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# The putative role of cytokines in the induction of primary anti-phospholipid syndrome in mice

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## SUMMARY

Antiphospholipid syndrome (APLS) is characterized by thrombocytopenia, thromboembolic phenomena and recurrent fetal loss, associated with anti-cardiolipin antibodies (ACA) and/or lupus anticoagulant. The syndrome may be primary or may be associated with other conditions such as systemic lupus erythematosus (SLE). In this study we induced primary APLS following immunization of BALB/c mice with a human monoclonal ACA (H-3). Analysis of the cytokine profile of the mice with experimental APLS indicated low production of IL-2, IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) by concanavalin A (Con A)-stimulated splenocytes of H-3 immunized mice. It seems that the low levels of IL-3 and GM-CSF have a potential role in the fetal loss of the APLS. Whatever the mechanism of IL-3 and GM-CSF in preventing fetal loss, these results may have therapeutic bearing on the reproductive outcome in women and other species with APLS.

**Keywords** anti-phospholipid syndrome cytokines lymphokines IL-2 IL-3 GM-CSF anti-cardiolipin antibodies autoimmunity autoantibodies

## INTRODUCTION

Primary anti-phospholipid syndrome (PAPS) is characterized by the presence of anti-cardiolipin antibodies or other anti-phospholipid antibodies and/or lupus anticoagulant, thrombocytopenia, recurrent thromboembolic phenomena, recurrent fetal loss and other diverse manifestations [1–5]. Recently, we have shown the pathogenetic role of the anti-cardiolipin antibodies (ACA) [6], by inducing an experimental model of anti-phospholipid syndrome (APLS) in naive mice following passive transfer of human and mouse monoclonal and polyclonal ACA to the tail vein of BALB/c and ICR mice [6] and following active immunization with a human monoclonal ACA(H-3) [7]. The APLS was exemplified by serological markers (ACA, prolonged activated partial thromboplastin time (APTT)), haematological findings (thrombocytopenia) and clinical manifestations (recurrent fetal resorptions—the equivalent of human fetal loss and smaller fetuses and placentae).

Cytokine studies of autoimmune conditions pointed to basically common profiles, namely, a decreased production rate of IL-2, and defects in peripheral blood lymphocyte response to phytohaemagglutinin (PHA) or concanavalin A (Con A) [8]. Conversely, in most autoimmune conditions, an increased level of serum IL-2 receptors was reported [9]. Less consistent results on the production of other cytokines in autoimmune states have been reported [10–16]. Cytokines were also found to have numerous roles in normal and pathologic processes of reproduction and pregnancy [17]. Interferon-gamma (IFN-$\gamma$), tumour necrosis factor (TNF), IL-1 and IL-2 were found to enhance the number of resorptions in the CBA × DBA/2 mouse combination, while granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 were found to decrease resorption rate and correct placental size and fetal weight [18,19].

The aim of this study was to report on the cytokine profile (IL-2, IL-3, GM-CSF) in PAPS-induced mice by active immunization with a human ACA (H-3) [7,20]. The model is analogous to our experiments on the induction of systemic lupus erythematosus (SLE) in mice using human anti-DNA antibody in which low levels of IL-2 production were found [21–23]. Since IL-2 was found to be a 'negative' regulator in pregnancy while GM-CSF and IL-3 were defined as 'positive' ones, we wanted to find out whether changes in the cytokine profile could explain the pathogenic mechanisms of the recurrent fetal loss observed in the syndrome.

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## MATERIALS AND METHODS

*Mice*

BALB/c mice (8–10-week-old females) were purchased from the Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel.

*Antibodies*

H-3 (a kind donation of A. Hohmman, Flinder Medical Centre, South Australia) is a human monoclonal IgM [20], generated following fusion of peripheral blood lymphocytes of a healthy subject immunized with diphtheria and tetanus, with GM 4672 lymphoblastoid cell line [20,21]. The antibody binds to tetanus and diphtheria toxoids, cardiolipin, phosphatidylethanolamine and phosphatidylserine [20]. As control we used an irrelevant human IgM.

*Induction of PAPS*

BALB/c mice were immunized intradermally in the hind footpads [7,22] with 1 $\mu$g of either H-3 MoAb ($n=43$) or human IgM ($n=50$) in Freund's complete adjuvant (FCA) (Difco). Three weeks later, boost injections were administered with the same amount of antibodies in isotonic PBS in the hind footpads.

