Induction of Antiphospholipid Autoantibodies by Immunization with β2 Glycoprotein I (Apolipoprotein H)

Azzudin E. Gharavi, Lisa R. Sammaritano, Jiaying Wen, and Keith B. Elkon
Division of Rheumatic Diseases, The Hospital for Special Surgery-Cornell University Medical Center, New York 10021

Abstract

A subset of patients with systemic lupus erythematosus has autoantibodies to acidic phospholipids. Since lipids are poor immunogens, the mechanism responsible for the induction of these antibodies is unclear. Immunization of a normal rabbit and normal mice with purified human β2-glycoprotein I (apolipoprotein H) resulted in the production of high levels of two non-cross-reactive antibody populations, anti-apolipoprotein H, and antiphospholipid. The antiphospholipid antibodies had binding specificities indistinguishable from autoantibodies obtained from human and murine lupus. These findings suggest a novel mechanism for the induction of antiphospholipid autoantibodies. (*J. Clin. Invest.* 1992. 90:1105–1109.) Key words: systemic lupus erythematosus • antiphosphospholipid syndrome • autoantibodies • anticardiolipin cofactor • apo H

Introduction

Autoantibodies (aPL)[1] directed against phospholipids (PL) such as cardiolipin occur in systemic lupus erythematosus (SLE) and related disorders (1–5). These autoantibodies are associated with serious clinical complications, viz., thrombosis and intrauterine fetal death secondary to placental infarction (6–9). In spite of the relatively high frequency with which these antibodies are detected, the mechanism(s) responsible for their induction remains an enigma. Lipids are poor immunogens and usually require a protein or carbohydrate carrier to stimulate antibody production (10, 11). As in the case of anti-DNA antibodies (12, 13), aPL directed against acidic PL cannot be induced simply by immunization with phospholipids in Freund's adjuvant. Some aPL have been induced experimentally by complex methods including immunization with cardiolipin and heterologous serum (14), frequent intravenous injection of lipid coupled to methylated bovine albumin (15), intraperitoneal injection of lipid coupled to monoclonal aPL-coated *Staphylococcus aureus* (16), and intrasplenic immunization with *Salmonella minnesota* coated with lipid (17). aPL have also been induced in experimental animals by immunization with liposomes containing various lipids (dipalmitoyl phosphatidylcholine, cholesterol, dicetyl phosphate, and lipid A) (18, 19). Mice immunized with phosphatidylethanolamine in hexagonal II phase have been shown to produce antiphosphatidylethanolamine antibodies that cross-react with cardiolipin (20). The relevance of these findings to autoantibody production in humans with SLE is uncertain. In this report, we describe production of high levels of antiphospholipid antibodies by immunization with a protein antigen. This protein is β2-glycoprotein I (apolipoprotein H, apo H) a 50-kD molecular mass plasma component, which binds to acidic phospholipids (21) and is believed to have a regulatory effect on coagulation (22, 23). Since this protein has been shown to enhance the binding of aPL to PL, apo H has been termed the aPL cofactor (24, 25).

Methods

Purification and characterization of apo H

Human, bovine, and mouse apo H were purified by perchloric acid treatment of serum followed by ion-exchange chromatography on QAE-Sephadex A-50 (Pharmacia LKB Biotechnology Inc., Pitscataway, NJ) (26). Fractions were tested for aPL cofactor activity by ELISA (27). The preparation was analyzed for protein by SDS-PAGE and for lipids by chloroform methanol extraction followed by high performance thin layer chromatography (28).

Immunization protocol

Rabbit. A 2-mo-old New Zealand White rabbit was immunized with 150 μg of the purified human apo H in CFA. After two booster injections in incomplete Freund's, the serum was tested for anti-apo H and aPL activity by ELISA.

Mouse. NIH/Swiss mice (three per group) were immunized with 50 μg of apo H in CFA (test, group A) or 50 μg of HSA mixed with 100 μg of cardiolipin vesicles in CFA (control, group B). After two booster injections of antigen in incomplete Freund's adjuvant, the sera were tested for anti-apo H and aPL activity by ELISAs.

ELISAs. For measurement of anti-apo H activity, plates were coated with purified apo H (10 μg/ml) in PBS overnight, blocked with 2% BSA, and incubated with serial dilutions of test serum. Anti-rabbit or anti-mouse IgG alkaline-phosphatase conjugate diluted (1/1,000) was applied, and plates were developed with 1 mg/ml *p*-nitrophenol phosphate in diethanolamine buffer for 45–60 min. The aPL ELISA was performed as described previously (17).

Inhibition studies

A 1/1,000 dilution of rabbit or 1/100 dilution of mouse antisera (in 1% BSA/PBS) was preincubated overnight with purified apo H or car-

Address correspondence to Azzudin E. Gharavi, M. D., The Hospital for Special Surgery-Cornell University Medical Center, 535 East 70th Street, New York, NY 10021.

