行政院國家科學委員會專題研究計畫 成果報告

利用動物及細胞模式探討人類微小病毒B19結構蛋白獨立區域誘發自體抗體及自體免疫疾病產生的免疫機轉之研究
研究成果報告(精簡版)

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行政院國家科學委員會補助專題研究計畫 ■成果報告 □期中進度報告

利用動物及細胞模式探討人類微小病毒 B19 結構蛋白獨立區域誘發自體抗體及自體免疫疾病產生的免疫機轉之研究

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計畫主持人：徐再靜

共同主持人：曾博修

計畫參與人員：蔡鈞州 施靖瑜 張孟君

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

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□赴國外出差或研習心得報告一份

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■出席國際學術會議心得報告及發表之論文各一份

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執行單位：中山醫學大學免疫學研究所

中　華　民　國　97　年　9　月　10　日

中文摘要

在我們最近發表的研究結果顯示急性感染人類微小病毒 B19 (Human Parvovirus B19, B19)的病人血清中之抗牛心脂抗體和抗 β2GPI 抗體(抗磷脂質抗體)的表現可能和 B19 結構蛋白獨立區域(VP1u)有關。因此推測 VP1u 在 B19 感染、自體免疫和自體抗體、抗磷脂質抗體產生之間可能扮演著相當重要的角色。而為了再更深入了解 VP1u 引發自體免疫之相關機轉，我們利用抗 VP1u 兔子多株抗體免疫注射老鼠並分析及評估免疫後的老鼠之血清抗磷脂質抗體及血液抗磷脂質症候群之表現。此外更分析抗 VP1u 兔子多株抗體對人類內皮細胞之影響，以了解 VP1u 可能誘發自體免疫的免疫機轉。結果發現免疫注射 VP1u 抗原之兔子所產生的抗 VP1u 多株抗體會辨識牛心脂、β2GPI 及磷脂質等抗原。更進一步也發現免疫注射純化的抗 VP1u 兔子 IgG 抗體之老鼠會造成血小板減少、凝血時間延長及抗 β2GPI 抗體及抗磷脂質抗體的產生。此外，純化的抗 VP1u 兔子 IgG 抗體也能促進血管內皮細胞的細胞表面分子 ICAM-1 (CD54), VCAM-1 (CD106), E-selectin (CD62E), MHC class II (HLA-DR, DP, DQ)的表現及細胞激素 TNF-α 的產生。此外，也發現分別給予人類 anti-β2GPI IgG 和抗 VP1u 兔子 IgG 抗體刺激的人類血管內皮細胞 ECV-304 其 phosphorylated-P38 mitogen-activated protein kinase (P38 MAPK)和 iNOS 蛋白的表現均有明顯增加。這些結果將提供一些線索在抗 B19-VP1u 抗體與抗磷脂質症候群的相關研究。

關鍵詞: 人類微小病毒 B19、結構蛋白獨立區域 VP1u、自體抗體

Abstract

Human parvovirus B19 infection has been frequently described as a cause or trigger of various autoimmune diseases. In previous studies, we have postulated the association among human parvovirus B19 (B19)-VP1 unique region (VP1u), production of anti-beta2-glycoprotein I (anti-β2GPI) antibody and anti-phospholipid syndrome (APS)-like autoimmunity. However, the precise role of B19-VP1u in induction of APS is still obscure. In this study, autoantibodies against CL, β2GPI, and phospholipid (PhL) in sera from patients with B19 infection, were cross-reactive with B19-VP1u. Consistently, sera from rabbits immunized with recombinant B19-VP1u protein displayed raised detectable immunoglobulins against B19-VP1u, CL, β2GPI and PhL. Additionally, the mice immunized with anti-B19-VP1u IgG developed thrombocytopenia, prolongation of aPTT, and autoantibody against β2GPI and PhL. Moreover, purified IgG from rabbits immunized with recombinant B19-VP1u proteins can up-regulate ICAM-1 (CD54), VCAM-1 (CD106), E-selectin (CD62E), MHC class II (HLA-DR, DP, DQ) molecule expression, and TNF-α production in endothelial cells as compared to those endothelial cells cultured with control IgG. Additionally, significantly increased phosphorylated-P38 mitogen-activated protein kinase (P38 MAPK) and iNOS were observed in both human anti-β2GPI IgG and rabbit anti-B19-VP1u IgG treated-ECV-304 cells, respectively. These experimental results imply that antibodies against B19-VP1u play important roles in the immunopathological processes as well as human anti-β2GPI IgG that leads to development of APS by involving p38 phosphorylation and iNOS activation. It could

provide a clue in understanding the role of anti-B19-VP1u antibodies in APS manifestations.