*Detection of anti-cardiolipin antibodies*

Anti-cardiolipin activity in the sera of the immunized mice was detected by ELISA as follows: 96-well ELISA plates (NUNC-immunol) were coated with cardiolipin (Sigma) at a concentration of 50 $\mu$g/ml in ethanol. The plate was left open to the air at 4°C until evaporation. Following blocking of any remaining blockable sites with PBS and 5% bovine serum (PBS-BS), serial dilutions (1:200–1:3600) of the mice sera in PBS plus 2% BS were incubated for 2 h. Excess sera were washed three times with PBS. Bound antibodies were detected using 1:1000 dilution of goat anti-mouse immunoglobulin conjugated to alkaline phosphatase (Sigma) and the addition of its substrate *p*-nitro-phenyl-phosphate. Colour was read in a Titertek ELISA reader at 405 nm.

*Detection of anti-ds-DNA*

Anti-ds-DNA antibodies were determined according to Shoenfeld *et al.* [21]. Briefly, polystyrene plates with 96 flat-bottomed wells (Nunc) were coated sequentially with poly-L-lysine (50 $\mu$g/ml in water), dsDNA-2·5 $\mu$g/ml TBS (Tris Base Buffered Saline) and poly-L-glutamate (50 $\mu$g/ml). Washing between steps was performed using TBS with 0·05% Tween-20, to minimize non-specific binding. The detection of the autoantibodies in the sera was done as described for anti-cardiolipin.

*Detection of lupus anticoagulant*

The presence of lupus anticoagulant was evaluated in two ways: (i) the prolongation of APTT in a mixing test [5], adding one volume of plasma (whole blood mixed with Na-citrate 0·13 mol/*l*, in a 9:1 ratio), to one volume of actin and incubating for 2 min at 37°C. Another volume of 0·02 M $CaCl_2$ was added and the clotting time was recorded in seconds; (ii) prolongation of clotting time in the presence of Kaolin [5]. The procedure is identical to the one used in APTT, except for the use of Kaolin (2 mg/ml in PBS).

*Evaluation of pregnancy outcome*

The number of vaginal plugs (indicating mating), the number of pregnancies (indicating fecundity) and the number of live embryos per successful pregnancy were studied.

*Cytokine production*

Spleen cells ($2\times10^6$) were suspended in enriched RPMI 1640 medium with 10% fetal calf serum (FCS) (GIBCO, MA) and supplemented with 2 $\mu$g/ml of Con A (Miles Yeda, Israel). This number of cells and Con A level were chosen following experiments carried out with various numbers of cells and mitogen concentrations. With these conditions we achieved maximal cytokine production. Spleen cell cultures were incubated for 48 h. At the end of the incubation period, 95% of the cells in the culture were viable. Supernatants were collected and assayed for IL-2, IL-3 and GM-CSF.

*IL-2 and IL-3 assays*

The assays for IL-2 and IL-3 activity were based on the induction of the CTLL and 32Dcl-23 cell line proliferation respectively [23,24]. Samples for the assay of IL-2 and IL-3 were diluted in 96-well microtitre plates. 32Dcl-23 cells ($1\times10^4$) or $5\times10^4$ CTLL cells were added to each well to a final volume of 0·2 ml RPMI 1640 containing 10% FCS. All cultures were incubated for 48 h at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air. Each well was pulsed with 1 $\mu$Ci $^3$H-thymidine (New England Nuclear, Boston, MA) for the final 20 h of culture. The pulsed cells were harvested and the amount of $^3$H-thymidine uptake was measured in a LKB liquid scintillation counter. One unit of IL-2 or IL-3 activity was defined as the reciprocal log 2 dilution required to give 50% of the maximal proliferation of murine recombinant IL-2 or murine recombinant IL-3 (Genzyme, Boston, MA).

*GM-CSF activity assay*

GM-CSF was quantified by determining the number of colonies that developed from C57BL/6J mouse bone marrow cells cloned in the presence of the spleen supernatant fraction to be tested. The soft agar technique described by Pluznik & Sachs [25] was used to clone bone marrow cells. Briefly, supernatants containing CSF were aliquoted in 10% volume in a 35-mm Petri dish. A total of $10^5$ bone marrow cells in 1 ml of soft agar medium (0·3%) was cloned. After 7 days of incubation at 37°C in a humidified atmosphere of 5% $CO_2$ in air, the number of colonies that had grown in the soft agar was scored.

*Statistical analysis*

Statistical analysis was performed by Student's *t*-test.

## RESULTS

Two-month-old female BALB/c mice ($n=43$) were immunized with the human IgM anti-cardiolipin H-3 MoAb as previously detailed by us [7]. One month following the booster injection, high titres of anti-cardiolipin antibodies could be detected in the sera. The control group which was immunized with human IgM exhibited low anti-cardiolipin activity.

