行政院國家科學委員會專題研究計畫 成果報告

人類微小病毒 B19 結構蛋白獨立區域及抗人類微小病毒 B19 結構蛋白獨立區域抗體誘發自體免疫之分子機制
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

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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(ex:抗磷脂質抗體)產生之間可能扮演著相當重要的角色。於是我們進一步將抗 B19-VP1u 抗體以靜脈注射方式至正常 BALB/c 老鼠體內，則發現會誘發抗 B19 -VP1u 抗體，抗磷脂質抗體，及導致 BALB/c 小鼠血小板下降和血液凝固時間(aPTT)延長。另外，我們也發現抗 B19-VP1u 抗體，如同人類的抗磷脂質抗體和抗 β2GPI 抗體，會促進內皮細胞活化。而為了更進一步了解 VP1u 引發自體免疫之相關機轉，我們探討(1)VP1u 對老鼠巨噬細胞 Raw264.7 的影響，首先證明重組蛋白 B19-VP1u 具有 sPLA2 活性，並發現此 sPLA2 的活性會影響老鼠巨噬細胞 Raw264.7 之遷移、吞噬、相關發炎細胞激素(IL-6、IL-1β)及 MMP9 mRNA 的表現增加。此外，也發現 sPLA2 活性會促進磷酸化 ERK1/2 和磷酸化 JNK 蛋白質表現增加。另外，為了了解抗 B19-VP1u 抗體在免疫疾病個體之角色，我們將抗 B19-VP1u 抗體以靜脈注射方式至狼瘡小鼠體內，發現會誘發抗 B19 VP1u 抗體，抗磷脂質抗體和抗 dsDNA 抗體的表現，及導致狼瘡小鼠血小板下降和血液凝固時間(aPTT)延長。此外也發現施打抗 B19-VP1u 抗體之狼瘡小鼠肝臟的 MMP9 活性及 MMP9 蛋白質表現均有顯著增加，並且透過磷酸化 PI3K 和 ERK 蛋白質之機轉。以上這些研究結果將可提供一些線索在 B19-VP1u 及抗 B19-VP1u 抗體與自體免疫的相關研究。

關鍵詞: 人類微小病毒 B19、結構蛋白獨立區域 VP1u、自體免疫

Abstract

In our recent studies, increased anti-cardiolipin and beta-2GPI antibodies were detected in sera from patients with acute B19 infection (IgM+/IgG+/DNA+). Consistently, the induction of anti-phospholipid and anti-cardiolipin antibodies, prolonged aPTT, and thrombocytopenia was observed in BALB/c mice receiving anti-B19 VP1u IgG intravenously. Additionally, we also demonstrated the sPLA2 activity of B19-VP1u protein and the activation of endothelial cells by B19-VP1u IgG as well as the results of human anti-phospholipid antibodies (anti-cardiolipin antibody or anti-β2GPI antibody) as reported in patients with APS. In this study, we found that increased migration, phagocytic index, and mRNA expressions of pro-inflammatory cytokines (IL-6, IL-1β) and MMP-9 were observed in RAW264.7 cells treated with B19-VP1u proteins that had sPLA2 activity. Moreover, significant reduction of platelet count and prolonged thrombocytopenia time were detected in anti-B19-VP1u IgG group as compared to other groups, whereas significant increases of anti-B19-VP1u, anti-phospholipid (APhL), and anti-dsDNA antibody binding activity were detected in anti-B19-VP1u group. Additionally, significant increases of MMP9 activity and protein expression were detected in B19-VP1u IgG group. Notably, phosphatidylinositol 3-phosphate kinase (PI3K) and phosphorylated extracellular signal-regulated kinase (ERK) proteins were involved in the induction of MMP9. These findings could provide clues in understanding the role of B19-VP1u and in B19 infection and B19-related diseases.

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

Introduction

Human parvovirus B19 (B19) is a human-pathogenic parvovirus consisting of a small non-enveloped particle with a single-stranded linear 5.6-kb DNA genome [1, 2]. The icosahedral capsid consists of two structural proteins, VP1 (83 kDa) and VP2 (58kDa), which are identical with the exception of 227 amino acids at the amino-terminal end of the VP1-protein, the so-called VP1-unique region (VP1u) [3]. Although VP2 proteins predominate in the capsid of B19, VP1 is critical in eliciting an appropriate immune response in both human and animal [4-6]. Previous studies have shown that antiserum produced by immunizing rabbits with a fusion protein containing the entire unique region sequence of VP1 neutralized the binding activity of B19 [7]. Recently, a phospholipase A2 (PLA2) motif has been linked to the B19-VP1u [8-11] and mutation of B19-VP1u in the phospholipase domain causes a complete loss in enzymatic activity and viral infectivity (ex: D175A) [10-12]. However, the precise function of secreted phospholipases A (sPLA2) from B19-VP1u is still obscure. Therefore, we evaluated the enzymatic activity of recombinant B19-VP1u with PLA2 motif proteins and its effect on macrophages.

