Neurological Dysfunction Associated with Antiphospholipid Syndrome: Histopathological Brain Findings of Thrombotic Changes in a Mouse Model

LEA ZIPOREN a,b, SYLVIA POLAK-CHARCON c, D AMOS KORCZYNY a,d, IRIS GOLDBERG c, ARNON AFEK c, JURI KOPOLOVIC c, JOAB CHAPMAN b and YEHUDA SHOENFELD a,*,†

 a Research Unit of Autoimmune Diseases, Department of Medicine B, Sheba Medical Center, Tel-Hashomer 52621, Israel; b Departments of Neurology and Physiology & Pharmacology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv 69978, Israel; c Department of Pathology, Sheba Medical Center, Tel-Hashomer 52621, Israel; d Sieratzki Chair of Neurology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv 69978, Israel

The aim of this work was to study the pathological processes underlying neurological dysfunctions displayed by BALB/C mice induced with experimental antiphospholipid syndrome (APS), as we have previously reported. Experimental APS was induced in female BALB/C mice by immunization with a pathogenic monoclonal anticardiolipin (aCL) antibody, H-3 (n = 10), or an irrelevant immunoglobulin in controls (n = 10). Mice immunized with H-3 developed clinical and neurological manifestations of APS, including: embryo resorption, thrombocytopenia neurological defects and behavioral disturbances. In mouse sera, the titer of various autoantibodies were elevated, including: anti-phospholipids (aPLs), anti-β2 glycoprotein-I (β2GPI), anti-endothelial cell antibodies (AECA) and low titer of anti-dsDNA antibodies. Five months after APS induction, mice were sacrificed and brain tissue specimens were processed for hematoxylin and eosin (H&E), immunofluorescence staining and transmission electron microscopy (TEM). H&E staining of cortical tissue derived from all APS mice revealed mild inflammation, localized mainly in the meninges. Prominent IgG deposits in the large vessel walls and perivascular IgG leakage were observed by immunofluorescence. No large thrombi were observed in large vessels. However, EM evaluation of cerebral tissue revealed pathological changes in the microvessels. Thrombotic occlusion of capillaries in combination with mild inflammation was the main finding and may underlie the neurological defects displayed by mice with APS.

Keywords: Antiphospholipid syndrome; Autoantibodies; Mouse; Neurological manifestations; Thrombosis

Abbreviations: APS, antiphospholipid syndrome; SLE, systemic lupus erythematosus; CL, cardiolipin; PLs, phospholipids; β2GPI, beta-2-glycoprotein-I; AECA, anti-endothelial cell antibodies; EC, endothelial cells; HUVEC, Human umbilical vein endothelial cells; TEM, transmission electron microscopy; H&E, hematoxylin and eosin

INTRODUCTION

The antiphospholipid syndrome (APS) is an autoimmune disorder characterized by recurrent thrombotic (venous or arterial) events, repeated spontaneous abortions, and thrombocytopenia, in combination with persistently elevated titers of anti-phospholipid antibodies (aPL) or lupus anticoagulants (Hughes, 1993; Asherson et al., 1996; Wilson et al., 1999). It appears as a primary entity, or in the constellation of other autoimmune diseases, mainly systemic lupus erythematosus (SLE) (Asherson and Cervera, 1994; Asherson et al., 1996). A wide spectrum of other clinical manifestations with a multi-organ involvement has been reported in association with APS (Hojnik et al., 1996; Ziporen et al., 1996; Levy et al., 1997; Brey, 2000), including valvular heart disease, dermal, renal and neurological dysfunctions. The neurological impairments reported in association with APS (Brey, 2000) include: transient ischemic attacks (TIA’s) and strokes in young people (Levine et al., 1990; Ginsburg et al., 1992; Brey et al., 1993; Toubi et al., 1995), amaurosis fugax (Digre et al., 1989), dementia...
MATERIALS AND METHODS

Animals

Female BALB/c mice were obtained from the animal house in the Tel-Aviv University Medical School. The animals were raised under standard conditions, temperature 23 ± 1°C, 12:12 h light–dark cycle with ad-libitum access to food and water.