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1. *Abbreviations used in this paper:* aPL, autoantibodies directed against phospholipids; PL, phospholipids.

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diolipin vesicles. The sera were then tested for aPL activity or anti-apo H activity by ELISA. The results are expressed as percent inhibition:

$$1 - \left(\frac{\text{OD serum with inhibitor}}{\text{OD serum without inhibitor}}\right) \times 100.$$

Results

Apo H isolated from human plasma was essentially pure as determined by SDS-PAGE, Coomassie blue staining, and immunoblotting (Fig. 1). The minor contaminant of ~ 68 kD (Fig. 1) was identified as HSA on the basis of complete inhibition of binding of the anti-apo H antiserum to the 68-kD band when preincubated with HSA and by direct binding of a monospecific goat anti-HSA antibody (Tago Inc., Burlingame, CA) to this protein (results not shown). Contaminating lipids of human origin were not detected by high performance thin layer chromatography (not shown). Immunization of a normal rabbit with the purified human apo H unexpectedly produced high levels of aPL in addition to anti-apo H antibodies (Fig. 2). As controls, eight rabbit sera (six from nonimmunized and two from rabbits immunized with irrelevant protein antigens in CFA) were tested for IgG aPL activity by ELISA at serum dilutions of 1:100 and 1:1,000. The mean±SD (and range) of the optical densities were 0.190±0.102 (0.096–0.412) at a 1:100 dilution and 0.091±0.008 (0.086–0.110) at a 1:1,000 dilution, compared to 2.230 and 1.205, respectively, for the rabbit immunized with apo H. When cross-inhibition studies using cardiolipin vesicles and apo H were performed, two separate, non–cross-reacting populations of antibodies were demonstrated (Fig. 3).

Immunization of normal mice (group A) with a second preparation of human apo H also induced the production of anti-apo H and aPL antibodies (Fig. 4). In contrast, when normal mice (group B) were immunized with HSA into which cardiolipin was intentionally mixed, the mice produced high

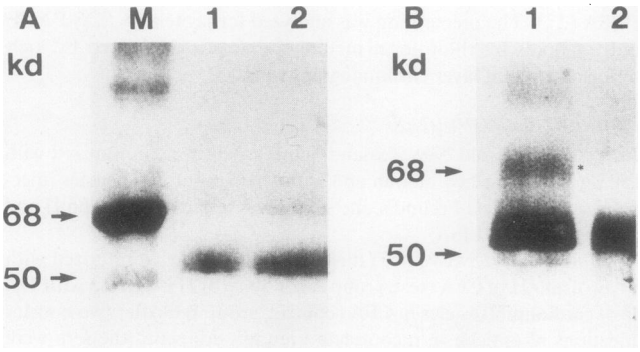

Figure 1. Characterization of purified apo H. (*A*) Coomassie blue stain of apo H. apo H was isolated from human serum and 10 μg was applied to an SDS 10% polyacrylamide gel under nonreducing conditions. Lane *1*, purified apo H, and lane *2*, reference apo H (kindly provided by Dr. E. M. Bevers, University of Limburg, Netherlands). The molecular weight markers (*M*), HSA (68 kD) and IgG chain (50 kD) are shown on the left. (*B*) Immunoblot of the same proteins (1 μg per line) shown in *A* using a reference rabbit anti-apo H serum (kindly provided by Dr. M. I. Kamboh, University of Pittsburgh, PA) at a 1:500 dilution. Trace contamination of the apo H preparation with HSA is observed as a minor band at 68 kD (*).

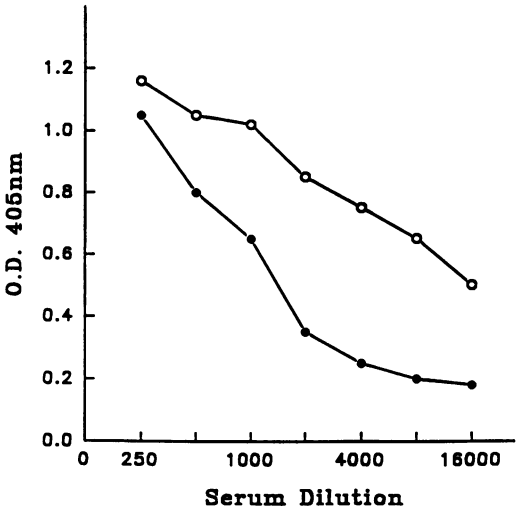

Figure 2. Detection of anti-apo H and aPL in a rabbit immunized with apo H. A 2-mo-old New Zealand White rabbit was immunized with 150 μg of the purified apo H in CFA. After two booster injections, the serum was serially diluted and tested for anti-apo H (○) and aPL (●) activity by ELISA. The preimmune serum had optical density values < 0.150 in both assays at a serum dilution of 1:100.