The infection of B19 has been postulated to the generation of various autoantibodies including anti-nuclear antibody (ANA), anticardiolipin antibody (aCL), and anti-phospholipid antibody (APhL) [13-17], as well as the anti-phospholipid syndrome (APS) [14]. Notably, a significant similarity existed in the specificity of APhL between patients with B19 infection or systemic lupus erythematosus (SLE) was reported [15-16]. Recent studies have suggested that B19 may exacerbate or even induce SLE [13-14]. In our current study, we evaluated the enzymatic activity of recombinant B19-VP1u with PLA2 motif proteins and its effect on macrophages. Moreover, we treated NZB/W F1 mice with passive transfer of rabbit anti-B19-VP1u antibody to investigate the effect of elicited anti-B19-VP1u antibody on diseases activity in SLE.

Materials and Methods

sPLA2 activity

B19-VP1u and B19-VP1uD175A proteins were assayed for sPLA2 activity by use of a colorimetric assay (sPLA2 Activity Kit; Cayman Chemical), in accordance with the manufacturer's instruction. Results are expressed as micromoles per minute per milliliter. Additionally, the incubation of B19-VP1u or B19-VP1uD175A proteins with rabbit anti-B19-VP1u, anti-B19-NS1 or normal rabbit IgG [18-20] was also assayed for sPLA2 activity.

Cell culture

Mouse macrophages RAW 264.7 cells (RAW 264.7) were originally obtained from American type culture collection (ATCC) (Manassas, Va, USA) and were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum at 37℃ and 5% CO2 incubator. RAW 264.7 cells were incubated with 400ng of B19-VP1u recombinant protein for 24 h at 37℃, 5% CO2. The cell lysate were then obtained and stored at －80℃ for further use.

Migration assay

To determine the effect of VP1u or VP1uD175A on cell motility, cells were seeded into a Boyden chamber (Neuro Probe, Cabin John, MD) on membrane filters, and migration of cells stimulated or un-stimulated with VP1u, VP1uD175A was measured as described. The sPLA2 from bee venom (bvPLA2; Sigma-Aldrich) was used as a positive control. A modified Boyden chamber assay using cell culture inserts with a 8-mm pore size polycarbonate filter in a 48-well format was used to perform an in vitro migration assay. Cells were seeded on the upper part of the chamber at a density of $2x10^5$ cells/well in 50 uL of serum free medium and then incubated for 16 h at 37℃. The bottom chamber contained standard medium with 10% FBS. The cells that had invaded to the lower surface of the membrane were fixed with methanol, washed with dd-H2O, and then stained with Giemsa (Sigma). Ten random fields were counted for each experiment under a light microscope.

Assessment of phagocytosis

For phagocytosis of Latex beads, $2x10^5$ of RAW264.7 cells were cultured in each well of a 16-well Lab-Tek®II Chamber Slide™ (Nunc, Denmark) overnight and then stimulated with 400ng of VP1u or VP1uD175A recombinant proteins for 16 h before incubation with FITC-labelled Latex beads (Sigma, Saint Louis Mo, USA). 100X FITC-labelled Latex beads were suspended in phosphate buffered saline (PBS) and opsonized by incubation with RAW264.7 cells for 4 h at 37℃. One hundred macrophages in five random fields were counted by observation under a light microscope, ZEISS AXioskop2 at a magnification of 200X. The phagocytic index was expressed as the number of phagocytosed particles divided by the total number of macrophages and expressed as a percentage.

Messenger RNA isolation and RT-PCR

All studies were carried out in a designated PCR-clean area. After treatments at 0-24 h, washed in Dulbecco's phosphate buffered saline (DPBS) twice and treated with Trizol buffer for total RNA extraction. The cDNAs encoding mouse IL-1β, IL-6, TNFα, and GAPDH were amplified according to our previous condition [21] and the specific RNA level of every sample was expressed as the product's intensity. cDNA encoding glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) was amplified and quantified for each sample.