H-3 Monoclonal Antibody

H-3 is a natural human monoclonal IgM-aCL carrying a pathogenic idiotype which is common in patients with autoimmune diseases (Sutjita et al., 1989) and when injected to naive BALB/c female mice induces primary APS (Bakimer et al., 1992; Ziporen et al., 1997). The antibody was affinity purified from the supernatant of a hybridoma cell line, kindly donated by Dr M. Sutjita, from the Flinders Medical Center, South Australia (Sutjita et al., 1989). Human IgM was used as control (Jackson Immunoresearch Laboratories, West Grove, PA).

Endothelial Cell Cultures: Human Umbilical Vein Endothelial Cells (HUVEC)

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cord veins as described previously (Damianovich et al., 1996) and used for cyto-ELISA and for FACS analysis to detect anti-endothelial cells antibodies (AECA) in the mice serum.

Induction of Experimental APS in Mice by Active Immunization with ACL (H-3)

BALB/c mice (8–10 weeks old females, weighing 25–30 g) were immunized intradermally in the hind footpads with either 10 µg H-3 (n = 10), or normal human IgM (n = 10), emulsified in complete Freund’s adjuvant (CFA, Difco Laboratories Inc., Detroit, MI). A boost injection with 10 µg of the H-3, or normal IgM in phosphate buffer saline (PBS) was administered 3 weeks later.

Serological and Clinical Evaluation of the Mice

Mice were bled from the retro-orbital plexus at one-month intervals. Blood cell counts were conducted using a single optical cytometer (HC Plus Cell Control; Coulter Electronics) for determination of thrombocytopenia. Sera were separated and tested by ELISA for the presence of autoantibodies.

ELISA

Serum samples were tested at 1:100 dilution for anticardiolipin (aCL), anti-phosphatidylserine (aPS), anti-phosphatidylserine (aPI), anti-phosphatydicholone (aPC), anti-β2GPI, anti-dsDNA, anti-IgM and AECA.

Ninety six well polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated with cardiolipin (CL), phosphatidylserine (PS), phosphatidylserine (PI), (or empty wells) at 50 µg/ml in ethanol, or with phosphatidyicholone (PC) in methanol/chloroform, then left open at 4°C until evaporation. For anti-human IgM detection, microtiter plates were coated with 2 µg/ml human IgM in 0.05 M NaHCO₃ buffer, pH 9.5, for overnight and washed three times with 0.05%TBS-Tween.
For the detection of anti-dsDNA, microtiter plates were coated with poly-L-lysine at 50 μg/ml in ddH2O for 30 min at RT, washed × 1 with TBS-Tween 0.1% and × 2 with TBS. Then, the wells were coated with calf thymus DNA at 2.5 μg/ml in TE for 2 h at RT, washed and neutralized with poly-L-glutamic acid at 50 μg/ml in TBS, for 1 h at 37°C.

Five percent bovine serum (BS) in tris buffer saline (TBS) was used as blocking agent, and 2% BS in TBS, as sample diluent. Washes were performed with TBS. The antibody binding was quantitated using alkaline phosphatase-conjugated goat anti-mouse IgG or IgM (Jackson Immunoresearch Laboratories, West Grove, PA), followed by p-nitrophenylphosphate substrate. Optical density (OD) was read at 405 nm.

For the detection of antibodies to β2 glycoprotein-I (β2GPI), microtiter plates were coated with 2.5 μg/ml purified human β2GPI (kindly donated by Dr A. Tincani, Brescia, Italy) in 0.05 M NaHCO3, pH 9.5 for 16–18 h at 4°C, followed by three washes with PBS-0.05% Tween 20. For blocking and sample dilution, 1% BSA-PBS-0.05% Tween 20 was employed.

Anti-endothelial Cell Activity

Anti-endothelial cell activity in the animal sera was measured in cyto-ELISA with unfixed HUVEC, according to a previously described protocol (Damianovich et al., 1996) in microtiter plates coated with confluent HUVEC endothelial cells. Briefly, HUVEC cells were seeded in gelatin coated 96-well microtiter plates (Nunclon) at 2.5 × 104 cells/well, and were allowed to grow to confluence for 1 or 2 days. Cells were washed with Hank’s balanced salt solution (HBSS) and incubated with a blocking buffer (HBSS/0.5% BSA) for 30 min at 37°C to prevent non-specific binding. After an additional wash cells were exposed to the tested sera or control antibody diluted in HBSS/10% FCS (dilution 1:25–1:200 of serum) for 60 min at room temperature. Cells were washed again and incubated with a second antibody, alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson Immuno Research Laboratories) followed by p-nitrophenyl phosphate disodium (pNPP, Sigma, St. Louis, MO) as a substrate. The OD was read at 405 nm by ELISA plate-reader (EAR 400 AT, SLT-Labinstruments, Austria).