Key words: Human Parvovirus B19 (B19), VP1 unique region protein (VP1u), autoantibodies

Introduction

B19 infection has been associated with the production of various autoantibodies, including anti-neutrophil cytoplasmic antibody, anti-phospholipid antibody (APhL), and anti-cardiolipin autoantibody (aCL) as well as various autoimmune diseases [1-6]. Notably, a remarkable similarity exists in the specificity of APhL between patients with B19 infection or SLE has been observed [1-3,5], and the anti-phospholipid syndrome (APS) has been hypothesized to have an infectious origin [7]. In our previous studies, we have postulated the association among human parvovirus B19 (B19)-VP1 unique region (VP1u), production of anti-beta2-glycoprotein I (anti-β2GPI) antibody and anti-phospholipid syndrome (APS)-like autoimmunity [8]. However, the relationship among B19 infection, APhL, and APS manifestation remains unclear. In this study, the binding activity of specific antibody with B19-VP1u is investigated. The relationship between B19-VP1u and autoantibody production was also examined by using immunized rabbits with purified recombinant B19-VP1u protein, and absorption experiments were performed to clarify the specificity of the raised antibodies in rabbits. Moreover, induction of passive APS by anti-B19-VP1u IgG antibodies and the effect of antibody against B19-VP1u on vascular endothelial cells were also evaluated.

Materials and Methods

Mice and induction of experimental APS by passive transfer

BALB/c female mice at age of 6 weeks were purchased from National Laboratory Animal Center, Taiwan and housed under supervision of the Institutional Animal Care and Use Committee at Chung Shan Medical University, Taichung, Taiwan. Induction of experimental APS by passive transfer was performed according to the method of Blank [9]. A group of 15 BALB/c mice (8 weeks of age) were infused with the rabbit anti-VP1u IgG antibodies (20ug per mouse) through the tail vein. Affinity-purified IgG from control rabbit that were immunized with adjuvant and PBS were also used as controls for immunization. After three weeks, a boost injection of 20ug of the immunoglobulin in PBS was administrated into the tail vein. The mice were sacrificed on day 30. APS clinical parameters (thrombocytomenia and prolonged activated partial thromboplastin time [aPTT]) were evaluated in the infused mice on day 30. Platelet counts from individual blood samples were quantified in whole blood using Systemex (KX-21, KOBE, Japan). The presence of lupus anticoagulants were evaluated by the prolongation of aPTT in a mixing tests, adding 1 volume of plasma from whole blood mixed with sodium citrate 0.123 mol/l in a 9:1 ratio to 1 volume of each cephalin and incubating for 2 minutes at 37℃. Another volume of 0.025M CaCl2 (Sigma, Saint Louis Mo, USA) was added, and the clotting time was recorded in seconds using Coatron M1 (TECO GmbH, Neufahrn NB, Germany).

Cell culture

ECV-304 cells (ECACC-92091712), an immortalized human vascular endothelial cell line and a spontaneously transformed human umbilical vein endothelial cell line, were cultured in DMEM supplemented with 10% fetal calf serum (FBS) (GIBCO-BRL, Carlsbad, California, USA), 2mM glutamine, 100U/ml penicillin and 100μg/ml streptomycin, in 5% CO_2 at 37°C in a humidified incubator. Treatments were carried out on 80 – 90% confluent cells. The effect of anti-B19-VP1u immunoglobulins at different doses on activation of ECV cells was performed in our preliminary tests and the dosage of 200ug/ml was used for Flow cytometric analysis, Cytokine ELISA, and Immunoblotting assay.