Gel zymography

RAW 264.7 cells were stimulated with VP1u/VP1uD175A recombinant proteins and the activities of MMP-2 and MMP-9 in medium were measured by gelatin-zymography assays. Ten microliters of ten-fold diluted serum or 20 μl supernatant were separated by an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels containing 0.1% gelatin. Gels were washed for 30min in 2.5% Triton X-100 to remove the SDS and then soaked in the reaction buffer containing 40mM Tris-HCl, pH8.0, 10mM CaCl2 and 0.02% NaN3 for 30 min. Gelatinolytic activity was visualized by staining the gels with 0.5% Coomassie brillant blue R-250, de-stained with methanol-acetic acid water, and relative MMP levels were quantitated by a gel documentation and

analysis system (AlphaImager, 2000, Alpha Innotech Corporation).

Mice and induction of experimental APS by passive transfer

Twenty-four female NZB/W F1 mice at age of 8 weeks were purchased from National Taiwan University, Laboratory Animal Center, Taiwan and housed under supervision of the Institutional Animal Care and Use Committee at Chung Shan Medical University, Taichung, Taiwan. The mice at age of 12 weeks were divided into four groups and were intravenously received rabbit anti-B19-VP1u IgG (20ug), normal rabbit IgG (20ug), rabbit anti-B19-NS1 IgG (20ug), and PBS through the tail vein, respectively. The mice were then sacrificed on day 30 by CO_2 asphyxiation and the heart blood samples were collected. APS clinical parameters, including thrombocytomenia and prolonged activated partial thromboplastin time [aPTT], were evaluated and the platelet counts were detected using Systemex (KX-21, KOBE, Japan). The presence of lupus anticoagulants were evaluated by the prolongation of aPTT in a mixing tests by 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).

ELISA

Direct antigen-specific ELISA kits were used to detect APhL IgG (Louisville APL Diagnostics, Inc. GA, USA) and anti-dsDNA IgG (INOVA Diagnostics, Inc. CA, USA) was performed as described in our recent publication [18-19]. The color reaction was performed as described above. For detecting the binding activity of anti-B19-VP1u antibody, recombinant B19-VP1u was coated in a 96 well plate and ELISA was performed as described in our recent report [19]. The cutoff value for each ELISA experiment was obtained (mean+3SD) and the absorbance above the value is regarded as positive.

Immunoblotting

Protein samples were separated in 12.5 or 10% of SDS-PAGE and electrophoretically transferred to nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). After blocking with 5% non-fat dry milk in (PBS), antibodies against AKT-p, Erk1/2-p, p38-p, JNK-p and NF-κB (p65-p), and actin (Upstates, Charlottesville, Virginia, USA) were diluted in PBS with 2.5% BSA and incubated for 1.5 hr with gentle agitation at room temperature. The membranes were then incubated with horseradish peroxidase (HRP) conjugated secondary antibody. Pierce's Supersignal West Dura HRP Detection Kit (Pierce Biotechnology Inc., Rockford, IL) was used to detect the antigen-antibody complexes. The blots were scanned and quantified by densitometry (Appraise, Beckman-Coulter, Brea, California, USA).

Statistical analyses

The paired t test and one-way ANOVA were used to analyze for statistical significance. A P value <0.05 was considered significant.

Results

Recombinant B19-VP1u proteins reveal sPLA2 activity and induces migration and phagocytosis

B19-VP1u protein has the PLA2 activity (0.19±0.03 μmol/min/mL), the recombinant B19-VP1u protein and B19-VP1uD175A protein, a mutant form of B19-VP1u losing the PLA2 activity. Moreover, a significant increase of migrated macrophages was observed in the experimental group that was treated with B19-VP1u proteins as compared to the control group. In contrast, significantly decreased numbers of migrated macrophages were observed in the experimental group that was treated with B19-VP1uD175A or the B19-VP1u +U0126 as compared to the B19-VP1u group (Fig 1A). Significant increases in the phagocytic index were detected in the B19-VP1u, B19-VP1uD175A, bvPLA2, and B19-VP1u+U0126 groups as compared to the control group (Figure 1B. However, a significantly lower phagocytic index was detected in the B19-VP1uD175A and the B19-VP1u with U10126 groups as compared to the B19-VP1u group, respectively

B19-VP1u induces IL-6, IL-1β mRNA expression and MMP9 activity

Significant increases in IL-6 and IL-1β mRNA expression were observed in RAW264.7 cells that were stimulated with B19-VP1u at 12 and 24 h. In contrast, no induction of IL-6 and IL-1β mRNA expression was detected in cells that were stimulated with either B19-VP1uD175A or bvPLA2. Quantified results of IL-6 and IL-1β levels are shown in the lower panel of Figure 2A. Significant increases in MMP9 activity was observed at 8, 12 and 24 h in the experimental group treated with B19-VP1u proteins as compared to the control group. However, significant decreases in MMP9 activity were observed in the experimental group that was treated with B19-VP1uD175A at 8, 12 and 24 h as compared to the B19-VP1u group. Quantified results are shown in the lower panel of Fig 2B.