Flow Cytometric (FACS) Analysis of Mouse Serum Binding to HUVEC

To detect AECA antibodies, serum pools of APS mice or controls were assessed for binding to EC by FACS. HUVEC cells (5 × 105) in PBS-BSA 2% were incubated with the mice pooled serum in 1:25 dilution (total volume 100 ml) for 45 min in 4°C. The cells were washed twice in PBS-BSA 2% and incubated with FITC-conjugated goat anti-mouse IgG (Sigma) for 30 min in 4°C in the dark. After 3 washes with washing buffer, the cells were filtered by a nylon-mesh filter and counted in a flow cytometer (Coulter, Hialeah, FL) equipped with a 500 mW argon laser and a helium–neon laser; 105 cells were tested in each experiment. The percentage of cells stained was determined.

Histological and Immunohistochemical Evaluation of Brain Tissue Specimens

Perfusion and Tissue Preparation

Mice were deeply anaesthetized with sodium pentobarbitone (Nembutal, Abbot, France), 50 mg/kg body weight, intra-peritoneal. A transcardiac perfusion was conducted with 100 ml physiologic, followed by a 10 ml mixture of 1% paraformaldehyde and 10 ml of 1.25% glutaraldehyde in PBS. A specimen from the frontal cortex of each brain was immersed in 2.5% glutaraldehyde for evaluation by electron microscopy (EM). One hemisphere of each brain was snap frozen in liquid nitrogen for immunofluorescence study, and the other hemisphere was dispersed in 4% formaldehyde buffer for hematoxylin and eosin (H&E) staining.

Immunofluorescence Studies for the Detection of Mouse IgG Deposits

Frozen brain tissue specimens derived from 10 APS mice and 10 control mice were transversely cut into 10 μm thick sections and placed on poly-L-lysine coated slides. Sections were stained with H&E and evaluated by direct immunofluorescence, as described previously (Hojnik et al., 1996). Briefly, slides were dried under airstream for 45 min, fixed in acetone for 5 min at −20°C, and rinsed three times for 5 min in BSA-TBS-Tween. FITC-conjugated primary antibody goat anti-mouse IgG (Sigma), or FITC-conjugated normal goat IgG as control, diluted in TBS, were applied for 30 min at room temperature (RT). The slides were rinsed three times for 5 min in BSA-TBS-Tween, stained with Evans blue, transferred to 95% ethanol for 2 min, dried and mounted with Entellan (Merck, Darmstadt, Germany), and examined under a fluorescence microscope (Olympus Vanox AH3).

Transmission Electron Microscopy (TEM)

Brain samples from APS and control mice were processed for TEM. Specimens (2 × 2 mm3) of brain tissue were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 h at RT. After several washings with the same buffer, the tissues were post-fixed with 2% OsO4 in cacodylate buffer for 2 h, washed, and dehydrated in a graded ethanol solutions (30, 50, 75, 96, 100%) at RT. The specimens were transferred to propylene oxide and subsequently embedded in Epon embedding mixture (Bio-Rad) at 55–65°C for 3 days in a flat mold. Thin sections of 60 nm were obtained with a Reichert ultra

