The Journal of Immunology

RESEARCH ARTICLE | SEPTEMBER 01 1999

GDKV-Induced Antiphospholipid Antibodies Enhance Thrombosis and Activate Endothelial Cells In Vivo and In Vitro[1] FREE

Azzudin E. Gharavi; ... et. al

J Immunol (1999) 163 (5): 2922–2927.
https://doi.org/10.4049/jimmunol.163.5.2922

GDKV-Induced Antiphospholipid Antibodies Enhance Thrombosis and Activate Endothelial Cells In Vivo and In Vitro[1]

Azzudin E. Gharavi,[2]* Silvia S. Pierangeli,† Margaret Colden-Stanfield,‡ Xiao Wei Liu,‡ Ricardo G. Espinola,§ and E. Nigel Harris*

Antiphospholipid (aPL) Abs are associated with thrombosis, pregnancy loss, and thrombocytopenia in patients with systemic lupus erythematosus or primary antiphospholipid syndrome (APS). β_2-Glycoprotein I (β_2GPI), a phospholipid-binding serum protein, is involved in aPL binding to phospholipids. aPL can be generated in mice by immunization with β_2GPI, and these Abs are thrombogenic and cause pregnancy loss in mice. The objective of this study is to determine whether aPL induced by immunization with the phospholipid-binding site of β_2GPI are thrombogenic and whether they activate endothelial cells (EC) in vivo and in vitro. Murine monoclonal aPL were generated from spleen cells of a mouse immunized with GDKV, a synthetic 15-aa peptide spanning Gly^{274}–Cys^{288} in the fifth domain of human β_2GPI, which represents the phospholipid-binding site of β_2GPI. The Abs generated had aPL and anti-β_2GPI activities. The effect of these Abs on thrombus formation and on EC activation in vivo was determined using a mouse model of thrombosis and microcirculation that enables examination of the adhesion of leukocyte to EC as an indication of EC activation as well as adhesion molecule expression using in vitro ELISA analysis. Mice injected with this monoclonal aPL showed a significant increase in leukocyte sticking and also produced larger thrombi that persisted longer. Exposure to GDKV-induced aPL for 4 h significantly increased surface Ag expression of E-selectin, ICAM-1, and VCAM-1. These data indicate that aPL induced by immunization with the phospholipid binding site of β_2GPI are thrombogenic and activate endothelial cells. ***The Journal of Immunology,*** **1999, 163: 2922–2927.**

Antiphospholipid (aPL)[3] occur in patients with systemic lupus erythematosus and related autoimmune disorders (1–5). These Abs are associated with serious clinical complications such as venous and arterial thrombosis, recurrent intrauterine fetal death, and thrombocytopenia. The mechanism(s) responsible for the production of aPL are not completely understood.

Pure lipids generally are poorly immunogenic, and immunization of animals with pure phospholipid (PL) in adjuvant does not induce aPL production (6–7). Some investigators, however, produced aPL experimentally in animals, using complex methods such as frequent i.v. injections of cardiolipin (CL) coupled to methylated BSA (8), i.p. immunization with CL coupled to monoclonal aPL-coated *Staphylococcus aureus* (9), and intrasplenic immunization with *Salmonella minnesota* coated with PL (10). APL Abs have also been induced in mice by immunization with vesicles containing various lipids and lipid A (11). The aPL in all these experiments were not shown to be pathogenic, and not all the above mentioned methods were reproduced by other investigators. In 1992, we described for the first time a simple method for the induction of very high levels of aPL in laboratory animals by immunization with heterologous, β_2-glycoprotein I (β_2GPI) (12). In 1990, three groups independently reported that a plasma protein is a cofactor for aPL binding to cardiolipin (13–15). This protein was identified as β_2GPI, also known as apolipoprotein H. β_2GPI is a heavily glycosylated single-chain normal plasma protein of 326 aa with a molecular mass of ~50 kDa. It is a non-complement-binding member of the complement control protein repeat superfamily that has in common four short consensus repeats (SCR) of ~60 aa each, also known as Sushi domains. Each domain is formed by four disulfide-bonded cysteines in a pattern of Cys 1–3 and Cys 2–4. β_2GPI also has a fifth SCR that forms a modified Sushi domain and contains a PL-binding site. β_2GPI is believed to be part of the epitope to which aPL Abs bind. Mice and rabbits immunized with β_2GPI produced high levels of aPL Abs in addition to Abs against β_2GPI (12). Binding of these aPL Abs to PL was enhanced by addition of β_2GPI similar to the autoimmune human aPL Abs (16). Our findings were reproduced by other investigators (17, 18) and β_2GPI-induced aPL Abs were shown to be pathogenic in certain strains of mice (19–22).

We demonstrated that immunization of mice with purified human or bovine β_2GPI, but not murine β_2GPI, induced aPL production (12). We hypothesized that in vivo binding of foreign β_2GPI to self-PL formed immunogenic complexes against which aPL were produced. To better understand the mechanisms involved in aPL production after immunization with β_2GPI, we tried to determine which part of β_2GPI is responsible for the induction of pathogenic aPL Abs. In the present study, we attempted to induce aPL in mice with synthesized PL-binding proteins constructed by coupling synthetic peptides

Departments of *Medicine, †Microbiology and Immunology, ‡Physiology, and §Microbiology, Morehouse School of Medicine, Atlanta, GA 30310

Received for publication December 3, 1998. Accepted for publication June 15, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was in part supported by National Institutes of Health Grant AR32929 (A.E.G.), by an American Heart Association Grant (South East affiliate), and by National Institutes of Health-National Institute of General Medical Sciences Grants RR03034 and 08248 (S.S.P.).

