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Autoantibodies against mouse bromelain-modified RBC are specifically inhibited by a common membrane phospholipid, phosphatidylcholine

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Summary. Sera from mice injected 4 days earlier with lipopolysaccharide lysed mouse RBC treated with bromelain (brom). This lytic activity was totally inhibited by including phosphatidylcholine at final concentrations of about 2 μg/ml, or more, in the lytic mixtures. In contrast, the lytic activity of antibodies against rat RBC was not inhibited, even at concentrations of phosphatidylcholine up to 2·5 mg/ml. Various components of the phoshatidylcholine molecule, and other lipids including the closely-related molecule dipalmitoyl phosphatidyl-dimethylethanolamine which is identical to diplamitoyl phosphatidylcholine, except for the absence of a CH_2 group on the polar head group, did not inhibit lysis by the autoantibodies. Autoantibodies against mouse brom RBC, but not antibodies against rat RBC, bound to, and could be eluted from, phosphatidylcholine molecules attached to an insoluble matrix. Liposomes of phosphatidylcholine prepared in the presence of phosphatidic acid or phosphatidylinositol did not inhibit the lysis of mouse brom RBC by autoantibodies to the same extent as liposomes of only phosphatidylcholine. This suggests that phosphatidylcholine is recognized by the autoantibodies only if presented in a certain configuration. We suggest that the function of these autoantibodies may be to facilitate the removal of membrane-damaged cells from the body. Such cells may arise by the process of ageing, or because of the effects of infectious agents such as viruses.

Abbreviations, brom, bromelain-modified; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PBSG, PBS with glucose; PFC, plaque-forming cells.

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INTRODUCTION

Cells in the spleens and in other lymphoid tissues of many strains of untreated healthy mice continuously secrete autoantibodies that react with antigens buried in the membranes of mouse RBC. These antigens, which cannot be detected on the surface of normal RBC, are exposed by treating the cells with proteolytic enzymes such as bromelain (brom) (Linder & Edgington, 1972; Cunningham, 1974; Cox, Baddams & Evans, 1977; Pages & Bussard, 1975). Similar, if not identical, antigens are present on the constant region of mouse IgG (Cunliffe & Cox, 1980). These autoantibodies are produced in significantly higher amounts in mice given anti-lymphocyte serum, lipopolysaccharide (LPS) or rat brom RBC (Cunningham, 1975; Cunliffe & Cox, 1980; Negoro *et al.*, 1979; Cox *et al.*, 1983), but injections of mouse brom RBC do not affect autoantibody levels (Cunningham, 1974; Cox *et al.*, 1977).

The functions and mechanisms of regulation of these autoantibodies produced continuously by nor-

mal mice including germ-free mice (Cunningham, 1976), are unknown. We set out to determine whether phospholipids would inhibit autoantibodies specific for mouse brom RBC after Bussard's group (Serban *et al.*, 1981) had established that monoclonal antibodies of the same specificity were inhibited by trimethylammonium-containing compounds such as choline. Here, the results show that the lytic activity of the autoantibodies is completely inhibited by very low amounts of the ubiquitous membrane phospholipid, phosphatidylcholine, but not by other phospholipids.

MATERIALS AND METHODS

Mice and antisera

Each batch of serum used in this investigation was obtained from 10 female inbred C_3H mice, aged 12–20 weeks. Mouse anti-brom RBC sera were obtained 5 days after the intraperitoneal (i.p.) injection of 30 μg per mouse of LPS. Mouse anti-rat RBC sera were obtained 5 days after the i.p. injection into each mouse of 0·2 ml of a 12% suspension of rat RBC.

Haemolytic assay

The methods for modifying RBC with bromelain and for measuring the haemolytic activity against mouse brom RBC or rat RBC have been described (Cox *et al.* 1977). The end-point was the last dilution of serum at which total lysis occurred (i.e. no pellet of RBC was visible) after incubation at 37° for 30 min and centrifugation. Rabbit serum was used as the source of complement in all assays. Haemolytic assays were performed by mixing dilutions of the appropriate antiserum, test chemical (added singularly) or buffer, RBC (final concentration 0·5%) and rabbit serum (final dilution 1/5) in a total volume of 100 μl in a 4·0 ml plastic tube. The rabbit serum was adsorbed with the target RBC before use. The tubes were incubated at 37° for 30 min with mixing every 10 min, centrifuged at 1100 ***g*** for 2 min and examined for lysis. The end-point was the last dilution of serum at which total lysis occurred (i.e. a buttom of RBC was not visible after centrifugation), provided that lysis did not occur in the tubes with only rabbit serum and RBC. This method of determining the amount of haemolysis was adopted because preliminary investigations showed that the results obtained using end-points of 50% lysis as determined spectrophotometrically at 540 nm gave results in which lysis, or inhibition of lysis, was of the same order as that observed when total lysis was estimated macroscopically.

