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SPECIAL ARTICLE

Adjuvant immunization induces high levels of pathogenic antiphospholipid antibodies in genetically prone mice: another facet of the ASIA syndrome

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Adjuvants may induce autoimmune diseases in susceptible individuals, a phenomenon recently defined as autoimmune/inflammatory syndrome induced by adjuvants (ASIA). Patients with both antiphospholipid antibodies (aPL) and the genetic coagulopathy factor V Leiden (FVL) are frequently found. We therefore evaluated whether adjuvant can induce aPL in heterozygous FVL mice. aPL were measured in naïve mice and at 1 and 5 months after immunization with either complete or incomplete Freund's adjuvant (CFA, IFA) in FVL and control C57/B6 background mice. We defined antibody levels 3 SD above the mean of C57/B6 mice immunized with adjuvant as positive (specificity of 99%). For β_2GPI-dependent aPL, 28.6% (6/21) of FVL mice 5 months after immunization with adjuvant (both IFA and CFA) were positive compared with 4.8% (1/22) of FVL mice 1 month after adjuvant and 0% of naïve FVL and C57/B6 mice (0/16, $p < 0.001$). aPL levels correlated with behavioral hyperactivity in the staircase test. FVL mice immunized with adjuvant did not develop β_2GPI-independent aPL. We hypothesize that the FVL aPL association is not a coincidence, but that chronic coagulation defects combined with external inflammatory stimuli analogous to adjuvant may induce aPL and also antiphospholipid syndrome, thus supporting the notion of ASIA. *Lupus* (2012) **21,** 210–216.

Key words: adjuvant; antiphospholipid syndrome; ASIA; autoantibodies; autoimmunity; experimental model; hypercoagulation

Introduction

Individuals may harbor several quiescent pathological conditions such as an immune dysregulation or genetic predisposition (e.g. HLADR4), rendering them prone to autoimmune disorders.[1–3] In the course of life, specific external stimuli may cause activation of the immune system, acting as a second hit, leading to overt clinical expression of an autoimmune disease. These external stimuli may be caused by environmental factors (infections, silica) or man-made ones such as pharmaceutical compounds, vaccines, and others. Autoimmune/inflammatory syndrome induced by adjuvants (ASIA) is a newly defined term for autoimmune syndromes caused by pharmaceutical and industrial compounds with immunogenic capabilities termed collectively adjuvants.[4–6]

Antiphospholipid syndrome (APS) is the most common acquired hypercoagulability disorder.[7,8] APS has many thrombogenic mechanisms, including disturbance of the coagulation cascade (by decreased activation of protein C and inhibition of β_2-glycoprotein I, anti-thrombin, and fibrinolysis); endothelial cell and platelet activation; and immune complex formation between antiphospholipid antibodies (aPL) and β_2-glycoprotein I (β_2GPI) on membrane phospholipids of endothelial cells and platelets.

Factor V Leiden (FVL) is a common hereditary hypercoagulability disorder. Patients with FVL have a mutant factor V, which is relatively resistant

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to degradation by activated protein C (APC). Numerous case reports describe patients with APS and FVL,[9–11] and these patients have a greater risk for thrombotic manifestations. In both disorders, a diminished inhibitory ability of APC results in a hypercoagulability state. It has been shown that APS patients with thrombosis were found to have a higher frequency of FVL than those without thrombosis.[12] We have recently demonstrated that genetically designed FVL mice had a more severe type of APS, compared with naïve mice, following immunization with β_2GPI.[13]

The coagulation and immune systems are closely related to each other, and a dysregulation of one may affect the other, perhaps due to a chronic activation of the coagulatory cascade, over-expression of coagulatory epitopes and the presence of an immune dysregulation. We aimed to evaluate a hypothesis that patients with thrombophilia are prone to autoimmunity. In the present study we examined the ability of adjuvant to induce autoimmunity in prone mice. Complete and incomplete adjuvants injected into FVL mice were found to induce pathogenic antiphospholipid (β_2GPI-dependent) antibody production.

Methods

Mice

The transgenic mice used in this study were kindly provided by Prof. David Ginsburg, University of Michigan, Ann Arbor, MI, USA. These mice carry the ortholog of human FVL mutation previously generated by a 'knock-in' of the R504Q mutation into the endogenous murine factor V locus by homologous recombination.[14] These mice were back-crossed to C57BL/6 mice for more than seven generations. Genotyping of the offspring for the FVL transgene was performed by PCR of DNA obtained from post-weaning tail biopsies with the primers previously described. The mice were raised under standard conditions, $23 \pm 1^{\circ}$C, 12-h light cycle (7AM–7PM) with ad libitum access to food and water. The Tel Aviv University Animal Welfare Committee approved all procedures.