[2] Address correspondence and reprint requests to Dr. Azzudin Gharavi, Department of Medicine, Morehouse School of Medicine, 720 Westview Drive, S.W., Atlanta, GA 30310-1495. E-mail address: aghara@pol.net

[3] Abbreviations used in this paper: aPL, antiphospholipid Ab; APS, antiphospholipid syndrome; β_2GPI, β_2-glycoprotein I; PL, phospholipid; CL, cardiolipin; EC, endothelial cell; WBC, white blood cell; KLH, keyhole limpet hemocyanin; SCR, short consensus repeat.

0022-1767/99/$02.00

Table I. *Amino acid sequences of GDKV and GDKV$_2$*[a]

	Sequence
GDKV	GDKVSFFCKNKEKKC
GDKV$_2$	GDKVSFFCKKKKKKC

[a] Double-underlined indicates hydrophobic residues.

representing the PL-binding region of the β_2GPI to carrier proteins such as BSA and also study whether these aPL Abs are pathogenic.

Materials and Methods

Synthetic peptides

A 15-aa peptide that spans Gly^{274}–Cys^{288} in the fifth domain of human β_2GPI, and which we called GDKV, was obtained (Q.C. Biochemical, Hopkins, MA). This part of β_2GPI was previously shown to bind CL and other anionic PL (23). This peptide contains four lysines flanked by hydrophobic residues (shown as double-underlined) (Table I). A modified version of GDKV in which all the residues between Cys^{281} and Cys^{288} were replaced with lysine was also prepared. This peptide binds PL more strongly than GDKV (24). This peptide was called GDKV$_2$ (Table I). Conjugates of these peptides to BSA and keyhole limpet hemocyanin (KLH) were also obtained. Binding of these peptides to PL was determined by measuring their competition with β_2GPI in binding to PL-coated plates.

Competitive PL-binding ELISA

Binding of these synthetic peptides to PL was confirmed by competitive PL-binding ELISA. Microtiter plates were coated with 50 μl/well CL in ethanol, 50 μg/ml, overnight at 4°C. Plates were washed three times with PBS (150 μl/well), and blocked for 2 h with 2% OVA in PBS (100 μl/well). Then, duplicates of 50-μl aliquots of serial dilutions of human β_2GPI in 2% OVA/PBS (12.5–800 nM final concentration) were added to each well, followed by equal volumes of PBS alone (control) or PBS containing peptide, 6 μM (final concentration). Plates were incubated for 3 h at 4°C, followed by three washes with PBS. Binding of β_2GPI to CL was detected with a monoclonal anti-β_2GPI Ab, produced in our laboratory (22), which did not react with GDKV, diluted 1:100 in 2% OVA/PBS, followed by alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO), 1:1000 (one h incubation each), and the color reaction was obtained by adding the specific substrate (*p*-nitrophenyl phosphate). The degree of competition with β_2GPI and thus the binding of the peptide to CL were determined by comparing the optical densities of wells containing β_2GPI alone (controls) with those of wells containing β_2GPI plus peptides. The percentage inhibition was calculated as: % inhibition = $[(OD_{control} - OD_{test})/OD_{control}] \times 100$.

Direct binding of GDKV peptides to phospholipid

After the immunization experiments, when mouse antisera to GDKV peptides were available, binding of these peptides to CL-coated plates was directly confirmed by ELISA. Plates were coated with CL blocked with 2% OVA/PBS and incubated with GDKV peptides in PBS at 20 μg/ml or PBS alone. Mouse antisera to GDKV and GDKV$_2$ were diluted 1:100 in 1% OVA/PBS, added to the corresponding wells, and incubated for 1 h, followed by alkaline phosphatase-conjugated anti-mouse IgG and then *p*-nitrophenyl phosphate as described above.

Immunization with synthetic peptides

Groups of 12 female NIH/Swiss mice were immunized with the following Ags in Freund's adjuvant: human β_2GPI, 30 μg/mouse (positive control); free GDKV, GDKV conjugated to KLH, GDKV conjugated to BSA; GDKV$_2$-BSA; KLH; or BSA alone.

Dose determination of immunogens

Induction of aPL after immunization with β_2GPI appears to be a function of the ability of this protein to bind acidic PL. Thus, the dose of GDKV and GDKV$_2$ was based in part on their m.w. as well as their PL-binding ability. After two booster injections with 2- weekly intervals, mice were bled, and sera were tested by ELISA for Ab activity.

Determination of aPL, anti-β_2GPI, and anti-peptide Abs

Anticardiolipin activity was determined in the sera of the mice and supernatant of the hybridoma by a standard anticardiolipin ELISA, using alkaline phosphatase-conjugated anti-mouse IgG, as described elsewhere (12). Supernatant was tested at 1:5 and 1:10 dilutions, and the mouse sera were tested at a 1:50 dilution in 10% adult bovine serum (10% ABS-PBS). The color reaction was stopped when a positive control of ~100 IgG antiphospholipid units reached an OD of 1.0. Similarly, the presence of Abs to β_2GPI, peptides, or BSA was determined with the use of plates coated with these Ags, blocked with 2% OVA/PBS. This buffer was also used as a diluent for the mouse sera when tested for anti-BSA activity. A rabbit antiserum to human β_2GPI, produced in our laboratory (12), was used as a positive control for anti-β_2GPI Abs.

Monoclonal Abs

To study the pathogenic effects of GDKV-induced aPL Abs, mAbs were developed. Spleen cells from a GDKV-BSA-immunized aPL-producing mouse were fused with P3X 63Ag 8.653 (American Type Culture Collection, Manassas, VA CRL 1580), a nonsecreting myeloma cell line, using polyethylene glycol (25). Ab-secreting hybridoma clones were rendered monoclonal by limiting dilutions method, and the mAbs were tested for aPL and anti-β_2GPI activity by ELISA. One GDKV-induced mAb with aPL and anti-β_2 GPI activity was used in the in vivo thrombus enhancement and endothelial cell activation studies.