Chemicals and buffers

All chemicals were from the Sigma Chemical Co. (St Louis, MO) except for tetramethylammonium chloride (Aldrich Chemical Co., WI). The purity of the chemicals, as indicated by the suppliers, is shown in the legend for Fig. 1. Water-insoluble chemicals were prepared as liposomes in the following way. The chloroform/methanol solvent (9:1) used for storage was evaporated to dryness under a stream of air. Phosphate-buffered saline with glucose (PBSG) (for use with mouse brom RBC), or Sorensen's PBS (for rat RBC), was added to give a final concentration of test chemical of 10 mg/ml. Preparations were then sonicated at Setting 8, (8·5 A), on a Dawe Soniprobe Type 1130A for 30 min at 18–22°. Two-dimensional thin-layer chromatography was used to show that this treatment of phosphatidylcholine did not alter its primary structure. Dilutions were prepared in buffers appropriate to the RBC type with which the test chemicals were to be used.

Phosphatidylcholine-affinity column

Phosphatidylcholine was attached to Sepharose 4B by the method of Takahashi, Sugarhara & Ohsaka (1981). The anti-rat RBC serum (Day 5) was heat-inactivated (56°/30 min) and absorbed with mouse brom RBC (200 μl of packed cells and 2·0 ml of serum at 37° for 30 min) to remove any antibodies against mouse brom RBC. One ml of the anti-brom mouse-RBC serum or of the absorbed heat-inactivated anti-rat RBC serum was added to the column and allowed to interact for 30 min at 4°. Unbound material was washed from the column with cold Tris-HCl 0·05 M, (pH 7·5 at 25°), with 0·5 ml fractions being collected. Fractions with an absorbance (280 nm) of greater than 0·1 were pooled, dialysed against PBSG overnight at 4°, concentrated using an Amicon 8050 ultrafiltration cell and YM30 diaflo membranes, and then assayed for haemolytic activity as described above, in the presence of increasing concentrations of phosphatidylcholine.

The percentage haemolysis in each tube was determined by measuring the absorbance at 540 nm of the supernatant and of the lysed pellet of red cells. The supernatant was removed and added to 500 μl of PBS; 500 μl of water was added to the pellet.

$$\% \text{ haemolysis} = \frac{100 \times A_{540\ nm}\ (\text{supernatant})}{A_{540\ nm}\ (\text{supernatant}) + A_{540\ nm}\ (\text{pellet})}.$$

For each serum, Fractions 1–30 represented the material passing through the column, and Fractions 51–58 represented the material eluted from the column. Elution from the column was done with cold Tris-HCl plus 0·1 M choline chloride.

RESULTS

Four days after injecting mice with LPS, sera diluted to 1 in 320 caused total lysis of mouse brom RBC in the presence of complement in a haemolytic assay (Fig. 1). The addition of phosphatidylcholine at a final concentration of 2 μg/ml completely imhibited the lytic activity of autoantibodies specific for mouse brom RBC. Significant inhibition was still observed when phosphatidylcholine was added at a final concentration as low as 20 ng/ml. In contrast, the lysis of rat RBC by mouse antibodies collected 5 days after immunizing mice with rat RBC was not inhibited by phosphatidylcholine, even up to concentrations of 2·5 mg/ml (Group a, Fig. 1). The lytic activity of the autoantibodies and of the antibodies against rat RBC was inhibited by an anti-mouse μ serum, but not by an anti-mouse γ serum (data not shown). This is strong evidence against the possibility that phosphatidylcholine was directly inhibiting the activation of complement by IgM antibodies because, if this were the case, it would be expected that the anti-rat RBC antibodies would also be inhibited.