Results

Induction of passive APS by anti-B19-VP1u antibody

BALB/c mice were immunized with purified anti-B19-VP1u antibodies from sera of rabbits as described in materials and methods. The titers of the aCL, APhL, and anti-β2GPI antibodies in the sera of the immunized mice were evaluated after one month of immunization. The sera of mice immunized with anti-B19-VP1u IgG from rabbit antisera, revealed significant increased titer of APhL and anti-β2GPI antibody as compared to the sera from mice immunized with IgG from control rabbits. The immunoglobulin isotype of APhL and anti-β2GPI antibodies in sera from mice immunized with rabbit anti-B19-VP1u immunoglobulin were found to be IgG but not IgM. Additionally, no increased titer of aCL antibodies in sera from each group of mice was observed. Moreover, the mice immunized with anti-B19-VP1u IgG also revealed thrombocytopenia and prolonged aPTT but not in the control mice immunized with IgG or PBS.

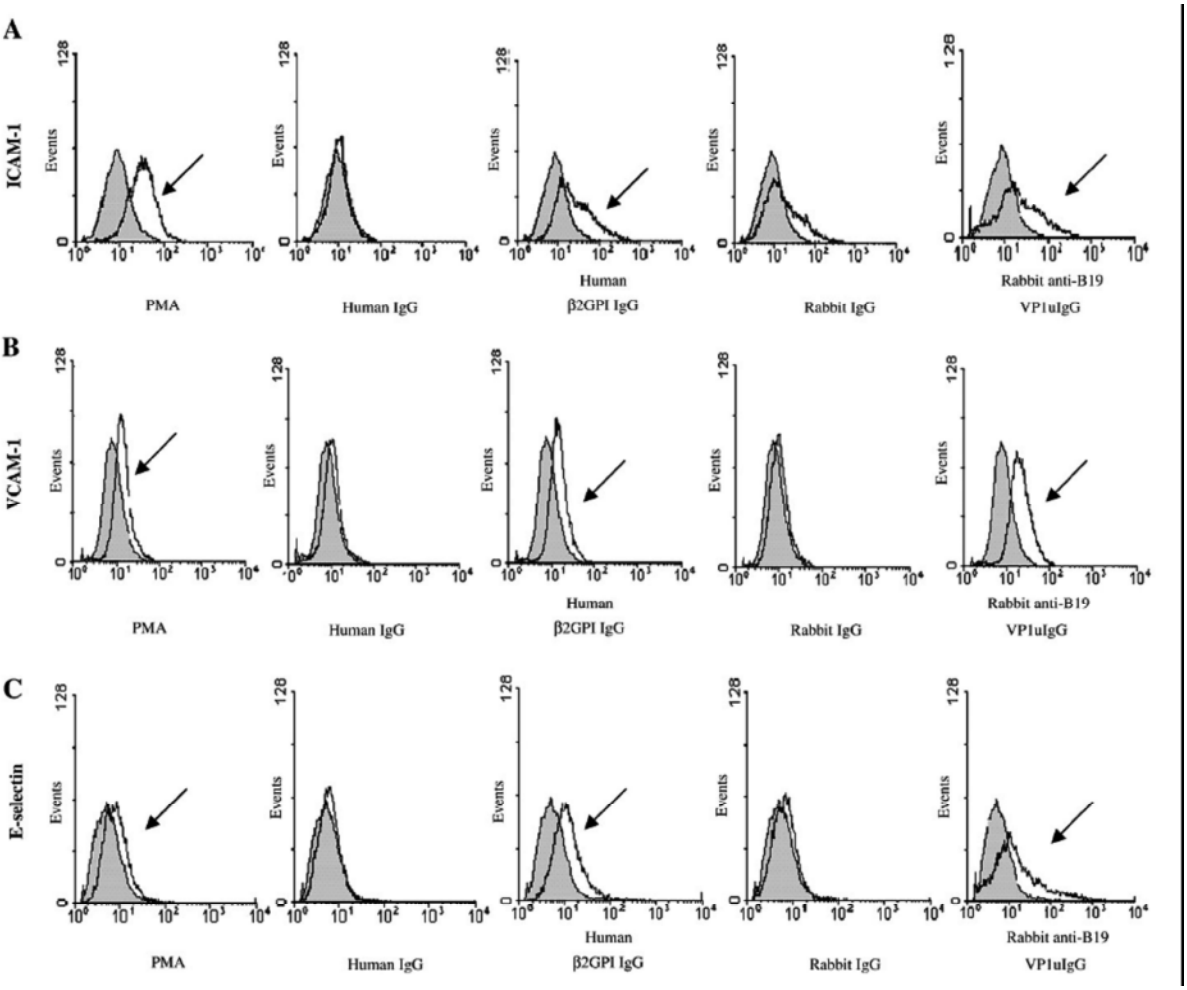
Mice infused with various rabbit antibody or reagent