Figure 1 represents the titres of ACA, anti-dsDNA and anti-H-3 antibodies in the immunized mice with H-3 antibody. The ACA was found to be inhibited by its binding to cardiolipin only by cardiolipin (Fig. 2a), while the anti-DNA antibody was

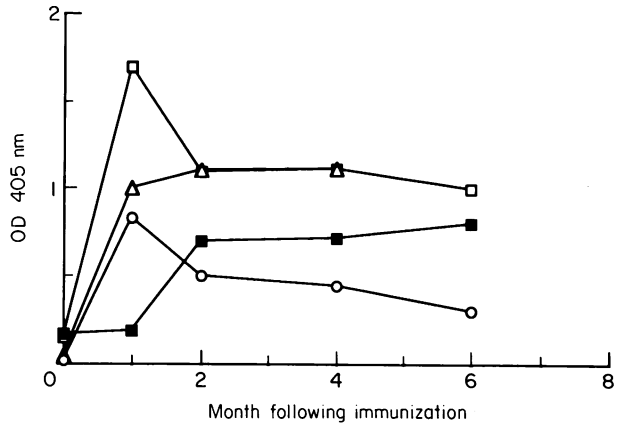


**Fig. 1.** Titres of anti-cardiolipin, anti-DNA and anti-H-3 and anti-human IgM antibodies in sera of mice immunized with H-3 human anti-cardiolipin MoAb. □, Anti-cardiolipin; ■, anti-DNA; △, anti-H-3; ○, anti-IgM.

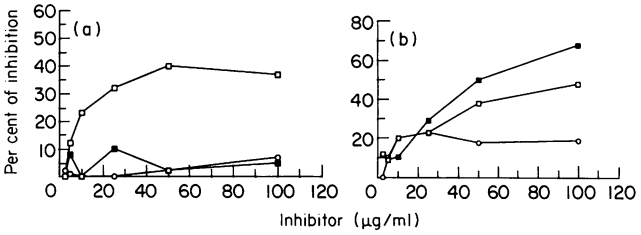


**Fig. 2.** (a) Inhibition assay of anti-cardiolipin antibody (ACA) of mice serum immunized with H-3. (b) Similar study of inhibition of the anti-DNA antibody. ACA was competed with only by cardiolipin, while the anti-DNA antibodies were found to be polyspecific and to be inhibited by both cardiolipin and DNA. ■, ds-DNA; □, cardiolipin; ○, bovine serum albumin.

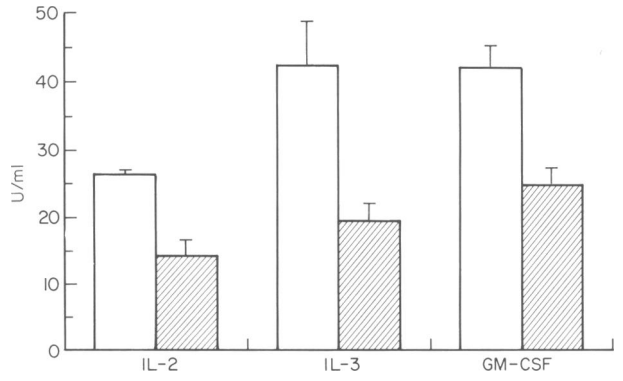


**Fig. 3.** IL-2, IL-3 and GM-CSF secretion *in vitro* by spleen cells stimulated with 2 μg/ml concanavalin A (Con A). Representative mean ± s.d. of 17 controls and 13 H-3-injected mice are shown. Since GM-CSF was determined as colonies/ml, for graphic purposes each colony was defined as 1 unit. □, Control; ▨, experiment.

found to be polyspecific, and its relation with DNA was competed for by prior incubation with both DNA and cardiolipin (Fig. 2b), pointing to its polyspecific characteristics. In concordance with the high titres of anti-cardiolipin antibodies in the sera, prolongation of the APTT was noted (57 ± 11 s), in comparison with the normal APTT measured in the plasma of mice immunized with the control IgM (30 ± 4 s). Similar results were obtained in the Kaolin clotting time test where plasma from mice immunized with H-3 has been clotted after 121 ± 6 s in comparison with 75 ± 3 s observed in plasma of mice immunized with IgM.

A lower fecundity rate was observed in the females immunized with H-3 (21% *versus* 48%, $P < 0.05$), although there was no decrease in the number of vaginal plugs (indicating pregnancy). A high percentage of resorptions could be found in animals immunized with H-3 (25 ± 13 *versus* 3 ± 5).