Study design

Female C57BL/6 (C57/B6) and heterozygous FVL mice were divided into three groups: a naïve group ($n = 9$ for C57/B6 and $n = 7$ for FVL mice), a complete Freund's adjuvant (CFA)-immunized group ($n = 11$ for C57/B6 and $n = 15$ for FVL mice), and a group immunized with incomplete Freund's adjuvant (IFA, FVL mice only $n = 7$). Mice were immunized once at 3 months of age. For serological evaluation all the immunized mice were bled 1 and 5 months after immunization. Naïve mice were bled at 6–7 months of age.

Serological evaluation

The mice were bled by retro-orbital sinus puncture. The sera were separated by centrifugation and stored at -70°C until assayed. The sera were tested by standard ELISA for the presence of autoantibodies as previously described.[15] These included serum-dependent (β_2-GPI) and independent antibodies to cardiolipin (CL) and phosphatidylserine (PS), antibodies to β_2-GPI and double stranded DNA (dsDNA).

Staircase test

This test of activity (stair-climbing measure) and explorative behavior/anxiety (rearing measure) was performed as previously described by us.[16,17]

Statistical analysis

Levels of antibodies were compared by means of one-way analysis of variance (ANOVA) which were followed by least significant difference (LSD) post hoc tests. Linear regression was performed to examine the correlation between antibody levels and behavioral measures. Statistical tests were performed using the SPSS-PC software package. All determinations were made with a 95% confidence interval and were considered significant at the $p < 0.05$ level.

Results

FVL mice immunized with CFA develop autoantibodies 5 months after immunization

We compared the effect of adjuvant immunization on the levels of autoantibodies in heterozygous FVL and C57/B6 background naïve mice. Antibodies against CL (β_2GPI-dependent) were measured in non-immunized (naïve) and CFA-immunized mice, 1 and 5 months after immunization. As can be seen in Figure 1, CFA-immunized FVL mice developed significantly higher levels of aCL(β_2GPI) antibodies 5 months after immunization compared with the level at 1 month after

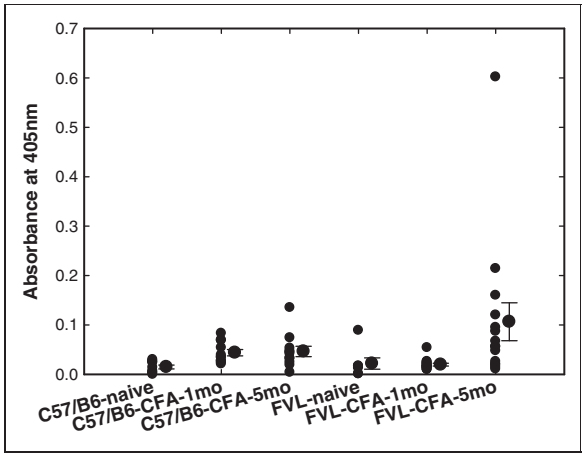

Figure 1 Autoantibody levels in sera of FVL and C57/B6 mice immunized with adjuvant (CFA). aCL(β_2GPI) antibodies were measured in C57/B6 and FVL non immunized naïve mice ($n=9$ and $n=7$, respectively) and C57/B6 and FVL mice immunized with CFA ($n=11$ and $n=15$, respectively). Titers were measured 1 and 5 months post immunization. The levels of antibody represent individual and mean absorbance values ± S.E. in the ELISA assay. FVL mice immunized with CFA develop autoantibodies 5 months after immunization.

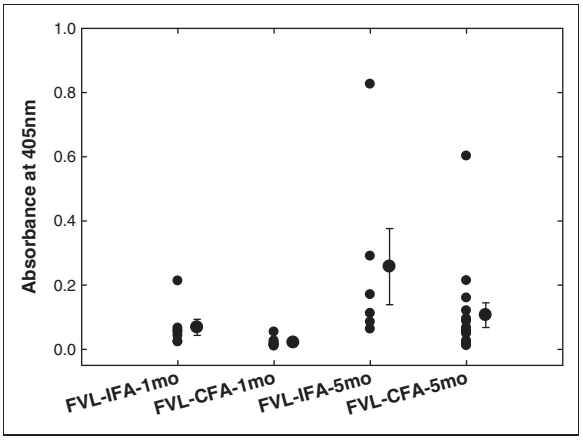

Figure 2 Autoantibodies induced by CFA vs. IFA immunization in FVL mice. aCL(β2GPI) antibody levels were measured in FVL mice immunized with complete (CFA) or incomplete Freund's adjuvant (IFA), 1 month (1 mo) and 5 months (5 mo) post immunization ($n=15$ and $n=7$, respectively). The levels of antibody represent individuals and mean absorbance values ± S.E. in the ELISA assay. No significant difference was found between IFA- and CFA-immunized mice.