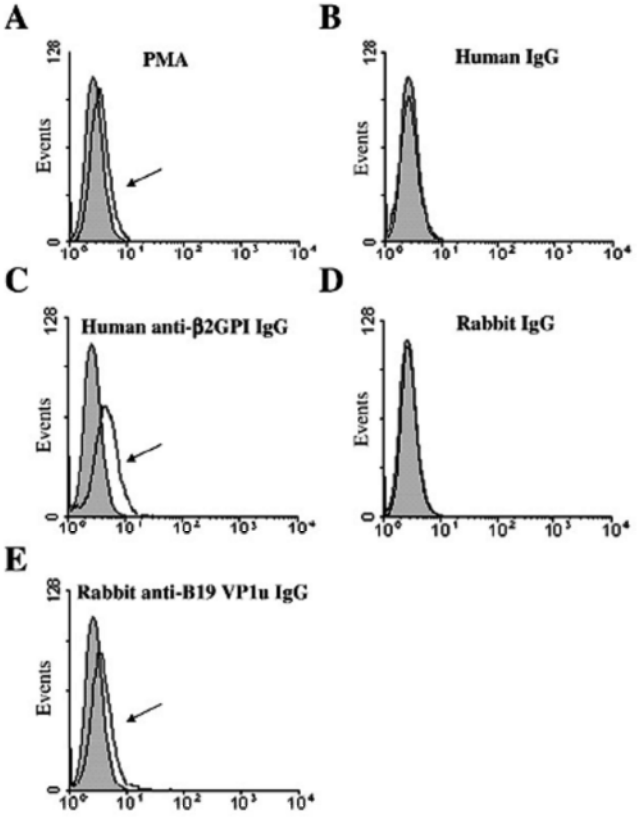
	Antibodies or reagent infused into mice				
	B19-VP1u (n=15)	Control IgG (n=10)	B19-NS1 (n=10)	PBS (n=10)	No treatment (n=10)
Body weight[a]	20.3±0.2	21.5±0.3	20.9±0.5	19.5±0.3	19.9±0.2
B19-VP1u binding activity[b]	0.505±0.05*	0.183±0.02	0.122±0.03	0.176±0.01	0.147±0.02
CL binding activity[b]	0.033±0.03	0.004±0.03	0.006±0.02	0.004±0.01	0.003±0.01
PhL binding activity[b]	0.208±0.01*	0.032±0.02	0.012±0.04	0.021±0.01	0.009±0.01
β2GPI binding activity[b]	0.252±0.02*	0.023±0.01	0.020±0.07	0.014±0.01	0.011±0.01
aPTT[c]	74.3±29.1*	30.4±1.8	30.8±7.2	27.3±2.1	25.9±4.3
Platelet count[d]	37±6.4*	51±7.0	60.2±8.8	72±13.0	61.8±9.6

VP1u: VP1 unique region protein; CL: cardiolipin; PhL: phospholipid; β2GPI: beta2 glycoprotein I; aPTT: activated partial thromboplastin time.
[a] The unit of body weight is gram.
[b] The unit of the data is optical density and present as mean±SD.
[c] The unit of the data is second.
[d] The unit of the data is 10^3 cells/mm^3.
* Indicates $P<0.05$ as compared to group PBS or No treatment.

Effect of rabbit anti-B19-VP1u antibodies on the expression of ICAM-1, VCAM-1, E-selectin, and MHC class II

Significant up-regulated expression of ICAM-1, VCAM-1, E-selectin, and MHC class II (HLA-DR, DP, DQ) was observed in ECV-304 cells treated with human anti-β2GPI IgG and rabbit anti-B19-VP1u IgG, respectively.