The possibility that the LPS acted as an antigen eliciting antibodies which cross-reacted with mouse

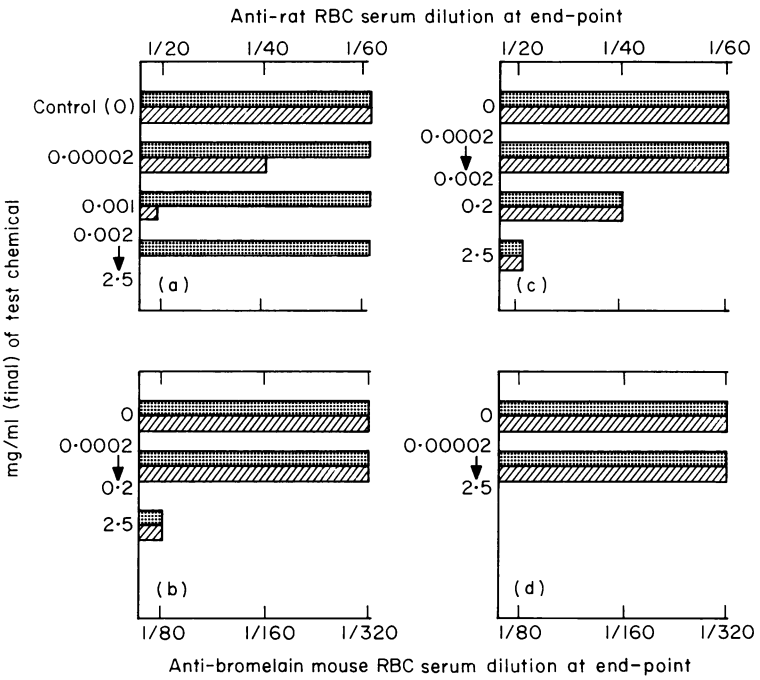

Figure 1. Phosphatidylcholine specifically inhibited lysis of mouse brom RBC by mouse autoantibodies. The following 'group' designation refers to chemicals (tested singularly) which caused similar effects in the haemolytic assays (side-chains were of mixed fatty acids unless specified): (▩), anti-rat RBC; (▨), anti-mouse bromelain RBC. The chemicals (and their approximate purities) were: Group a, phosphatidylcholine dipalmitoyl (98%) and phosphatidylcholine (99%); Group b, tetramethylammonium (Cl^-; 97%) tetraethylammonium (Cl^-; purity not given), choline (Cl^-; 99%); phosphorylcholine (Cl^-, Ca salt; purity not given), phosphatidylinositol (Na salt; 98%), phosphatidylglycerol (ammonium salt; 99%), phosphatidic acid (Na salt; 98%), sphingomyelin (99%), L-α-glycerophosphorylcholine (free base; 98%), and L-α-phosphatidyl-dimethyl-ethanolamine dipalmitoyl (99%); Group c, phosphatidylserine (98%), and lipopolysaccharide (*E. coli* 0111:B4, Difco); Group d, lysophosphatidylcholine lauroyl (98%), phosphatidylethanolamine (98%), L-α-phosphatidylethanolamine dipalmitoyl (98%), L-α-phosphatidyl-monomethyl-ethanolamine dipalmitoyl (99%).

brom RBC is unlikely for several reasons. Firstly, LPS did not specifically inhibit the lysis of mouse brom RBC by the autoantibodies when added to haemolytic assays (Group c, Fig. 1). Secondly, the Day-4 autoantibody-containing serum from mice injected with LPS did not haemagglutinate or lyse LPS-coated mouse RBC at dilutions of 1/20 or greater (data not shown), in contrast to a serum from mice injected with LPS five times (Cox & Keast, 1974). Thirdly, plaque-forming cells (PFC) specific for mouse brom RBC from the spleens of untreated mice (Cunningham, 1974; Cox *et al.*, 1977) or in peritoneal cells cultured *in vitro* for 3–4 days (Pages & Bussard, 1975; Steele & Cunningham, 1978; Cox *et al.*, 1979) were inhibited specifically by phosphatidylcholine (13,900 PFC/10^8 viable spleen cells reduced to 1600 PFC/10^8 in the presence of 1·4 μg/ml phosphatidylcholine). Other phospholipids did not inhibit the PFC, and PFC against trinitrophenol or fluorescein were not inhibited by phosphatidylcholine (data not shown). Fourthly, serum from mice injected with rat brom RBC, which has significantly higher levels of autoantibodies specific for mouse brom RBC compared to serum from normal mice (Negoro *et al.*, 1979; Cox *et al.*, 1983) were specifically inhibited by phosphatidylcholine, but not by other phospholipids (data not shown).