Purification of mouse mAb from the hybridoma supernatant

Mouse mAb was purified from the supernatant of the hybridoma by affinity chromatography, utilizing an anti-mouse IgG or IgM affinity column. The purified mAbs were dialyzed against Tris-buffered saline, pH 7.4, and the concentration of the protein was determined by the Lowry method (Sigma). Samples were filtered sterile before injection into the mice.

In vivo experiments

The effects of aPL Abs on thrombosis was studied by examining the dynamics of thrombus formation in the exposed femoral vein (26–28) and their ability to activate endothelial cells in vivo was determined by examining white blood cell (WBC) adhesion to endothelium in an exposed cremaster muscle (29, 30). Both experiments were performed in the same treated mouse (30).

Animals and injection protocol. Normal male CD-1 (outbred) mice weighing 30–40 g (Charles River Laboratories, Wilmington, MA) were used for these studies. The animals were housed in the Animal Care (American Association of Laboratory Animal Care-approved) facilities of the Morehouse School of Medicine. Animals were handled by trained personnel according to Institutional Animal Care and Use Committee guidelines. Mice were initially injected i.p. with monoclonal GDKV-induced aPL Ab in normal saline, at 0 and 48 h, 10 μg mAb per mouse. The control group received equal amount of a murine mAb of irrelevant specificity. The surgical procedure were performed 72 h after the first Ab injection.

Analysis of thrombus dynamics: effects of aPL on thrombus formation. Analysis of thrombus dynamics in a mouse model has been described previously (26–28). In brief, mice were anesthetized 72 h after the first injection with the aPL or the control IgG, and the right femoral vein was exposed. The vein was pinched with a standard pressure to introduce an injury and to induce a clot. Clot formation and dissolution in the transilluminated vein were visualized with a microscope equipped with a closed-circuit video system (including a color monitor and a recorder). Thrombus size (in square micrometers) was measured when the thrombus reached the maximum size by digitizing the image and tracing the outer margin of the thrombus; the times (in minutes) of formation (from appearance to maximum size) and disappearance (from maximum size to disappearance) of the thrombus were measured as well. Three to five thrombi were successfully induced in each animal, and mean values were computed. Mean thrombus area and mean times for formation, disappearance, and total times were then computed for each group of injected animals. The person performing the surgery and measurements (X.W.L.) was blinded as to what treatment had been given to each animal.

Analysis of endothelial cell activation in the microcirculation of the exposed cremaster muscle of mice. Activation of endothelial cells was assessed by direct visualization and quantitation of adhering ("sticking") WBC to endothelial cells in the microcirculation of the exposed cremaster muscle of mice as described elsewhere (29, 30). In brief, 72 h after the first injection, mice were anesthetized and placed in the dorsal position, the animal's right scrotum was incised, and the cremaster muscle and testicle were gently exposed and placed on a microscope slide. The evaporative fluid and heat loss was minimized as described previously (29, 30). The dynamic events in the microcirculation of the mouse's exposed cremaster muscle (thickness, 120 μm only) can be directly visualized without the use of vital dyes. Thus, the lumen of venules and capillaries can be directly

viewed and the interaction of individual blood cells (such as WBC, erythrocytes, or platelets) with the luminal surface can be quantitatively assessed. After a stabilization period of 30 min, the leukocytes that remained stationary on the endothelium for at least 30 s were considered adhering ("sticking"). Such adhering WBC were counted under the microscope in five different venules of 25–35 μm diameter; the means were calculated and compared between treated and control groups.

In vitro exposure of endothelial cells to GDKV-induced aPL Abs

Confluent monolayers of HUVEC (10^4 cells/well) seeded in collagen-coated 96-well plates were incubated with complete RPMI culture medium or GDKV-induced murine monoclonal aPL Abs (neat supernatant) in RPMI culture medium for 4 h at 37°C. As a positive control, some HUVEC monolayers were treated with LPS (3 μg/ml) in complete RPMI for 4 h to increase the surface expression of E-selectin, ICAM-1, and VCAM-1. After paraformaldehyde fixation, adhesion molecule expression was assessed with a colorimetric ELISA previously described (31, 32). Color development was stopped at 3 M H_2SO_4 at 20 min, and the OD was read at 492 nm wavelength on a SpectraMax 250 ELISA plate reader (Molecular Devices, Sunnyvale, CA). The degree of specific Ag expression was calculated by subtracting nonspecific binding of the secondary Ab from all test values.

Statistical analysis

An independent *t* test was used to compare the Ab levels in different groups of mice. The unpaired Student *t* test was used to compare the means of thrombus sizes and times (total disappearance) and adhering WBC numbers between treated and control groups. Statistical significance was achieved when $p \leq 0.05$. Statistically significant differences on surface Ag expression of endothelial adhesion molecules on HUVEC monolayers exposed to control medium or to GDKV-induced Abs were evaluated with the unpaired Student *t* test. Statistical significance was achieved when $p \leq 0.05$.

Results

Phospholipid binding by β_2GPI and peptides

Binding of β_2GPI to CL-coated plates was dose dependent and followed a hyperbolic curve. In the presence of 6 μM GDKV, this binding was decreased, which indicates the competition of GDKV with β_2GPI in binding to CL. The concentration of β_2GPI required for 50% saturation increased from 71 nM to 173 nM. Thus, 6 μM GDKV was equivalent to 102 (173 − 71 = 102) nM β_2GPI in binding to CL; making it ~59-fold less effective than β_2GPI. $GDKV_2$ also competed with β_2GPI in binding to CL in a similar manner, but with somewhat higher avidity. In the presence of 6 μM $GDKV_2$, the concentration of β_2GPI required for 50% saturation increased from 71 nM to 252 nM. Thus, at 6 μM, $GDKV_2$ was equivalent to 181 (252 − 71) nM β_2GPI in binding CL, making it almost 33-fold less effective than β_2GPI. The percent inhibition of 100 nM β_2GPI binding to CL by GDKV and $GDKV_2$ was 43 and 56%, respectively. These findings confirmed the binding of these peptides to CL, which was previously reported by other investigators (23, 24).