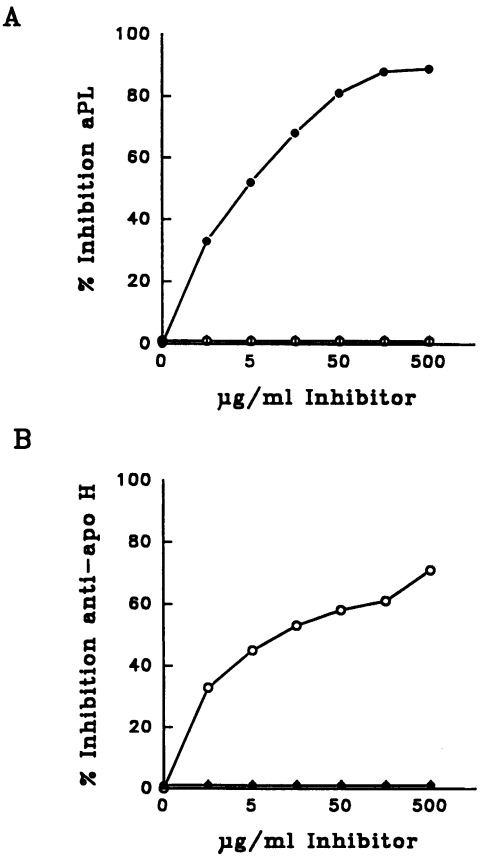

Figure 3. Immunization with apo H induces two, separate, non–cross-reactive antibody populations. The rabbit serum as shown in Fig. 2 was diluted 1:1,000 in 1% BSA/PBS and then preincubated overnight with increasing quantities of purified apo H (○) or cardiolipin vesicles (●). The sera were then tested for aPL activity (Fig. 2 *A*) or anti-apo H activity (Fig. 2 *B*) by ELISA. The results are expressed as percent inhibition. ●, PL; ○, apo H.

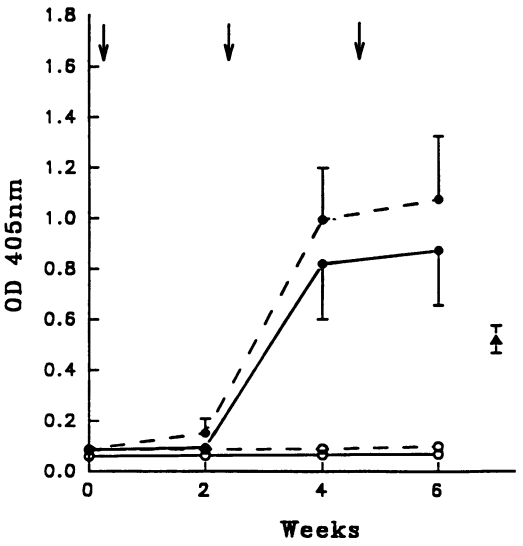

Figure 4. Induction of aPL in mice by immunization with apo H. NIH/Swiss mice (three per group) were immunized with purified apo H in CFA (test, group A [●]) or HSA mixed with cardiolipin vesicles in CFA (control, group B [○]). After two booster injections, the sera were tested for anti-apo H (——) and aPL (- - -) activity by ELISA at a 1:50 dilution. The mean (±SE) antibody activity for each group is shown. The mean (±SE) aPL activity of six, 4 to 5-mo-old MRL/lpr mouse sera (diluted 1:50) is indicated (▲). Arrows represent initial and booster immunizations.

levels of anti-HSA but no aPL antibodies (Fig. 4). Similarly, when cardiolipin was mixed with apo H and injected into three NIH/Swiss mice using the identical immunization protocol, the immunized mice produced similar levels of anti-apo H and aPL to mice immunized with apo H alone (not shown). Compared to spontaneous aPL in MRL/lpr mice (29), apo H-induced murine aPL were of higher levels (Fig. 4).

As in the case of aPL in autoimmune diseases (30, 31), both rabbit and mouse apo H-induced aPL bound to other acidic phospholipids such as phosphatidylserine but did not bind to neutral phospholipids (phosphatidylcholine) (not shown). Similarly, the apo H-induced aPL demonstrated enhanced binding to phospholipids when apo H was added to the ELISA wells, suggesting an antibody preference for an apo H-phospholipid complex.