Fractions of the phosphatidylcholine molecule, other phospholipids and related compounds either caused no inhibition (Group d, Fig. 1) or inhibited the autoantibodies in concentrations that were about 2000 times higher than those at which phosphatidylcholine caused inhibition (Groups b and c, Fig. 1). However, at these higher concentrations, antibodies against rat RBC were also inhibited, which contrasts with the specific autoantibody inhibition caused by phosphatidylcholine (Group a, Fig. 1).

Autoantibodies against mouse brom RBC bound to phosphatidylcholine immobilized on an insoluble matrix (Fig. 2). These autoantibodies could be eluted from the column and their lytic activity inhibited by adding phosphatidylcholine to the assays (Fig. 2). Most of the anti-rat RBC antibodies passed through the column; those antibodies that were bound and then eluted were not inhibited by phosphatidylcholine (Fig. 2).

In Fig. 1, the inhibition of haemolysis by various components of the phosphatidylcholine molecule and related compounds is shown. The results show that the entire phosphatidylcholine molecule is required for maximum inhibition of lysis. However phosphatidylcholine sonicated with another non-inhibitory phospholipid, phosphatidylinisitol, was much less effective at inhibiting the lytic activity of the autoantibodies (Table 1). In the mixed liposomes, 10–1000 times more phosphatidylcholine was required to achieve the inhibition observed when phosphatidylcholine was used alone (Table 1). Mixing with phosphatidic acid also reduced the inhibitory effects of phosphatidylcholine to about the same extent as that observed with phosphatidylinisitol.

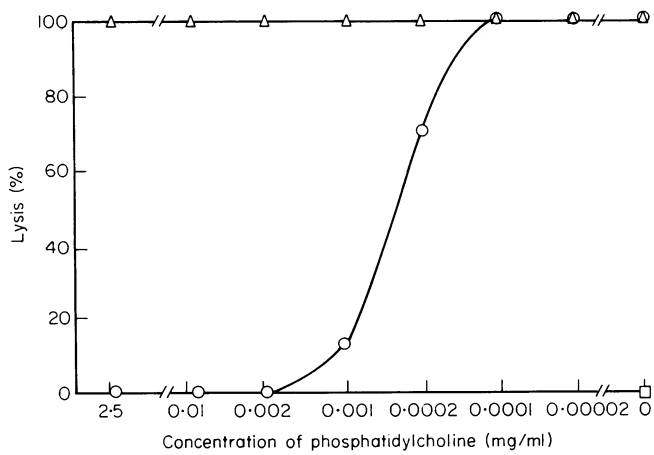

Figure 2. Autoantibodies against mouse brom RBC bind to phosphatidylcholine on an insoluble matrix: (△), anti-rat RBC washings and eluate with rat RBC; (□), anti-mouse brom RBC washings with mouse brom RBC; (○), anti-mouse brom RBC elutate with mouse brom RBC.

Table 1. Inhibition of autoantibodies by phosphatidyl-choline abrogated by preparing liposomes with other lipids

Lipid preparation	Maximum lipid concentration (mg/ml) which did not inhibit lysis of:*	
	Mouse brom RBC†	Rat RBC†
Phosphatidylcholine (PC)	2×10^{-5}	$>2\times10^{-1}$
Phosphatidylinositol (PI)	2×10^{-1}	2×10^{-1}
Phosphatidic acid (PA)	2×10^{-1}	2×10^{-1}
PC/PA (1:1) } Mixed before sonication	$1\times10^{-2}/1\times10^{-2}$‡	2×10^{-1}
PC/PA (10:1) } Mixed before sonication	$2\times10^{-4}/2\times10^{-5}$	$>2\times10^{-1}$
PC/PI (10:1) } Mixed before sonication	$2\times10^{-4}/2\times10^{-5}$	$>2\times10^{-1}$
PC/PA (10:1) Mixed after sonication	$2\times10^{-5}/2\times10^{-6}$	$>2\times10^{-1}$

* Assays for haemolysis were performed as described in the Materials and Methods.