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RESULTS

Serological Markers and In Vivo Assessment of Mice

Mice were evaluated for clinical and serological manifestations of APS (Ziporen et al., 1997). As previously published (Bakimer et al., 1992; Ziporen et al., 1997), all the mice that had been immunized with H-3 antibody demonstrated elevated titers of circulating aPLs, including anti-CL, anti-PS and anti-PE antibodies, anti-β2-GPI antibodies, AECA, and anti-dsDNA, measured by specific ELISA assays (Bakimer et al., 1992; Ziporen et al., 1997). Control mice developed only anti-human IgM (hIgM). Results of antibody levels at the time point the mice were sacrificed, 5 months post immunization with aCL, are summarized in Table I. Other manifestations of APS were also found. Thrombocytopenia was detected in the APS mice (Bakimer et al., 1992; Ziporen et al., 1997). Number of platelets (average no. ± SD) was 653 ± 179 × 10³/μl in APS mice, compared to 1076 ± 217 × 10³/μl in the control mice, p < 0.05. Fetuses resorption were measured, as previously described (Bakimer et al., 1992; Ziporen et al., 1997), 12/32 resorptions were detected in APS mice, compared to 5/30 in the controls, p < 0.05. Behavioral and neurological tests were conducted on all the mice, demonstrating behavioral changes in the APS mice including hyperactive behavior, defected performance of neurological reflexes and motor incoordination, as previously described (Ziporen et al., 1997).

Flow Cytometric Analysis Findings

To assess specific binding activity to endothelial cells (EC), in addition to ELISA test, we performed flow cytometric analysis with HUVEC cells. Pooled serum from 10 APS mice was found to bind 89% of HUVEC cells tested (Fig. 1A), compared to 27% binding activity of pooled sera from 10 control mice, as shown in Fig. 1B. These results indicate that a specific binding reactivity to

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**TABLE I** Antibodies level (average ± SD) in the serum of APS mice and controls, as tested in ELISA (1:100 dilution), 5 months after the induction of APS

| Mouse group | CL (O.D.405) | PS (O.D.405) | PI (O.D.405) | PC (O.D.405) | AECA (O.D.405) | dsDNA (O.D.405) | β2-GPI (O.D.405) | hIgM (O.D.405) |
|-------------|--------------|--------------|--------------|--------------|----------------|----------------|----------------|-------------|
| APS         | 1.3 ± 0.2    | 1.4 ± 0.15   | 1.2 ± 0.19   | 0.3 ± 0.04   | 1.05 ± 0.18    | 0.35 ± 0.05    | 1.2 ± 0.23      | 1.1 ± 0.09 |
| Control     | 0.13 ± 0.02  | 0.2 ± 0.025  | 0.1 ± 0.02   | 0.15 ± 0.01  | 0.15 ± 0.03    | 0.12 ± 0.02    | 0.25 ± 0.03     | 1.15 ± 0.02 |

CL: cardiolipin, PS: phosphatidylserine, PI: phosphatidylinositol, PC: phosphatidylcholine, AECA: anti-endothelial cell antibodies, dsDNA: double stranded DNA, β2-GPI: β2-glycoprotein-I, hIgM: human IgM antibodies.
EC is present in the sera of APS mice, compared to controls. These are either specific AECA that bind EC (Bordron et al., 1998), or are aPL antibodies that crossreact with ECs through β2GPI attached to EC (Del Papa et al., 1995).

**Pathological Findings**

**Histological and Immunofluorescence Findings**

**H&E Staining**

To observe gross pathological changes in cortical specimens, H&E staining was performed on specimens derived from the cortex of APS and control mice. Mild inflammatory reactions were observed in 4 out of 10 cortical specimens of APS mice. Mononuclear infiltrates were demonstrated in the meninges and subependymal areas. In cortical specimens derived from control mice no such infiltrates were discerned (Fig. 2A, B). By this staining, no thrombus was observed in the brain tissue specimens.

**Immunofluorescence Staining**

To show immune reactants deposited in the cerebral tissue, immunofluorescence staining was performed using FITC-conjugated anti-mouse IgG. In APS mice, prominent deposits of mouse IgG were shown in large and small cerebral blood vessel walls along the endothelial lining (Fig. 3A, B). Mild perivascular leakage of IgG was also observed in the vicinity of affected blood vessels (Fig. 3A, B). No such staining was observed in the control mice brain.