Previously [7] we have detailed the rest of the clinical findings (e.g. fecundity rate: 21% *versus* 48% in controls; number of live embryos/pregnancy 4·8 ± 2·3 *versus* 8 ± 2).

Figure 3 shows that IL-2 and IL-3 production by splenocytes was significantly decreased ($P < 0.001$) in the H-3-injected animals in comparison with the control group. A marked decrease in GM-CSF secretion by spleen cells of H-3-injected mice was noted. GM-CSF secretion decreased from a mean (± s.d.) of 42·2 ± 2·9 in the control group to 24·7 ± 2·6 colonies/$10^5$ bone marrow cells in the H-3-treated animals ($P < 0.001$).

## DISCUSSION

In previous studies [6,7] we were able to confirm the pathogenicity of the anticardiolipin antibodies. Passive transfer of purified serum anticardiolipin and MoAbs [6] and active immunization with a human monoclonal ACA (H-3) [7] led to the induction of the APLS in mice exemplified by recurrent fetal loss, thrombocytopenia and the presence of the serological markers.

Various explanations and theories were offered to solve the enigma of recurrent thromboembolic phenomena in APLS in the presence of thrombocytopenia and lupus anticoagulant [1–5]. In the present study we attempt to describe a novel aspect of APLS which may explain at least the recurrent fetal loss frequently reported in women with ACA. In this study we measured *in vitro* cytokine production by spleen cells derived from immunized mice in order to explore the functional activity of these cells *in vivo*. Earlier studies on cytokines revealed that there is a correlation between *in vitro* cytokine production and the *in vivo* situation. The classic example is the low IL-2 production by cancer patients which can be modulated by recombinant IL-2 administration [26]. In the present study we detected low production levels of IL-2, IL-3 and GM-CSF by splenocytes of mice with active experimental APLS in comparison with control mice immunized with human IgM. The immunization with human IgM as control was carried out in order to exclude the possibility that the immunization process itself could induce these alterations. This low cytokine production was shown by incubating splenocytes of the immunized mice in the presence of the mitogen Con A. In almost all classical autoimmune conditions, low production, low levels and rates of response to IL-2 were reported [16]. In parallel, high concentrations of soluble IL-2 receptors in the serum were described [9].

In contrast, the low IL-3 and GM-CSF levels may have a cardinal role in the induction of fetal resorption. Earlier evidence indicates that trophoblast-like cells might be responsible for the production of GM-CSF [27]. GM-CSF has the ability to prevent spontaneous fetal resorption when injected intraperitoneally into pregnant mice. In addition it leads to an increase in placental and fetal size [28]. Recent work has suggested that GM-CSF may have autocrine effects within the placenta. It is released by JEG, JAR and BEWO, three different cell lines of human choriocarcinoma origin. The addition of GM-CSF to cultures of placental cytotrophoblasts from human full-term pregnancies stimulates both differentiation of syncytium and release of placental lactogen and chorionic gonadotrophin [17].

In this study we present new data to support this notion: it is not only the GM-CSF secreted by the placenta which is important for the preservation of the embryo and the normal pregnancy, but most probably, the cytokine produced by the splenocytes that contributes to the normal pregnancy. Though this confirms the results obtained in other studies on strains with spontaneous high resorption rate [28], it remains to be confirmed that injection of the missing cytokine may prevent the fetal loss. Such a confirmation may offer new therapeutic possibilities to young women suffering from APLS.

In addition to the low production of GM-CSF we found a low ability of splenocytes to generate IL-3 (Fig. 1). IL-3 is a multifunctional factor possessing a wide range of biological activities including the stimulation of stem cell differentiation into committed cells of the erythrocyte, granulocyte and megakaryocyte cell lineage [29]. Like GM-CSF, IL-3 was shown recently by Chaouat *et al.* [28] to increase the chances of fetal survival when injected into abortion-prone mice (CBA/J × DBA/2) reducing resorption rates from 52% to 22%. Furthermore, both IL-3 and GM-CSF were shown by the same authors to increase fetal and placental size and in particular to expand the spongiotrophoblast zone in the placenta. This may be due to a direct trophic influence on placental cells or to an indirect effect of natural killer-like cells or both. Moreover, IL-3 is capable of promoting T suppressor cell proliferation and down-regulation of LAK cell activity [29,30]. Since both IL-3 and GM-CSF are capable of inducing megakaryocyte differentiation [31], the thrombocytopenia observed in the present study may be attributed to decrease in the levels of these cytokines.

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