† Lysis by mouse anti-brom RBC sera (1/120) or mouse anti-rat RBC sera (1/20) described in the Materials and Methods.

‡ Concentrations for mixed lipids are listed as PC/PA or PC/PI.

DISCUSSION

The results show that a mouse RBC autoantibody is specifically inhibited by phosphatidylcholine, a common membrane component. Phosphatidylcholine may be inhibiting lytic activity of autoantibodies by one of two mechanisms; firstly, it may be identical or similar to the antigenic determinants recognized by the autoantibodies; secondly, phosphatidylcholine may be binding to a site on the mouse brom RBC, thereby masking the site recognized by the autoantibodies. The results support the first possibility.

Phosphatidylcholine was immobilized on an insoluble matrix according to the method of Takahashi *et al.* (1981). Autoantibodies against mouse brom RBC bound to, and could be eluted from, the column using a choline-containing buffer. Choline was chosen for elution because, in relatively high concentrations, it inhibited autoantibody activity (Serban *et al.*, 1981) (Group b, Fig. 1), and, since it is a small molecule, it could be easily removed from the eluted autoantibodies by dialysis. The autoantibodies eluted from the column (titre of 1/32 after concentration of fractions) lysed mouse brom RBC, and this lysis was inhibited by adding phosphatidylcholine to the assays (Fig. 2). In contrast, IgM antibodies against rat RBC passed through the column, except for a low titre (1/4); this activity, like that of the rat RBC antibodies passing through the column, was not inhibited by adding phosphatidylcholine to the assays (Fig. 2). Some anti-rat RBC antibodies bound to the column; however, this may be due to cross-reactivities between egg components and rat RBC, since the method of preparation of egg yolk lipoproteins required for the column would not have yielded only phosphatidylcholine, although this would have been the most abundant phospholipid (Takahashi *et al.*, 1981).

The second line of evidence which supports the idea that phosphatidylcholine can compete with the antigenic determinants on the mouse brom RBC for the autoantibody-combining site is from experiments in which phosphatidylcholine was added to autoantibody-coated RBC. These were obtained by mixing mouse brom RBC with mouse antisera specific for mouse brom RBC at 37° for 30 min. The RBC were recovered by centrifugation and washed. These RBC were lysed by adding rabbit serum, as a source of complement, whereas mouse brom RBC incubated with buffer or serum of a different specificity, before the addition of rabbit serum, were not. The autoantibody-coated RBC to which phosphatidylcholine had been added were not lysed by adding rabbit serum (data not shown). This suggests that the phosphatidylcholine molecules had displaced the autoantibodies from the RBC.

It was important to establish which parts of the phosphatidylcholine molecule inhibited the autoantibodies. Tetramethylammonium, choline, phosphorylcholine (in the form of chlorides), glycerophosphorylcholine, phosphatidic acid and lysophosphatidylcho-

line lauroyl, all integral parts of phosphatidylcholine, did not inhibit the autoantibodies in assays of haemolytic activity (Groups b and d, Fig. 1). These data suggest that the entire molecule is required for effective inhibition. However, the polar head group is important because phosphatidyl-dimethyl-ethanolamine dipalmitoyl did not inhibit specifically (Group b, Fig. 1), whereas phosphatidylcholine dipalmitoyl did cause specific inhibition (Group a, Fig. 1). Both of these phospholipids have identical fatty acid side-chains, the only difference being the presence of two or three CH_2 groups on the polar head group. Therefore, we conclude that choline, as the polar head group of the phospholipid, is essential for autoantibody binding. Serban *et al.* (1981) established that compounds containing trimethylammonium groups such as choline inhibit autoantibody-mediated lysis of mouse brom RBC. The extension of their finding presented here is that phosphatidylcholine inhibits autoantibodies in amounts that are about 2000 times less than the concentration of choline required for the same inhibition. Further, the autoantibodies used by Serban *et al.* (1981) were monoclonals derived from NZB mice, a strain with a propensity to develop autoimmune diseases, whereas the autoantibodies used here were from the serum of C_3H mice a strain not known to develop autoimmune diseases spontaneously.