A
ICAM-1
B
VCAM-1
C
E-selectin
Events
PMA
Human IgG
Human β2GPI IgG
Rabbit IgG
Rabbit anti-B19 VP1uIgG

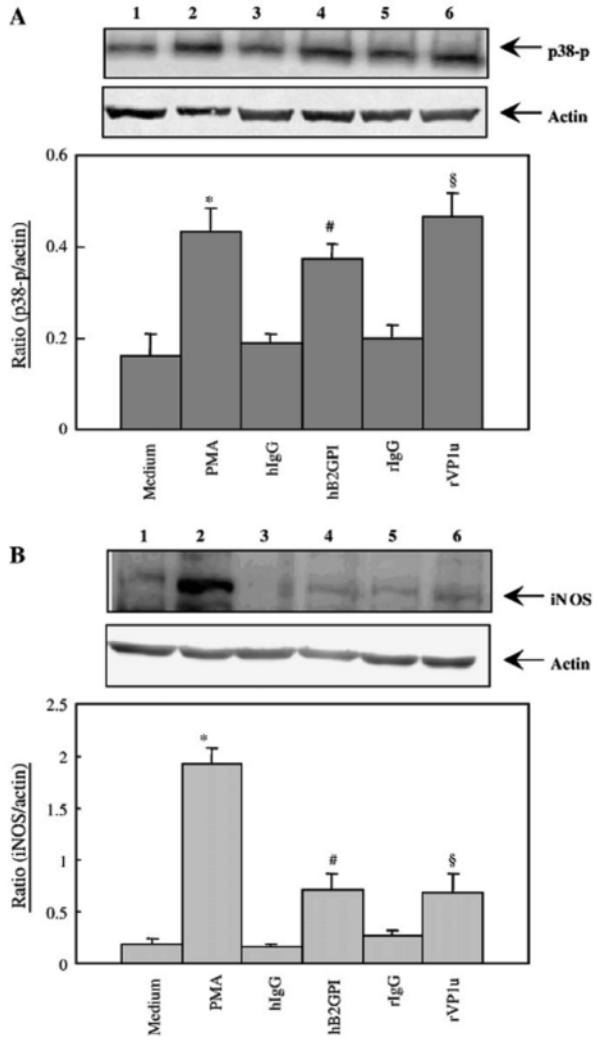

A
PMA
B
Human IgG
C
Human anti-β2GPI IgG
D
Rabbit IgG
E
Rabbit anti-B19 VP1u IgG
Events

Effect of anti-B19-VP1u antibodies on the production of TNF-α

The significant increase of TNF-α was observed in ECV-304 cells treated with rabbit anti B19-VP1u IgG as compared to those cells treated with control rabbit IgG. Similar result was obtained in ECV-304 cells treated with human anti-β2GPI antibody.

Effect of purified rabbit anti-B19-VP1u antibodies on the phosphorylation of p38 MAPK and on the expression of iNOS

Purified rabbit IgG against B19-VP1u induced significant increase in p38 phosphorylation and iNOS in ECV-304 cells. This result is similar to the result of treatment with human IgG against β2GPI and is consistent with a previous report [10].



Conclusion

Sera from rabbits immunized with recombinant B19-VP1u protein displayed raised detectable immunoglobulins against B19-VP1u, CL, β2GPI and PhL. Additionally, the mice immunized with anti-B19-VP1u IgG developed thrombocytopenia, prolongation of aPTT, and autoantibody against β2GPI and PhL [11]. Moreover, purified IgG from rabbits immunized with recombinant B19-VP1u proteins can up-regulate ICAM-1 (CD54), VCAM-1 (CD106),

E-selectin (CD62E), MHC class II (HLA-DR, DP, DQ) molecule expression, and TNF-α production in endothelial cells as compared to those endothelial cells cultured with control IgG. Additionally, significantly increased phosphorylated-P38 mitogen-activated protein kinase (P38 MAPK) and iNOS were observed in both human anti-β2GPI IgG and rabbit anti-B19-VP1u IgG treated-ECV-304 cells, respectively [12].

References

[1] Lehmann HW, von Landenberg P, Modrow S. Parvovirus B19 infection and autoimmune disease. Autoimmun Rev 2003; 2:218-223.

[2] Von Landenberg Philipp, Lehmann HW, Modrow S. Human parvovirus B19 infection and antiphospholipid antibodies. Autoimmun Rev 2007; 6: 278-85.

[3] Loizou S, Cazabon JK, Walport MJ, Tait D, So AK. Similarities of specificity and cofactor dependence in serum antiphospholipid antibodies from patients with human parvovirus B19 infection and from those with systemic lupus erythematosus. Arthritis Rheum 1997; 40:103-108.