immunization or without immunization, and compared with C57/B6 immunized or non-immunized mice ($p<0.021$ for the effect of group, ANOVA). Post hoc analysis revealed that the FVL-CFA-5 month group had significantly higher levels of antibodies compared with the other groups ($p<0.043$ post hoc by LSD) which were not significantly different from each other ($p>0.3$ post hoc by LSD).

Autoantibodies induced by CFA vs. IFA immunization in FVL mice

We examined whether the increases levels of aPL were linked to the type of adjuvant used, CFA or IFA, (Figure 2). Both CFA (0.02 ± 0.005 Absorbance Units) and IFA (0.07 ± 0.02)-immunized FVL mice spontaneously developed aCL(β_2GPI) ($p=0.07$ for adjuvant type effect). For other antibodies tested (aCL, PS(β_2GPI), PS, β_2GPI, and dsDNA) no significant difference was found between IFA- and CFA-immunized mice ($p>0.11$ ANOVA). There was a significant elevation from 1 month to 5 months after immunization in most of the antibodies tested (except CL, PS, and dsDNA) in both IFA- and CFA-immunized mice ($p<0.02$ for month effect). There was no significant interaction between adjuvant type and time for all antibodies ($p>0.11$ for adjuvant type × month effect, ANOVA).

FVL mice immunized with adjuvant specifically develop β_2GPI-dependent autoantibodies

Since the IFA and CFA-immunized FVL mice were essentially similar, we combined these two groups for the purpose of analyzing the levels of autoantibodies to relevant phospholipids in this group (Figure 3). Five months after immunization there was a significant increase ($p<0.001$ non-parametric Kruskal–Wallis test) in the level of β_2GPI-dependent autoantibodies (aCL(β_2GPI), anti-PS(β_2GPI) and anti-β_2GPI Abs) in the adjuvant-immunized FVL mice compared with 1 month adjuvant-immunized FVL mice, naïve FVL mice and adjuvant-immunized C57/B6 mice. In contrast there was a non-significant decrease in β_2GPI-independent autoantibodies (aCL and anti-PS antibodies) in the adjuvant-immunized FVL mice compared with naïve FVL and C57/B6 mice (Figure 3). There was no significant difference in the level of anti-dsDNA antibodies between the groups, although there were a number of mice in the 5 months adjuvant-immunized FVL group with a significant level of anti-dsDNA antibodies. We defined mice as positive when they had antibody levels 3 SD above the mean of C57/B6 mice immunized with adjuvant (specificity of 99%) and analyzed their proportion. For β_2GPI-dependent aCL antibody we found that 28.6% (6/21) of FVL mice immunized with adjuvant for 5 months were positive compared with 4.8% (1/22) of FVL