The fact that choline, in soluble forms such as lysophosphatidylcholine, glycerophosphorylcholine phosphorylcholine or choline, did not inhibit autoantibodies at concentrations as low as those at which phosphatidylcholine did, suggests that the fatty acid residues must be important for antibody binding. However, as phosphatidic acid did not inhibit the autoantibodies, and because the phosphatidylcholine preparations used would have contained a variety of fatty acid side-chains (Singleton *et al.*, 1965), it seems highly likely that the fatty acids by themselves are not the major site of binding. Since the phospholipids form liposomes, it is possible that the configuraton of the phosphatidylcholine in the liposomes leads to a unique steric structure of choline residues with a characteristic charge which is recognized by the antibody-combining site. Alternatively, the fatty acids may be a second site to which part of the antibody molecule binds, in addition to the specific binding of the combining site to the choline residue.

The results presented in Table 1 suggest that the actual configuration and/or charge of the phosphatidylcholine residues effects the binding by autoantibodies. Liposomes prepared by sonicating phosphatidylcholine with either phosphatidic acid or phosphatidylinositol were much less effective at inhibiting autoantibodies than phosphatidylcholine alone. Ten times the amount of phosphatidylcholine was required to inhibit autoantibodies when mixed with phosphatidic acid or phosphatidylinositol before sonication. Phosphatidic acid sonicated separately and then mixed with phosphatidylcholine liposomes inhibited autoantibody activity to the same extent as phosphatidylcholine alone. It is unlikely that phosphatidic acid or phosphatidylinositol were causing the inhibition in the mixtures because more than 1000 times the amount was required to achieve inhibition when these lipids were used alone (Fig. 1). It seems more likely that the negatively-charged phosphatidylinositol or phosphatidic acid, in liposomes with phosphatidylcholine, interfere with the binding between the polar head group of phosphatidylcholine and the autoantibodies. This suggestion is based on the observation that the phosphatidylcholine exchange protein, which catalyses the exchange of phosphatidylcholine between rat liver mitochondria and sonicated liposomes, was inhibited by the incorporation of negatively-charged lipids into the phosphatidylcholine-containing vesicles (Wirtz *et al.*, 1976).

The reasons for the production of autoantibodies against a common membrane phospholipid remain unknown. The idea that one of the functions of the adaptive immune system is to remove damaged or worn-out cells from the body has been attractive for the last four decades (Grabar, 1975; Fudenberg, 1966), although supporting evidence in the case of old RBC from mice has not been obtained (Cox & Cunliffe, 1979). We suggest that autoantibody production against phosphatidylcholine may be an example of adaptive immunity against damaged self-components, and propose that in a normal membrane the configuration and/or charge of phosphatidylcholine molecules is such that the autoantibodies do not bind. However if a membrane is damaged, for example by ageing, virus infection, or in the laboratory by proteolysis with enzyme such as bromelain, the autoantibodies recognize the configuration of phosphatidylcholine in the membranes.

Autoantibodies against mouse brom RBC also bind to IgG (Fc) (Cunliffe & Cox, 1980). It is not readily apparent why these antibodies should be inhibited by a chemically distinct molecule such as phosphatidylcholine. Phosphate-containing lipids were shown to inhibit the binding of monoclonal anti-DNA autoantibodies to DNA in final concentrations similar to those

at which autoantibodies against mouse brom RBC have been inhibited by phosphatidylcholine in the present study (Lafer *et al.*, 1981). The specificities observed were different to those of the autoantibodies against mouse brom RBC, however, because phosphatidylcholine did not inhibit, whereas diphosphatidylglycerol, phosphatidylglycerol and phosphatidic acid did. The sugar–phosphate backbone of the DNA may be of a similar structure to that of the phosphate group of some phospholipids and related molecule. There are other examples of antibodies binding to apparently unrelated antigens. For example, a human monoclonal antibody has been shown to react with IgG, DNP and DNA (Lafer *et al.*, 1981). Thus, the fact that mouse autoantibodies against mouse brom RBC also bind to mouse IgG (Fc) and to phosphatidylcholine may be a further example of unexpected cross-reactivities of antibodies for apparently unrelated molecules. Another possibility is that IgG molecules are contaminated with low amounts of phospholipids during secretion from the cells, and that the autoantibodies reacting with IgG are really binding to IgG-associated phosphatidylcholine. Experiments have been started to investigate these suggestions.

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