific effect by enhancement of cholesterol binding to the enzyme rather than any direct interaction with cholesterol [7]. It has been suggested that the effect of cholesterol in enhancing the flocculation test for anti-cardiolipin antibodies in syphilis is related to a spacing role of the sterol for the phospholipid [8]. Modulation of the interaction of human antibodies with cardiolipin and other acidic phospholipids by cholesterol provides an interesting model to examine possible structural aspects of cardiolipin–antibody interaction.

Materials and Methods

Sera were obtained from individuals with documented cases of syphilis through the courtesy of the Erie County Health Department, Division of Public Health. The fluorescent treponemal antibody absorption test for syphilis was positive in each patient and all sera were reactive in the venereal disease research laboratory (VDRL) flocculation test. Nitrocellulose paper was purchased from Bio-Rad Laboratories (Rockville Center,

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NY). Affinity purified goat and rabbit anti-human IgG were purchased from Jackson ImmunoResearch Laboratories (Avondale, PA). These antibodies could be used interchangeably. [1-^{14}C]Acetic anhydride (30–40 mCi/mmol) was obtained from New England Nuclear (Boston, MA). Anti-human IgG peroxidase conjugates and diaminobenzidine were purchased from Sigma Chemcial Company (St. Louis, MO). Cardiolipin (bovine heart), phosphatidic acid (egg yolk), and phosphatidylserine (bovine brain) were obtained from Sigma Chemical Company and Avanti Polar Lipids (Birmingham, AL) and were checked for purity in two-dimensional thin-layer chromatography as previously described [9]. Hydrofluor was supplied by National Diagnostics (Somerville, NJ).

Qualitative anti-phospholipid antibody assessment

Cardiolipin and cholesterol were dried under N_2, dissolved inchloroform, and then mixed in varying cholesterol: cardiolipin molar ratios. 10 μl of each mixture (containing a constant 27 nmol cardiolipin) were spotted on nitrocellulose papers (3.5 × 8 cm) and dried. The papers were agitated in 3% gelatin blocking buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM Mg^{2+}, 0.02% NaN_3, 0.1% Tween 20) for 90 min at 25°C. The papers were then incubated for 16 h in blocking buffer and patient serum (400 μl in a total volume of 20 ml). The strips were then washed three times in blocking buffer for 10 min and were incubated with goat anti-human IgG-peroxidase conjugate according to the manufacturers instructions, 1 : 1000 in 20 mM sodium phosphate buffer (pH 7.4), containing 150 mM sodium chloride (phosphate-buffered saline) at room temperature for 90 min. The papers were rewashed three times with phosphate-buffered saline and color development was visualized after addition to diaminobenzidine solution (50 mg/100 ml phosphate-buffered saline and 0.04% H_2O_2).

Quantitative anti-phospholipid antibody measurement

Nitrocellulose papers were cut in 1 cm diameter circles and spotted with 10 μl of cholesterol/phospholipid mixtures in the same molar ratios as for the qualitative experiments with the cardiolipin concentration held constant at 68.0 nmol. The circles and controls without phospholipid were placed in 50 ml conical centrifuge tubes containing 4 ml of 3% gelatin blocking buffer and were incubated for 1 h in a 33° angled centrifuge rack. Syphilitic serum (80 μl) was then added up to a final volume of 4.0 ml. The tubes were agitated for 16 h at 25°C, and the papers were then removed and washed three times for 15 min in 50 ml of blocking buffer. The circles were subsequently reacted for 1 h with affinity purified goat or rabbit ^{14}C-labelled anti-human IgG, which had been prepared by reaction with [^{14}C]acetic anhydride as previously described [10]. The papers were then washed three times, and were counted in a Packard Tricarb 460CD scintillation counter after addition of 10 ml hydrofluor. The extent of antibody binding was plotted as a function of cholesterol: cardiolipin molar ratios after subtraction of the level of background counts (no phospholipid) for each experimental point. This non-specific binding was low level. Non-specific binding of the labelled second antibody to the paper with and without phospholipid but without the human serum step was negligible.