Direct binding of GDKV peptides to phospholipid

Experiments with mouse antisera to GDKV peptides confirmed the binding of these peptides to CL-coated plates. The optical densitiesfor binding of anti-GDKV to wells containing CL alone and CL+GDKV were 0.322 vs 0.805, and for the anti-$GDKV_2$ were 0.472 vs 0.984. Binding to wells with CL alone indicates aPL activity in mouse sera and the additional binding to wells with CL plus peptide reflects binding of peptides to CL and binding of the antisera to peptides.

Ab production

As expected mice immunized with β_2GPI produced high levels of aPL in addition to anti-β_2GPI Abs. Mice immunized with GDKV-KLH, GDKV-BSA, or $GDKV_2$-BSA, in addition to producing high levels of Ab against the immunizing peptide and the carrier proteins, produced significantly (independent *t* test: $p < 0.05$) higher levels of aPL than did mice immunized with KLH, BSA, free GDKV, or the preimmune control mice (Fig. 2). However, the levels of aPL in mice immunized with whole β_2GPI were much higher than the aPL levels in mice immunized with β_2GPI-derived peptides conjugates. This may be explained in part by the greater affinity of β_2GPI for PL compared with these peptides affinity for PL or β_2GPI being more immunogenic than these peptides. Mice immunized with GDKV-KLH showed significantly elevated levels of anti-β_2GPI Abs, when compared with mice immunized with KLH or GDKV alone or nonimmunized controls (Fig. 2). Similarly, mice immunized with GDKV-BSA and $GDKV_2$-BSA also had elevated levels of anti-β_2GPI activity (data not shown). Mice immunized with BSA or KLH alone showed high levels of Abs to these proteins (data not shown) together with slightly elevated levels of aPL, probably due to polyclonal B-cell activation (Fig. 1).

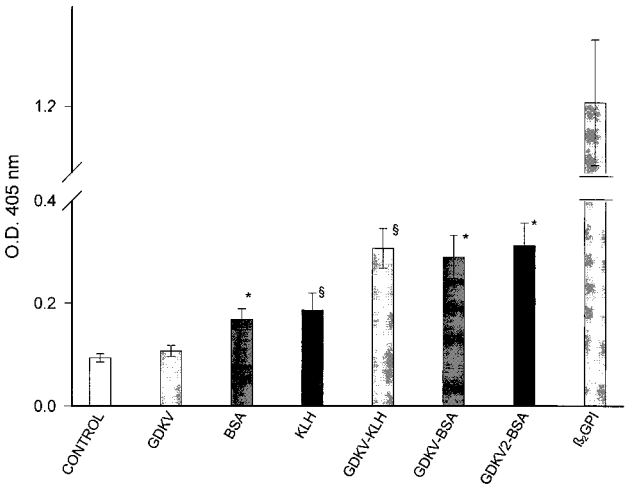

FIGURE 1. aPL activities in mice immunized with GDKV peptides. Mouse sera were diluted 1:50 in 10% ABS-PBS and tested by ELISA for aPL Abs. Mice immunized with peptide-KLH or peptide-BSA conjugates had significantly higher levels of aPL activity compared wit those immunized with KLH alone, BSA alone or nonimmunized controls. *, $p \leq 0.05$, statistically significant different from BSA control mice. §, $p \leq 0.05$, significantly different from KLH control mice.

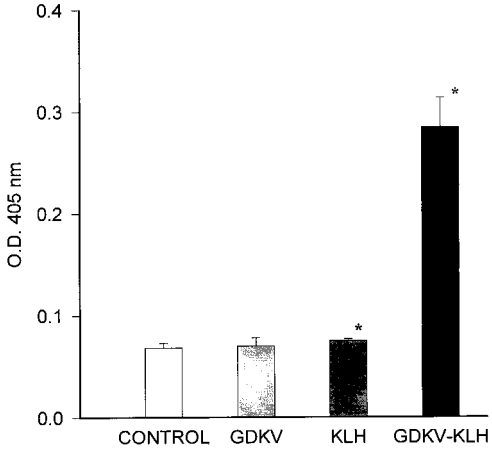

FIGURE 2. Anti-β_2GPI activities in mice immunized with GDKV-KLH conjugate. Mouse sera were diluted 1:50 in 2% OVA/PBS and tested by ELISA for anti-β_2GPI Abs. Mice immunized with GDKV-KLH had significantly higher levels of anti-β_2GPI (*, $p \leq 0.05$) compared with those immunized with KLH, GDKV alone, or nonimmunized controls. *, $p \leq 0.05$, different from control mice.