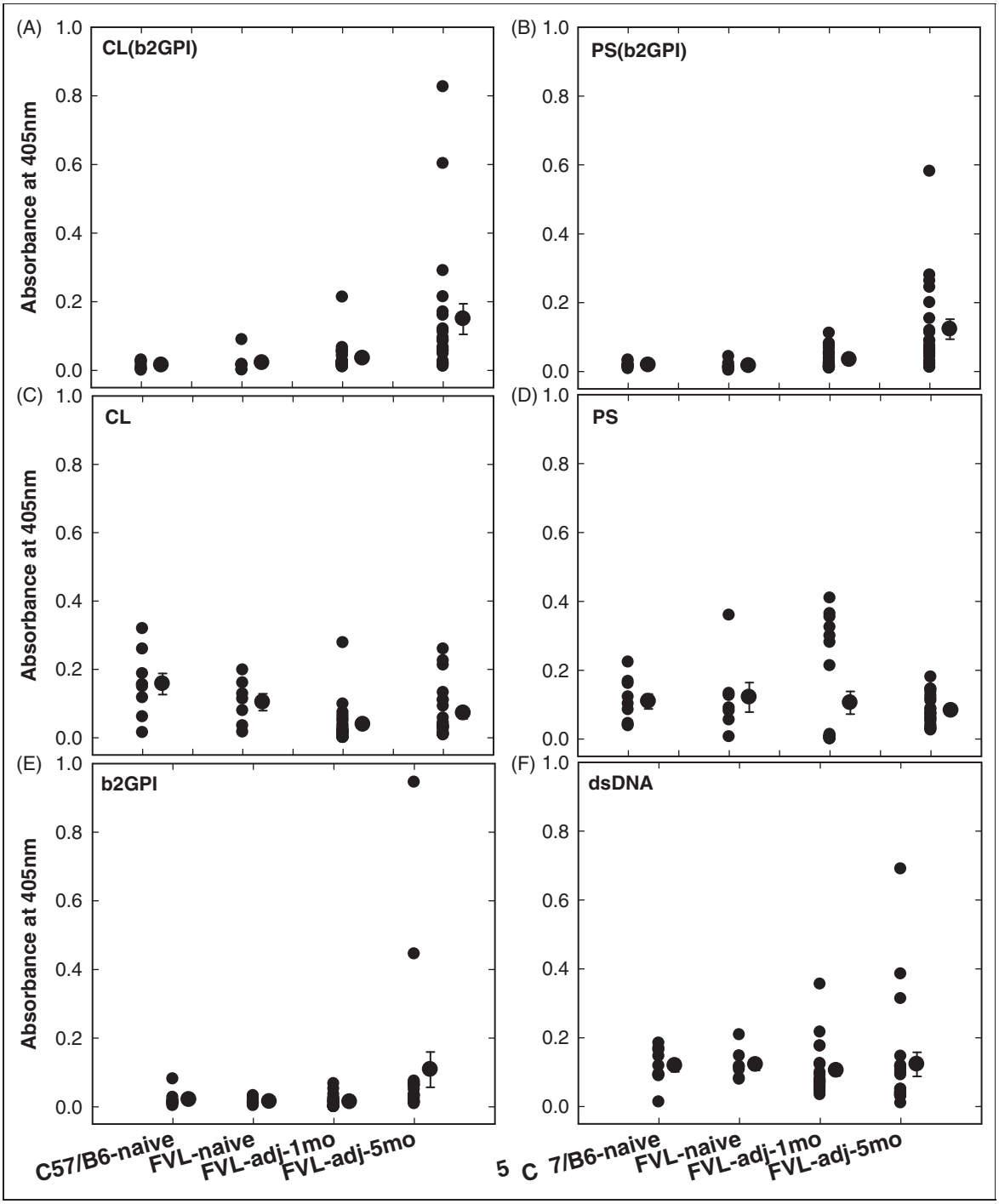

Figure 3 FVL mice immunized with adjuvant specifically develop β2GPI-dependent autoantibodies. Autoantibody titers to β_2GPI-dependent-CL (A), to β_2GPI-dependent-PS (B), to non-β_2GPI-dependent-CL (C), non-β_2GPI-dependent-PS (D), to β_2GPI (E), and to ds-DNA (F) were measured in sera of naïve C57BL/6 (C57/B6, $n=9$) and FVL mice ($n=7$) and adjuvant immunized FVL mice 1 and 5 months post immunization ($n=22$ and $n=21$ respectively). The antibody titers represent individual and mean absorbance values ± S.E. in the ELISA assay. The adjuvant-immunized FVL mice developed high levels of β_2GPI-dependent antibodies over time.

mice immunized with adjuvant for 1 month and compared with 0% of naïve FVL and C57/B6 mice (0/16, $p<0.0001$, Chi square test) (Figure 3A). The same analysis done for β_2GPI-independent aCL antibodies revealed no positive mice in any group tested (Figure 3C).

In order to control for a non-specific general pro-inflammatory effect of the FVL genotype we

also tested antibodies against an additional non-specific antigen, albumin. Anti-albumin antibodies were very low and similar in all groups of mice tested (data not shown).

Correlation between behavioral performance and autoantibodies level

In order to examine the pathogenic potential of the aPL, CFA-immunized mice were tested for behavior in the staircase test. We found a significant correlation between the number of stair-climbing events (a measure of locomotor activity) and aCL(β_2GPI) level ($p = 0.002$, Pearson correlation). There was no significant correlation between the number of rearing events (a measure of exploratory behavior and anxiety) and aCL(β_2GPI) level ($p = 0.59$, Pearson correlation).