[4] Chou TN, **Hsu TC**, Chen RM, Lin LI, Tsay GJ. Parvovirus B19 infection associated with the production of anti-neutrophil cytoplasmic antibody (ANCA) and anticardiolipin antibody (aCL). Lupus 2000; 9:551-554.

[5] von Landenberg P, Lehmann HW, Knoll A et al. Antiphospholipid antibodies in pediatric and adult patients with rheumatic disease are associated with parvovirus B19 infection. Arthritis Rheum 2003; 48:1939-1947.

[6] Lehmann HW, Plentz A, Von Landenberg Philipp, Muller-Godeffroy E, Modrow S. Intravenous immunoglobulin treatment of four patients with juvenile polyarticular arthritis associated with persistent parvovirus B19 infection and antiphospholipid antibodies. Arthritis Res Ther 2004; 6: R1-R6.

[7] Lim W, Crowther MA, Eikelboom JW. Management of antiphospholipid antibody syndrome: a systematic review. JAMA 2006; 295:1050-1057.

[8] Tzang BS, Tsay GJ, Lee YJ, Li C, Sun YS, **Hsu TC***. The association of VP1 unique region protein in acute parvovirus B19 infection and antiphospholipid antibody production. **Clin Chim Acta.** 2007; 378: 59-65. (*corresponding author) (SCI)

[9] Blank M, Cohen J, Toder V, Shoenfeld Y. Induction of anti-phospholipid syndrome in naive mice with mouse lupus monoclonal and human polyclonal anti-cardiolipin antibodies. Proc Natl Acad Sci U S A 1991; 88:3069-73.

[10] Vega-Ostertag M, Casper K, Swerlick R, Ferrara D, Harris EN, Pierangeli SS. Involvement of p38 MAPK in the up-regulation of tissue factor on endothelial cells by antiphospholipid antibodies. Arthritis Rheum 2005; 52:1545-54.

[11] Tzang BS, Lee YJ, Yang TP, Tsay GJ, Shi JU, Tsai CC, **Hsu TC***. Induction of antiphospholipid antibodies and antiphospholipid syndrome-like autoimmunity in naive mice with antibody against human Parvovirus B19 VP1 unique region protein. **Clin Chim Acta** 2007; 382: 31-36. (*corresponding author) (SCI)

[12] Tzang BS, Tsai CC, Chiu CC, Shi JY, **Hsu TC***. Up-regulation of adhesion molecule expression and induction of TNF-α on vascular endothelial cells by antibody against human parvovirus B19 VP1 unique region protein. **Clin Chim Acta** 2008; 395: 77-83. (*corresponding author) (SCI)

計畫成果自評部份

研究內容與原計畫相符程度 100%，為探討人類微小病毒 B19 結構蛋白獨立區域、自體免疫疾病和自體抗體的相關性。結果讓我們更進一步了解到抗 B19-VP1u 抗體與抗磷脂質症候群的密切關係，達預期目標 80%。這些線索可提供對於人類微小病毒 B19 -VP1u 在人類微小病毒 B19 感染時病人抗磷脂質抗體產生及抗磷脂質症候群扮演重要的的角色。這些研究結果已發表在 *Clinica Chimica Acta* (SCI)雜誌[15-16]。

出席國際學術會議心得報告

計畫編號	96-2314-B-040-005
計畫名稱	利用動物及細胞模是探討人類微小病毒 B19 結構蛋白獨立區域誘發自體抗體及自體免疫疾病產生的免疫機轉之研究
出國人員姓名 服務機關及職稱	徐再靜助理教授　中山醫學大學免疫學研究所
會議時間地點	2007.10.07 ~2007.10.12　希臘羅德島
會議名稱	第三屆國際自體免疫會議 **(3rd International Conference on Autoimmunity: Mechanisms and Novel Treatments)**
發表論文題目	Induction of antiphospholipid antibodies and antiphospholipid syndrome-like autoimmunity in naive mice with antibody against human parvovirus B19 VP1 unique region protein