Results

In the more than 50 syphilitic sera tested, all had detectable antibodies to cardiolipin, phosphatidic acid, and phosphatidylserine [11]. In the qualitative tests there was no reactivity with phosphatidylcholine (egg or dicaproylphosphatidylcholine), or phosphatidylethanolamine (data not shown). Fig. 1 shows the results of two qualitative binding experiments in which a syphilitic serum was reacted with cardiolipin and phosphatidylserine respectively with increasing concentrations of cholesterol impregnated in nitrocellulose papers. Inspection of the staining intensity reveals that anti-cardiolipin antibody binding began to decrease at molar ratios of cholesterol : phospholipid in the 2.5–5.0 range and was undetectable in the 10.0–20.0 range. Anti-phosphatidylserine binding was suppressed at a lower molar ratio.

Figs. 2–4 show the effects of increasing cholesterol : phospholipid molar ratios on quantitative antibody binding to different phospholipids in sera from eight different syphilitic patients. In

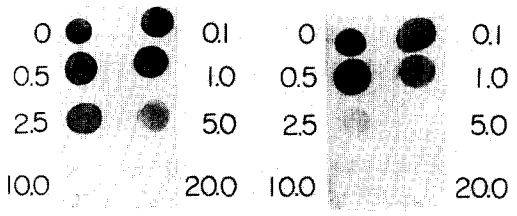

Fig. 1. Cholesterol-induced suppression of anti-phospholipid binding. Nitrocellulose organic blots contained, respectively, 27 nmol cardiolipin (left panel) and phosphatidylserine (right panel) in 10 μl chloroform, as well as cholesterol at the indicated molar ratios. Incubation in serum (1 : 50 in blocking buffer) was followed by washing and incubation in peroxidase-linked anti-human IgG. Visualization was accomplished with diaminobenzidine and hydrogen peroxide.

each case binding is plotted as the amount of anti-IgG bound as a function of increasing molar ratio of cholesterol to the particular phospholipid with the concentration of the latter held constant. The results of three separate experiments, in which anti-cardiolipin binding was measured in different syphilitic sera, are shown in Fig. 2. In each case as the ratio increased, binding to cardiolipin began to decline at the 0.5–1.0 molar ratio point. Binding consistently reached a minimum at a ratio of

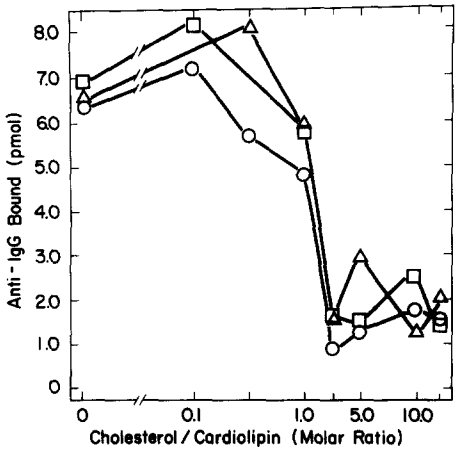

Fig. 2. Suppression of anti-cardiolipin antibody binding by cholesterol. Cardiolipin (68 nmol) and increasing cholesterol at the indicated molar ratios were spotted on 1 cm nitrocellulose circles in 10 μl chloroform followed by incubation with three separate sera (80 μl, separate symbols) from syphilitic patients in 4.0 ml of blocking buffer. After washing the circles were incubated with excess ^{14}C-labelled anti-human IgG.

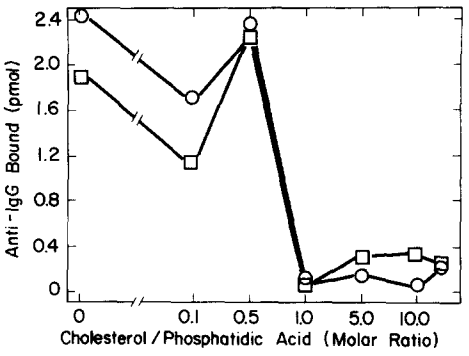

Fig. 3. Suppression of anti-phosphatidic acid binding by cholesterol. Details as in Fig. 2.

2.5 and remained relatively constant thereafter at approx. 15% of the original level. The results of four additional experiments with different sera showed the same pattern.