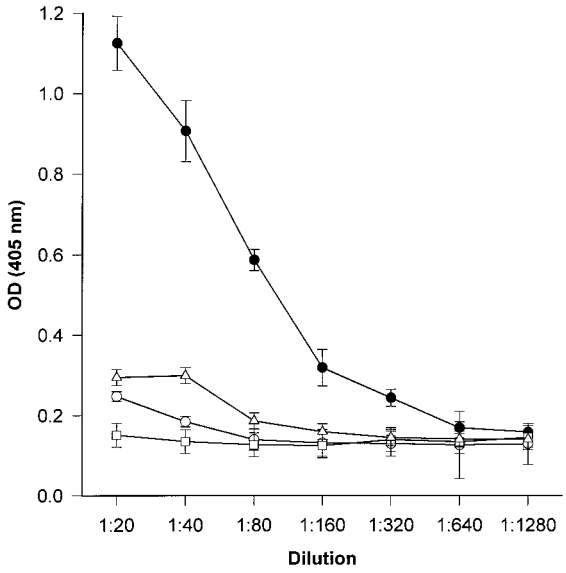

FIGURE 3. Titration of aPL activity. The GDKV-induced aPL (●) and mouse sera immunized with BSA (△), or KLH (○), as well as the nonimmunized mouse sera (□) were serially diluted in 10% ABS and tested by ELISA for aPL activity.

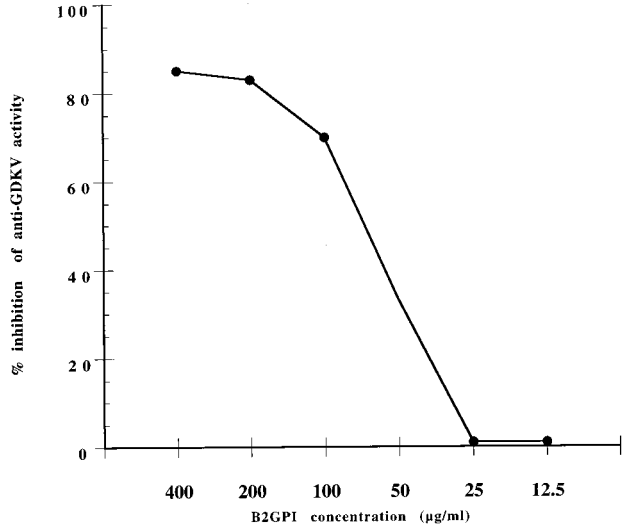

FIGURE 4. Inhibition of anti-GDKV activity by β_2GPI. Anti GDKV was diluted 1:80 in 1% OVA/PBS and incubated with increasing concentrations of human β_2GPI for 1 h at room temperature and then overnight at 4°C before testing by ELISA for anti-GDKV activity. There was up to 85% inhibition of anti-GDKV activity by β_2GPI.

However, animals immunized with free GDKV (with no carrier protein) did not produce any Ab against PL, β_2GPI (Figs. 1 and 2), or even GDKV (data not shown). The titration curves for GDKV-induced aPL, and those in mice immunized with BSA, or KLH, as well as nonimmunized mice are shown in (Fig. 3). Interaction of β_2GPI with GDKV and inhibition of anti-GDKV activity by β_2GPI was also determined. Anti-GDKV was diluted 1:80 in 1% OVA/PBS and incubated with increasing concentrations of human β_2GPI (12.5–400 μg/ml) for 1 h at room temperature and then overnight at 4°C before testing by ELISA for anti-GDKV activity. β_2GPI inhibited the binding of the Ab to the GDKV-coated plate in a dose-dependent manner, and there was up to 85% inhibition of anti-GDKV binding by β_2GPI at 400 μg/ml (Fig. 4).

In vivo studies

The GDKV-induced mAb used in the in vivo experiments in addition to anti-GDKV activity had both aPL and anti-β_2GPI activity (mean OD units, 0.565 ± 0.102 and 0.652 ± 0.095, respectively), in preparations containing 550 ng/ml murine Ig. Control murine mAb at similar concentration had negligible aPL and anti-β_2GPI activities: 0.123 ± 0.092 and 0.189 ± 0.098, respectively. Seventy-two hours after the first i.p. injection (immediately before the surgical procedures), mice injected with GDKV-induced aPL showed significantly elevated levels of aPL and anti-β_2GPI Abs (data not shown). Animals injected with control monoclonal preparation were negative for aPL and anti-β_2GPI.

Effect of mAbs on thrombus formation and on activation of endothelial cells in vivo

Mice injected with GDKV-induced aPL Ab produced larger thrombi that persisted significantly longer when compared with controls (Table II).

Mice injected with GDKV-induced monoclonal aPL also showed greater numbers of WBC sticking to endothelial cells of the venules than did mice injected with control murine monoclonal IgG (Figs. 5 and 6). This increased adhesion of leukocytes to endothelial cells is an indication of endothelial cell activation in vivo.

GDKV-induced aPL Abs enhance surface expression of endothelial adhesion molecules

As a correlate to the in vivo microcirculation experiments with GDKV-induced aPL Ab, surface Ag expression of ICAM-1, E-

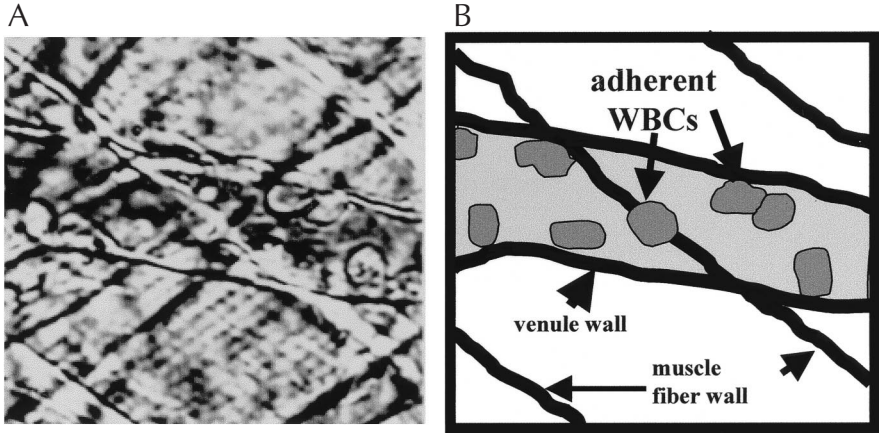

FIGURE 5. Adhesion of WBC to endothelium in mouse cremaster muscle. *A*, Close view of the exposed cremaster muscle under microscope showing WBC adhering to endothelial cells in a postcapillary venule (diameter, 18 μm). *B*, WBC adhesion to endothelium in the mouse cremaster muscle postcapillary venules.