會議經過

2007 年 10 月 6 日搭乘長榮班機由台北經巴黎轉機至希臘雅典，但因颱風影響班機使得巴黎至希臘的轉機延誤而以致行李遺失 96 小時。但由於能獲邀參加此次國際會議實爲難得，因此在調適心理後繼續轉搭班機前往會議地點希臘羅德島參加第三屆國際自體免疫會議:機轉與新治療。本次經飛行及轉機時間約 24 小時後於 10 月 7 日抵達會議地點希臘羅德島 Aldemar Paradise Village Conference Center。經辦理報到後取得相關會議資料，便立刻參與開幕式。而這個會議最主要是集合世界各地基礎與臨床的自體免疫學專家共同爲自體免疫學研究進行相關領域整合及交流而使得自體免疫學的研究進展能更迅速。10 月 8 日-10 月 11 日的會議議程將分爲幾個主軸做廣泛及深入的分析，包括七大主題: (I)Innate Immunity and Autoimmunity (II) Genetics of Autoimmunity (III) Antigen presentation/ role of B lymphocytes in autoimmunity (IV)T cell activation and Signaling (V) T cell Differentiation, Memory and Homeostasis (VI) Immunotherapy: Animal Models (VII) Immunotherapy: Human trials 由美國 Farber D 和 Scott D 教授主持帶領大家一起進入及討論自體免疫的發生及影響和未來治療的方向。各國自體免疫學者及與會人

士均踴躍發言並且提出意見交換，會議相當熱烈討論，而在中場會議休息時間，大家仍在場外進行討論，把握難得的機會和世界各地的免疫學者互相交流。本會議約有將近二百名學者參與。

與會心得

我所發表的壁報論文在壁報間展示，主要和自體免疫及感染有關。在這次會議上也認識了不少世界級免疫大師，包括來自歐美各名校哈佛、耶魯、劍橋等知名國際大學教授、博士後研究、博士生等優秀研究人員，在相互介紹及交換名片下，也相約下次能有機會共同進行學術交流。這些年在自體免疫研究中的新發現包括樹突狀細胞和調控 T 細胞接參與自體免疫反應，影響細胞激素調節和影響病毒細菌的感染表現。此外也發現 IL6 和 TGFbeta 會誘使 Th17 T 細胞分化產生進而參與及導致自體免疫疾病產生。深覺自體免疫機轉的研究分工將更精細，期待有更突破的治療方式能早日能發展並治癒此疾病。因此此次南歐會議之行的收穫相當豐富。而這次能順利的出國進行學術交流和參與自體免疫盛會，吸取新知，實在很不容易。所幸有國科會大力的支持經費與鼓勵我們新一代的研究學者出國進行學術訪問。在此特別致上最衷心的感謝。相信在不久的將來，自己的這些努力辛苦所呈現的研究成果能在學術上受到肯定，也期待日後繼續能有機會參與國外的重要學術會議。

Induction Of Antiphospholipid Antibodies And Antiphospholipid Syndrome-Like Autoimmunity In Naive Mice With Antibody against Human Parvovirus B19 VP1 Unique Region Protein

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Abstract

Previous studies have postulated a connection between human parvovirus B19 (B19) infection and anti-phospholipid antibodies (APhL). Indeed, B19 infection and anti-phospholipid syndrome (APS) also exhibit congruent symptoms. However, the precise role of B19-VP1 unique region (VP1u) in pathogenesis of autoimmunity is still obscure. To elucidate the roles of VP1u in B19 infection and autoimmunity, the reactivity of B19-VP1u proteins with various autoantibodies were evaluated by ELISA and Immunoblotting. Rabbits were immunized with purified recombinant B19-VP1u protein to generate anti-sera. Absorption experiments were then performed to determine the binding specificity of rabbit anti-sera against B19-VP1u, cardiolipin (CL) and beta-2- glycoprotein I (β2GPI). Moreover, the effects of passive transfer of polyclonal rabbit anti-B19-VP1u IgG antibodies on platelets, activated partial thromboplastin time (aPTT), and autoantibodies were assessed. Autoantibodies against CL, β2GPI, and phospholipid (PhL) in sera from patients with B19 infection, were cross-reactive with B19-VP1u. Consistently, sera from rabbits immunized with recombinant B19-VP1u protein displayed raised detectable immunoglobulins against B19-VP1u, CL, β2GPI and PhL. Additionally, the mice immunized with anti-B19-VP1u IgG developed thrombocytopenia, prolongation of aPTT, and autoantibody against β2GPI and PhL. These experimental results suggested the association between B19-VP1u and production of anti-β2GPI antibodies, APhL, and APS-like autoimmunity. Altogether, it may provide a clue in understanding the role of B19-VP1u in inducing autoantibodies and B19-associated APS manifestations.