The effect of cholesterol on binding to phosphatidic acid and phosphatidylserine resulted in similar binding inhibition. In the two sera which were incubated with cholesterol and phosphatidic acid (Fig. 3) the inhibition was in close parallel and maximum depression (approx. 90%) were seen at molar ratios of 0.5–1.0. A further experiment showed a nearly superimposable binding curve although the absolute values were lower. Reproducibility was also seen in the case of binding to phosphatidylserine (Fig. 4). In the three syphilitic sera tested with this phospholipid, the levels of

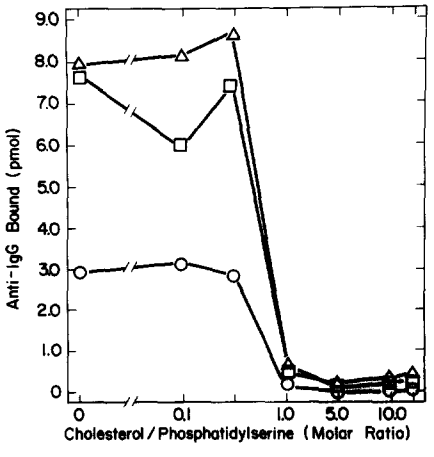

Fig. 4. Suppression of anti-phosphatidylserine antibody binding by cholesterol. Details as in Fig. 2.

anti-IgG binding varied at the control points and lower cholesterol concentrations but in each case began to diminish above a ratio of 0.5 and reached almost 90% inhibition at a ratio of 1.0 where it remained.

Discussion

Cholesterol is an important component of many biological membranes, but its specific role in membrane structure/function has yet to be fully elucidated [12]. Apparently very little work has been done on cardiolipin-cholesterol interactions in model systems, perhaps because they tend not to be found in substantial concentration together in the same membranes [8].

The results of the present experiments demonstrated that there was consistent inhibition by cholesterol of human antibody binding to different phospholipids in sera from different syphilitic subjects. Surprisingly, there was impressive uniformity in these sera, both with regard to the magnitude of suppression and with regard to the molar ratio at which it occurred with each phospholipid. Overall, anti-cardiolipin binding tended to be higher but uniformly began to decline at the 0.5–1.0 molar ratio range and showed maximum depression at 2.5. A similar pattern was evident for phosphatidic acid and phosphatidylserine at even lower cholesterol : phospholipid ratios. Cholesterol has been used along with phosphatidylcholine to amplify the flocculation reactions of anti-cardiolipin antibodies with cardiolipin in syphilis [8], but the molecular basis of the cholesterol effect is not clear. The mechanism may be unrelated to the interaction of cardiolipin and antibody described here.

Why the quantitative antibody measurement with labelled anti-IgG appeared to show inhibition of anti-cardiolipin and anti-phosphatidylserine at a lower molar ratio than the assessment by peroxidase-linked anti-IgG is not clear. In any case there was consistency in that cholesterol-induced suppression of anti-phosphatidylserine antibody binding was observed at a lower cholesterol : phospholipid ratio than that for anti-cardiolipin by both methods.

Concentration-dependent disruption of membrane phospholipid and bilayer stability by cholesterol may occur through different mechanisms [13]. At relatively low cholesterol levels site-specific hydrogen bond formation between cholesterol and the cardiolipin fatty acid residue occurred. At higher cholesterol concentrations cardiolipin disruption was thought to take place as a result of head group disengagement. In other studies mixtures of unsaturated cardiolipin in membranes were disrupted by cholesterol as a result of assumption of dynamic wedge or cone-shaped structures [14,15]. This indirect evidence would point to the provisional hypothesis that cholesterol-induced changes in the physical array of the cardiolipin molecules render the binding of anti-cardiolipin antibody molecules unfavorable. These effects of cholesterol an anti-phospholipid antibody binding are complementary to the observations outlined in the companion paper (divalent cation effects on antibody binding) [16]. The disruptive role of cholesterol in a system of biological interest may provide another reason for the dissimilar membrane distribution of cardiolipin and cholesterol. Cholesterol and related sterols could be useful as molecular probes for the interaction of cardiolipin with anti-cardiolipin antibodies.

Acknowledgements

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