To determine whether autologous apo H could induce aPL, three groups (groups C–E) of NIH/Swiss mice (three mice per group) were immunized with human, bovine, or mouse apo H (50 μg per injection) using the immunization protocol described above. Human, bovine, and mouse apo H preparations were virtually identical on SDS-PAGE and produced similar cofactor activity in the aPL ELISA. After two booster injections, only the sera of mice immunized with heterologous apo H (human and bovine) demonstrated aPL activity, while mice immunized with autologous apo H had no aPL activity (OD 0.793±0.396, 0.985±0.425, vs. 0.174±0.015, respectively).

Finally, to determine whether phospholipids present in mycobacteria in CFA were necessary for the induction of aPL, three groups of NIH/Swiss mice (three mice per group) were immunized with purified human apo H (50 μg per injection) using three different adjuvants: incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI) (32) containing only mineral oil and emulsifier (33) (group F); Super Carrier-alum (Pierce Chemical Co., Rockford, IL) containing cationized BSA (34), and aluminum hydroxide (group G); and CFA (Difco Laboratories) (group H). In all cases, the same adjuvant was used for the initial immunization and for boosting (except for group H which was boosted with incomplete Freund's adjuvant). All other conditions (dose, route, and timing of immunization) were identical to those described for groups A–E. Results of immunization with human apo H in different adjuvants are shown in Table I. Both groups F and G (immunized with phospholipid-free adjuvants) produced high levels of IgM and IgG anti-apo H as well antiphospholipid antibodies comparable to group H which was immunized with phospholipid containing adjuvant. Sera from age- and sex-matched nonimmunized NIH/Swiss mice were used as controls. The specificity of aPL induced by these adjuvants was confirmed by inhibition with PL micelles (not shown).

Discussion

Immunization with exogenous apo H appears to be a potent method for inducing aPL antibodies. Several mechanisms could explain the induction of aPL in these experiments. Trace contamination of apo H with human PL is an unlikely explanation since PL were not detected in the immunogen and addition of PL to an irrelevant foreign protein (HSA) failed to induce aPL under identical conditions. Although mycobac-

Table I. Induction of aPL in Mice Immunized with Human apo H in Three Different Adjuvants

Adjuvant	Antiphospholipid		Anti-apo H	
	IgM	IgG	IgM	IgG
	OD		*OD*	
Incomplete Freund's adjuvant n = 3 (group F)	0.317±0.057	0.738±0.378	0.348±0.128	0.512±0.214
Super Carrier-alum n = 3 (group G)	0.270±0.101	0.693±0.436	0.142±0.044	0.538±0.315
CFA n = 3 (group H)	0.335±0.068	0.872±0.329	0.445±0.069	0.442±0.212
None n = 10 (Preimmune sera)	0.265±0.061	0.219±0.038	0.108±0.014	0.134±0.014

NIH/Swiss mice were immunized with 50 μg purified human apo H, in incomplete Freund's adjuvant (group F), Super Carrier-alum (group G), and complete Freund's adjuvant (group H). 7 d after the third booster, mice were bled and tested for IgG and IgM antiphospholipid and anti-apo H by ELISA. The results are expressed as optical density values.

teria in CFA provide a source of PL, immunization with phospholipid-free adjuvants, Freund's incomplete adjuvant or Super Carrier-alum (cationized BSA and alum), also induced aPL. Since apo H is a PL-binding protein, an idiotype/antiidiotype mechanism is possible. However, the simultaneous appearance and parallel levels of antibodies to apo H and to PL in individual sera (Fig. 4) argue against this possibility. We therefore propose that aPL autoantibodies are induced by the binding of apo H to endogenous PL in vivo. Since aPL are relatively low affinity antibodies (35) and because antibody binding to PL is enhanced in the presence of apo H, a similar mechanism, viz., PL-protein interaction, may explain aPL production in SLE. Since SLE antibodies do not appear to bind directly to human apo H (24), another PL binding protein may be the key immunogen in the phospholipid syndrome in SLE. Identification of this protein may explain both the induction of aPL and may provide a clue to the pathogenesis of the associated thrombotic disorder. Furthermore, since we have demonstrated a requirement for foreign apo H in the induction of aPL, immunization by foreign PL-binding proteins may explain the production of aPL in infectious diseases (36, 37).

Autoantibodies cannot be readily induced by immunization with self antigens. Even in autoimmune-prone strains of mice, immunization with nucleic acid (13) or ribonucleoprotein self antigens (38) fails to break tolerance. Autoantibodies have been induced experimentally by other strategies. Immunization with foreign or modified self antigens induces antibodies that cross-react with self antigens (38–42). Antiidiotypic antibodies that bind to self cell surface receptors can be produced by immunization with antibodies (idiotypes) directed against the receptor ligand (43–45). The experiments described in the present report indicate that autoantibodies can be generated by a third mechanism, viz., immunization with a foreign autoantigen-binding protein. This mechanism may have relevance to the induction of autoantibodies to antigens other than PL.

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