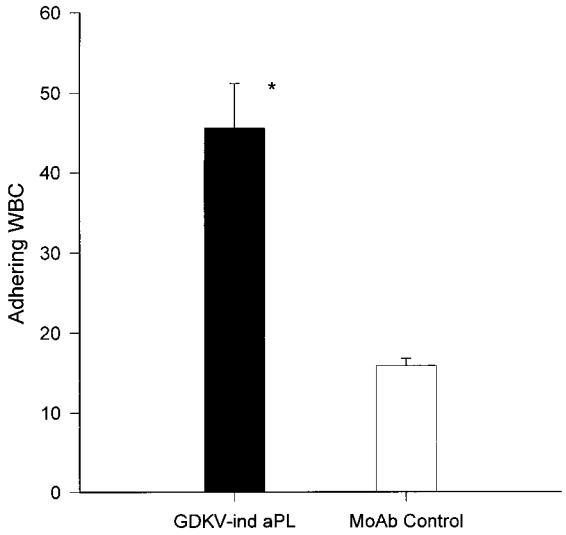

FIGURE 6. Effects of GDKV-induced aPL on endothelial cells in vivo. WBC adhesion to endothelial cells of the postcapillary venules in the cremaster muscle of mice treated with GDKV-induced monoclonal aPL Ab or controls was measured as indicated in *Materials and Methods.* Results are expressed as mean ± SD of the numbers of WBC adhesion. *, $p \leq 0.05$, significantly different from control treated mice.

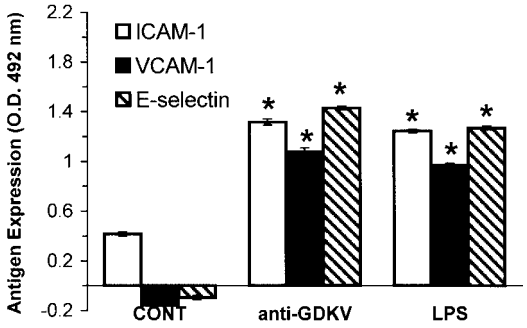

FIGURE 7. Effects of GDKV-induced aPL Ab on endothelial cells in vitro. ICAM-1, VCAM-1, and E-selectin surface Ag expression on HUVEC monolayers exposed to complete RPMI culture medium (CONT), GDKV-induced aPL Ab (anti-GDKV), or LPS in complete RPMI culture medium for 4 h. Each point is the mean ± SEM of quadruplicate wells. *, $p \leq 0.05$, significantly different from corresponding control group.

selectin, and VCAM-1 were quantitated after a 4-h exposure to GDKV-induced aPL Ab. ELISA analysis revealed that control HUVEC monolayers constitutively expressed ICAM-1, whereas VCAM-1 and E-selectin were not detectable (Fig. 7). However, HUVEC monolayers that were exposed to GDKV-induced aPL Ab for 4 h expressed significantly higher levels of each adhesion molecule. GDKV-induced aPL Ab was as potent in up-regulating expression of the adhesion molecules as exposure of HUVEC monolayers to LPS for 4 h (110–112% of LPS-induced expression).

Discussion

Previous studies by our group showed that immunization with heterologous β_2GPI induced aPL Abs in mice with properties similar to those found in patients with autoimmune diseases (12, 16). The β_2GPI-induced aPL have been shown to be pathogenic in certain strains of mice, causing intrauterine fetal death, thrombocytopenia, and thrombosis (18–21).

The present study demonstrated that induction of aPL is not restricted to immunization with the whole β_2GPI molecule, and peptides representing the PL-binding site of the β_2GPI (GDKV) or peptides with sequences similar to that region that have PL-binding properties ($GDKV_2$) may also induce aPL production. $GDKV_2$ is a hypothetical peptide and is not derived from β_2GPI but has stronger PL-binding properties than GDKV, the β_2GPI-derived peptide. $GDKV_2$ has been used in these studies to show that aPL may be induced by immunization with other PL-binding peptides that are not part of β_2GPI. However, because these PL-binding peptides are too small to be immunogenic by themselves, they need to be coupled to larger protein molecules to become immunogenic. We hypothesize that in vivo a complex of PL-peptide-carrier protein is required for the induction of aPL. In the case of immunization with β_2GPI, which does not need a carrier protein, it is possible to speculate that PL binds to the PL-specific site (GDKV) in the fifth domain of β_2GPI, and the rest of the β_2GPI serves as a carrier protein induces aPL and anti-β_2GPI production.

Similar to the β_2GPI-induced aPL (18, 21), the GDKV-induced aPL were shown to be pathogenic. They caused enlargement of the in vivo induced thrombi in murine femoral veins. The GDKV-induced aPL are associated with anti-β_2GPI activity, and their binding to PL is enhanced by β_2GPI; therefore, it is conceivable that their thrombogenic effect be due to their interference with the regulatory function of β_2GPI on coagulation. The GDKV-induced aPL in this study not only caused enhanced thrombus formation but also caused increased adherence of leukocytes to endothelial cells in vivo. The latter observation may suggest the endothelial cell activation by aPL as a mechanism for their pathogenicity (30, 33).