Discussion

The central and intriguing finding in the present study is the induction of high levels of aPL in FVL mice following adjuvant immunization alone. In addition, both complete and incomplete adjuvant (with or without *M. tuberculosis*) elicited the same response, which may indicate that this effect is driven by a general inflammatory reaction, by the adjuvant itself, rather than by specific antigens. The capability of adjuvants (CFA and IFA) to induce the production of autoantibodies was reported first by Noel Rose and colleagues in 1959.[18] This was the first report of an experimental autoimmune disease induced in animals. In this study the authors found that injection of thyroglobulin with CFA or IFA induced production of thyroglobulin-specific autoantibodies in rabbits; however, an actual autoimmune disease was induced only with CFA only.[19] In the present study we found that immunization with CFA or IFA induced specific pathogenic (β_2GPI-dependent) autoantibodies in genetic hypercoagulation-prone FVL mice. Interestingly, CFA-immunized FVL mice exhibited hyperactivity behavior which correlated with autoantibodies level. These results are in line with hyperactivity behavior displayed in experimental APS models.[16,17,20,21] Altogether, immunization of FVL mice with adjuvant induced an autoimmune response manifested by specific autoantibodies and a pathological behavior – we consider this to be an experimental model of ASIA syndrome.

Other models of experimental ASIA were described in this issue of *Lupus*. Injection of CFA to NZB/W × F1 lupus-prone mice caused acceleration of the autoimmune response, resulting in worsening of glomerulonephritis and shortening of survival time.[22] In another study, injection of tetanus toxoid after pretreatment with CFA or glycerol adjuvant induced autoantibodies and APS-related reproductive dysfunction in BALB/c mice but not in C57BL/6 mice.[23] Injection of pristane and other naturally occurring hydrocarbons was found to induce autoantibodies and eventually the clinical manifestations of systemic lupus erythematosus in BALB/c mice.[24]

Our results, demonstrating that aPL levels were elevated in FVL mice exclusively 5 months after immunization with adjuvant (a chronic effect), may indicate that expression of the FVL gene does not have an immediate stimulatory effect on the immune system. These results are similar to findings of a prospective cohort, which followed healthy volunteers who were immunized with recombinant DNA hepatitis B vaccine or influenza vaccination; in this study a progressive increase of aCL titers during 6 months was observed in a few subjects. Although there was no statistically significant production of aPL after vaccination with hepatitis B vaccine in healthy adults, a long-term aPL response in genetically predisposed individuals could not be excluded.[25–27] In another study, low rate of aCL positivity was demonstrated in healthy individuals 3 months after influenza vaccination.[28] Thus, we suggest that susceptible individuals (genetically prone) have an increased risk of developing a sustained autoimmune response, and subsequently produce autoantibodies after vaccination or exposure to adjuvant.

The specificity of the antibody response seems also significant, being directed at antigens specific for APS including β_2GPI-dependent aCL and anti-β_2GPI. Non-specific antibodies, such as β_2GPI-independent antiphospholipid antibodies, were not elevated. The most reasonable explanation for this late and prolonged elevation of aPL levels in FVL mice is an ongoing process such as the chronic exposure of the immune system to activated components of the clotting system, in particularly β_2GPI. In another related study we found an increase of aPL levels associated with behavioral and cognitive dysfunction in FVL mice immunized with β_2GPI (experimental APS, eAPS). These effects were linked to gene dosage and were thus significantly more pronounced in homozygous $FVL^{Q/Q}$ in comparison with heterozygous $FVL^{Q/+}$ mice.[13] We therefore propose the following hypothesis for the mechanism of specific generation of pathogenic aPL in the FVL mice: Autoantibodies

to coagulation factors and associated proteins are commonly described in patients with APS.[29,30] β_2GP1 itself is intimately associated with the coagulation process. In a situation such as FVL, in which there is a chronic uncontrolled increased activation of the coagulation system, the immune system is continuously exposed to antigens altered by and specifically associated with coagulation. A continued exposure may pose an immune burden, leading to breakdown of self-tolerance, and autoimmunity. This is analogous to antibodies generated by exposure to high levels of apoptotic cells generated in animals with deficiencies of clearance such as a complement deficiency and Fas deficiency, or in cancer, which are strongly associated with autoimmunity. This hypothesis suggests that the clinical association of FVL and APS is not merely a coincidence, but that chronic coagulation defects combined with external inflammatory stimuli analogous to adjuvant may induce aPL and also APS. We suggest therefore that chronic coagulation defects should be added to apoptotic cell clearance defects, cancer, and infection as significant factors leading to autoimmunity.[2,3,31] The hypothesis would explain the linkage of APS with FVL found in a familial study.[32] It would also predict that in humans the FVL genotype would be associated with higher levels of aPL and perhaps also APS.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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