**FIGURE 2** H&E staining of a representative brain specimen derived from APS mouse immunized with H-3- monoclonal aCL. (A) Subependymal infiltration of mononuclear cells is observed (arrow). (B) Infiltration of mononuclear cells is also seen in the meninges. (A,B) Original magnification, ×200.
TEM Findings

To analyze further the changes in the cortical tissue of APS mice, TEM examination was conducted. Vasculopathy of the cortical vessels was found in specimens derived from 4 out of 10 APS mice immunized with H-3 aCL (Ziporen et al., 1997). In cortical specimens of APS mice, up to 35% of capillaries shown in the tested field were found contracted, occluded by microthrombi and/or swollen EC, compared to 5% contracted capillaries in the control specimens, correlating with significant neurological and behavioral dysfunction, as previously reported (Ziporen et al., 1997). As shown in Fig. 4, in the brain of APS mice the capillaries were found contracted and occluded by swollen EC leading to the formation of slit-like lumens (Fig. 4A, B), compared to wide-open lumens in most of control vessels. Aggregation of activated platelets with cytoplasmic microvesicles and the formation of platelet microthrombi were also observed in the capillaries of APS specimens (Fig. 4C). Activated macrophages were shown in the vicinity of occluded vessels (Fig. 4D). Extravasated red blood cells were seen (Fig. 4E) in areas in which the parenchyma next to the occluded capillaries showed ischemic damage (Fig. 4F).

DISCUSSION

An experimental model for APS has been previously established in our laboratory (Bakimer et al., 1992; Z. LEA et al. 72).
Ziporen et al., 1997), by immunizing BALB/C mice with monoclonal pathogenic aCL (H-3), carrying a pathogenic idiotype (H-3). The mice developed several clinical manifestations characteristic of the human disorder, such as thrombocytopenia, fetal loss and elevated levels of autoantibodies, including aPL, AECA and anti-β2GPI antibodies (Bakimer et al., 1992; Ziporen et al., 1997). Recently, we have shown that neurological dysfunction and hyperactive behavior are also associated with experimental APS, supporting the concept that neurological involvement is one of the manifestations of APS (Coull et al., 1987; Leach et al., 1989; Lie, 1989; Levine et al., 1990; Westerman et al., 1992; Hugston et al., 1993; Lie, 1994), we aimed to investigate this unresolved question utilizing our experimental model for primary APS in mice.

Some evidence exists in the literature regarding histopathological processes associated with experimental APS that is secondary to lupus in lupus-prone mice (Kier, 1990; Smith et al., 1990; Brey and Teale, 1992; Hess et al., 1993; Aron et al., 1995), including the MRL/lpr, its congenic strain MRL++ mice immunized with β2GPI (Smith et al., 1990; Brey and Teale, 1992; Hess et al., 1993; Aron et al., 1995), and NZB/NZW F1 mice (Kier, 1990). In these mice neurological defects and cognitive impairment correlated with the finding of perivascular lymphocytic infiltrates in the choroid plexus, or around cerebral and hippocampal blood vessels and occlusive vasculopathy (Kier, 1990; Smith et al., 1990; Brey and Teale, 1992; Hess et al., 1993; Aron et al., 1995). However, the correlation with aCL was not elucidated in these mice models of secondary APS.
In the current study we have extended histopathological assessment to brain tissue of naive BALB/C mice, not genetically prone to develop an autoimmune disease, that develop primary APS and neurological defects upon induction with pathogenic idotype (Bakimer et al., 1992; Ziporen et al., 1997).

Experimental APS was generated in female BALB/C mice that developed elevated levels of autoantibodies, including aPLs, anti-β2GPI and AECA antibodies and low levels of a dsDNA, as tested by ELISA (Table I). Prominent binding of mice serum to HUVEC endothelial cells was further confirmed by FACS analysis (Fig. 1). Clinical manifestations of APS were confirmed, as previously reported, and significant neurological defects were demonstrated including hyperactive behavior, impaired neurological reflexes, and motor incoordination (Ziporen et al., 1997).

The neurological deficits displayed by APS mice were found related mainly to the cortical level (Ziporen et al., 1997). Therefore, we focused our attention on pathological changes in the cortex of APS, compared to control mice by a series of histopathological and ultrastructural analyses.

H&E staining of cortical specimens derived from APS mice revealed mainly mild inflammation, shown as small infiltrates of lymphocytes localized in the meninges and in subependymal areas (Fig. 2A, B). No wall thickening in the capillaries was evident as aggregates in the cerebral tissue and interfere with functional synaptoneurosomes, "Antiphospholipid antibodies permeabilize and depolarize brain synaptoneurosomes", Stroke 18, 1107–1112.

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