The levels of aPL induced by β_2GPI were higher than those of aPL induced by GDKV or $GDKV_2$ using the same protocol. This may be explained partly by the greater PL-binding properties of whole β_2GPI compared with these peptides. The other explanation may be the presence of more PL-binding sites other than GDKV on the β_2GPI, that cause formation of larger, PL-containing, and immunogenic complexes with foreign β_2GPI. Another possibility is that the polyclonal Ab responses generated in the mice immunized with β_2GPI would include Abs directed to multiple other epitopes in the intact molecule. In addition, other investigators have observed Abs to other regions of the β_2GPI molecule in patients with aPL. Furthermore, the levels of aPL in patients with APS are never as high as those in animals immunized with β_2GPI, and they are usually more comparable with those seen in mice immunized with PL-binding peptides.

Table II. *Dynamics of thrombus formation (area and time of thrombus) in mice injected with murine monoclonal aPL induced by GDKV*

Samples	No. of Animals Tested	Thrombus Area (μm^2) (mean ± SD)	Thrombus Disappearance Time (min) (mean ± SD)	Thrombus Total Time (min) (mean ± SD)
GDKV-induced aPL	9	2345.0 ± 456.9*	6.8 ± 1.5*	12.9 ± 1.6*
Control	9	867.9 ± 234.5	3.5 ± 0.5	5.4 ± 1.8

*, Statistically significant different from control, $p < 0.05$.

Some investigators have suggested the Id-anti-Id network as a cause for the induction of aPL autoantibodies and APS in mice (18, 34), based on the hypothesis that following immunization with β_2GPI or PL-binding peptide-carrier protein complex Abs against the PL- binding region would be produced that will be similar to PL, and anti-idiotypic Abs against them may have anti-PL activity.

Not all PL-binding proteins are capable of aPL induction by immunization, and it is likely that PL may need to bind in some specific way to form an immunogenic, aPL-inducing complex. For example, placental anticoagulant I (annexin V) which binds PL very avidly and much more effectively than β_2GPI does not induce aPL production by immunization and induces only anti-annexin V in animals (35).

Altogether, these data indicate that immunization with foreign β_2GPI, or its PL-binding site, can induce high levels of pathogenic aPL. However, it is unlikely that this would be the cause of aPL production in patients with APS, but incidental exposure to other PL-binding foreign proteins such as viral or bacterial products, with structural and functional similarities to the PL-binding site of β_2GPI may induce pathogenic aPL and anti-β_2GPI production and cause APS.

References

1. Gharavi, A. E., and W. A. Wilson. 1997. Antiphospholipid antibodies. In: *Dubois' Lupus Erythematosus.* 5th Ed. D. J. Wallace and B. H. Hahn, eds. Williams and Wilkins, Baltimore, pp. 471.
2. Sammaritano, L. R., A. E. Gharavi, and M. D. Lockshin. 1990. Antiphospholipid antibody: immunologic and clinical aspects. *Semin. Arthritis Rheum. 20: 81.*
3. Harris, E. N., A. E. Gharavi, and G. R. V. Hughes. 1985. Antiphospholipid antibodies. *Clin. Rheum. Dis. 11:591.*
4. Gharavi, A. E., E. N. Harris, R. A. Asherson, and G. R. V. Hughes. 1987. Anticardiolipin antibodies: isotype distribution and phospholipid specificity. *Ann. Rheum. Dis. 46:1.*
5. Harris, E. N., A. E. Gharavi, M. L. Boey, M. B. Patel, C. G. Mackworth-Young, S. Loizou, and G. R. V. Hughes. 1983. Anticardiolipin antibodies: detection by radioimmunoassay and association with thrombosis in systemic lupus erythematosus. *Lancet 2:1211.*
6. Marcus, D. M., and G. E. Schwarting. 1976. Immunological properties of glycoproteins and phospholipids. *Adv. Immunol. 23:203.*
7. Alving, C. R. 1986. Antibodies to liposomes, phospholipids, and phosphate esters. *Chem. Phys. Lipids 40:303.*
8. Inoue, K., and S. Nujima. 1967. Immunochemical studies of phospholipids. I. Reactivity of various synthetic cardiolipin derivatives with Wasserman antibody. *Biochim. Biophys. Acta 144:409.*
9. Rauch, J., H. Tannenbaum, B. D. Stollar, and R. S. Schwartz. 1984. Monoclonal anticardiolipin antibodies bind to DNA. *Eur. J. Immunol. 14:529.*
10. Igarashi, K., M. Umeda, L. Tokita, K. Soo Nam, and K. Inoue. 1991. Effective induction of antiphospholipid and lupus anticoagulant in normal mouse. *Thromb. Res. 61:135.*
11. Schuster, B. G., M. Neidig, M., B. M. Alving, and C. R. Alving. 1979. Producing antibodies against phosphocholine, phosphatidylcholine, and sphingomyelin and lipid A by injection of liposomes containing lipid A. *J. Immunol. 122:900.*
12. Gharavi, A. E., L. R. Sammaritano, J. Wen, and K. B. Elkon. 1992. Induction of antiphospholipid autoantibodies by immunization with β_2 glycoprotein I (apolipoprotein H). *J. Clin. Invest. 90: 1105.*
13. McNeil, H. P., R. J. Simpson, C. J. Chesterman, and S. A. Krilis. 1990. Antiphospholipid antibodies are directed against a complex antigen that includes lipid binding inhibitor of coagulation: β_2 glycoprotein I (apolipoprotein H). *Proc. Natl. Acad. Sci. USA 87:4120.*
14. Galli, M., P. Comfurius, H. C. Hemker, M. H. Debaets, P. J. C. van Breda-Vreisman, T. Barbui, R. F. A. Zwaal, and E. M. Bevers. 1990. Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor. *Lancet 335:1544.*
15. Matsuura, H., T. Igarashi, M. Fujimoto, K. Ichikawa, and T. Koike. 1990. Anticardiolipin cofactor(s) and differential diagnosis of autoimmune disease. *Lancet 336:177.*
16. Gharavi, A. E., L. R. Sammaritano, J. L. Bovastro, and W. A.Wilson. 1995. Specificities and characteristics of β_2 glycoprotein I-induced antiphospholipid antibodies. *J. Lab. Clin. Med. 125:775.*
17. Pierangeli, S. S., and E. N. Harris. 1993. Induction of phospholipid-binding antibodies in mice and rabbits by immunization with human β_2 glycoprotein I or anticardiolipin antibodies alone. *Clin. Exp. Immunol. 78:233.*
18. Blank, M., D. Fadin, A. Tincani, J. Kopolovic, I. Goldberg, B. Gilburd, F. Allegri, G. Balestrieri, G. Valesini, and Y. Shoenfeld. 1994. Immunization with anticardiolipin cofactor (β_2-glycoprotein I) induces experimental antiphospholipid antibody syndrome in naïve mice. *J. Autoimmun. 7:441.*
19. Aron, A. L., M. L. Cuellar, R. L. Brey, S. McKeon, L. R. Espinoza, Y. Shoenfeld, and A. E. Gharavi. 1995. Early onset of autoimmunity in MRL/++ mice following immunization with β_2 glycoprotein I. *Clin. Exp. Immunol. 101:78.*
20. Garcia, C. O., A. Kanbour-Shakir, H. Tang, J. F. Molina, L. R. Espinoza, and A. E. Gharavi. 1997. Induction of experimental antiphospholipid syndrome in PL/J mice following immunization with β_2 GPI. *Am. J. Reprod. Immunol. 37: 118.*
21. Pierangeli, S. S., X. W. Liu, G. H. Anderson, J. H. Barker, and E. N. Harris. 1996. Thrombogenic properties of murine anti-cardiolipin antibodies induced by $\beta_2$glycoprotein 1 and human IgG antiphospholipid antibodies. *Circulation 94:1746.*
22. Gharavi, A. E., S. S. Pierangeli, A. E. Gharavi, H. Tang, X. Liu, X, and E. N. Harris. 1998. Thrombogenic properties of antiphospholipid antibodies do not depend on their binding to β_2 glycoprotein I (β_2GPI) alone. *Lupus 7:341.*
23. Hunt, J., and S. Krilis. 1994. The fifth domain of β_2 glycoprotein I contains a phospholipid binding site (Cys 281–Lys288) and a region recognized by anticardiolipin antibodies. *J. Immunol. 152:653.*
24. Matsuura, E., M. Igarashi, Y. Igarashi, K. Ichikawa, and T. Koike. 1994. Molecular studies on phospholipid binding sites and cryptic epitopes appearing on β_2 glycoprotein I structures recognized by anticardiolipin antibodies. *Lupus 3:313.*
25. Kohler, G., and C. Milstein. 1975. Continuous culture of fused cells secreting antibody to predefined specificity. *Nature 256: 495.*
26. Stockmans, F., H. Deckmyn, J. Gruwez, J. Vermylen, and R. Acland. 1991. Continuous quantitative monitoring of mural, platelet-dependent thrombus kinetics in the crushed rat femoral vein. *Thromb. Haemostasis 65:425.*
27. Pierangeli, S. S., J. H. Barker, D. Stikovac, D. Ackerman, G. Anderson, J. Barquinero, R. Acland, and E. N. Harris. 1994. Effect of human IgG antiphospholipid antibodies on an in vivo thrombosis model in mice. *Thromb. Haemostasis 71:670.*
28. Pierangeli, S. S., X. W. Liu, G. H. Anderson, J. H. Barker, and E. N. Harris. 1995. Induction of thrombosis in a mouse model by IgG, IgM and IgA immunoglobulins from patients with the antiphospholipid syndrome. *Thromb. Haemostasis 74:1361.*
29. Peter, F. W., D. A. Schuschke, W. Z. Wang, G. H. Anderson, R. J. P. M. Franken, J. M. Gu, S. S. Pierangeli, and J. H. Barker. 1998. Do leukocytes contribute to impaired microvascular tissue perfusion after arterial repair? *Microsurgery 18:23.*
30. Pierangeli, S. S., M. Colden-Stanfield, X. Liu, J. H. Barker, G. L. Anderson and E. N. Harris. 1999. Antiphospholipid antibodies from antiphospholipid syndrome patients activate endothelial cells in vitro and in vivo. *Circulation 99:1997.*
31. Colden-Stanfield M., D. Radcliffe, E. B.Cramer, and E. K.Gallin. 1993. Characterization of influenza-virus-induced luekocyte adherence to human umbilical vein endothelial cell monolayers. *J. Immunol. 151:310.*
32. Colden-Stanfield M., J. F. Kalinich and E. K. Gallin. 1994. Ionizing radiation increases endothelial and epithelial cell production of influenza virus and leukocyte adherence. *J. Immunol. 153:5222.*
33. Simantov, R., J. M. LaSala, S. K. Lo, A. E. Gharavi, L. R. Sammaritano, J. E. Salmon, and L. R. Silverstein. 1995. Activation of cultured vascular endothelial cells by antiphospholipid antibodies. *J. Clin. Invest. 96:2211.*
34. Bakimer R., P. Fishman, M. Blank, B. Srednc, M. Djadetti, and Y. Shoenfeld. 1992. Induction of primary APS in mice by immunization with a human monoclonal anticardiolipin antibody (H-3). *J. Clin. Invest. 89:1558.*
35. Sammaritano, L. R., A. E. Gharavi, and M. D. Lockshin. 1992. Phospholipid binding of antiphospholipid antibodies and placental anticoagulant protein. *J. Clin. Immunol. 12:27.*