B19-VP1u increases the phosphorylation of ERK 1/2 and JNK

Notably, the phosphorylation of ERK 1/2 and JNK proteins was significantly increased in RAW264.7 cells that were stimulated with B19-VP1u as compared to those stimulated with B19-VP1uD175A or control (Figure 3A and 3B). Additionally, significant decreases of phosphorylated ERK 1/2 and JNK proteins were observed in the presence of U0126, an ERK inhibitor, or SP600125, a JNK inhibitor (Figure 3A and 3B). Quantified results are shown in the lower panels of Fig 3A and 3B.

Enhanced APS-like syndrome in NZB/W F1 by passive transfer of purified rabbit anti-B19-VP1u antibody

Significant decreases of platelet counts and aPTT were observed in sera from NZB/W F1 mice that were received purified rabbit anti-B19-VP1u IgG, compared to those mice that were received normal rabbit IgG, rabbit anti-B19-NS1 IgG, or PBS, respectively. Additionally, ELISA experiments were performed to elucidate the effect of anti-B19-VP1u IgG by analyzing the binding activities of APhL antibodies in NZB/W F1 mice that were received purified rabbit anti-B19-VP1u IgG. Elevated titers of anti-B19-VP1u and APhL and anti-dsDNA antibodies were detected in serum from NZB/W F1 mice that were received purified rabbit anti-B19-VP1u IgG as compared to those mice that were received normal rabbit IgG, anti-B19-NS1 IgG, or PBS, respectively (Fig.4).

Enhanced MMP-9 activity and expression in liver from NZB/W F1 by passive transfer of purified rabbit anti-B19-VP1u antibody

Significant increase of MMP9 activity was observed in liver of NZB/W F1 mice that were received rabbit anti-B19 VP1u IgG as compared to PBS, Control IgG, or B19-NS1 IgG group, respectively (Fig. 5A). However, no significant variation was detected in MMP-2 activity among all experimental groups (Fig. 5A). The quantified results of MMP-9/MMP-2 ratio were shown in lower panel of Fig 5A. Moreover, Western blots were performed to examine the expression of MMP9 and MMP2. Significant increase of MMP-9/MMP-2 ratio was detected in B19-VP1u group as compared to PBS, Control IgG, or B19-NS1 IgG group, respectively (Fig 5B). Quantified results were shown in the lower panel of Fig 5B.

Increased phosphorylation of ERK 1/2 and PI3K proteins in NZB/W F1 by passive transfer of purified rabbit anti-B19-VP1u antibody

Notably, the PI3K and phosphorylation of ERK 1/2 proteins were observed in liver from NZB/W F1 mice that were treated with rabbit anti-B19 VP1u IgG, as compared to PBS, Control IgG, or B19-NS1 IgG group, respectively (Fig 6). Quantified results were shown in lower panels of Fig. 6A and 6B.

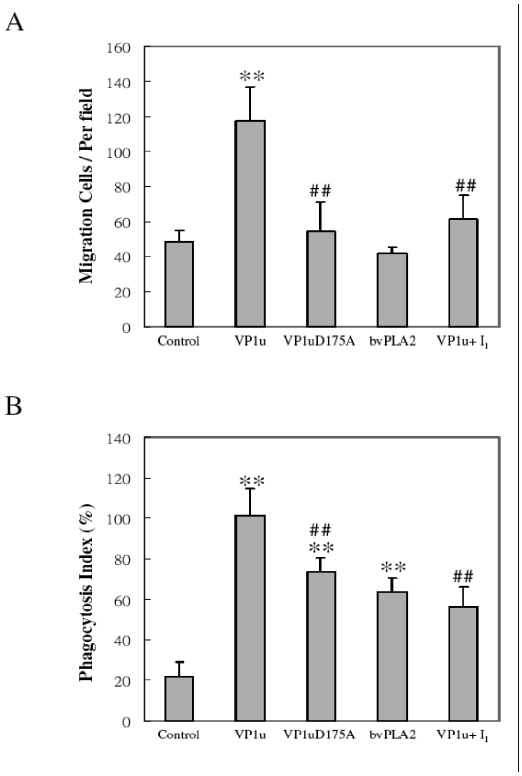

Fig. 1

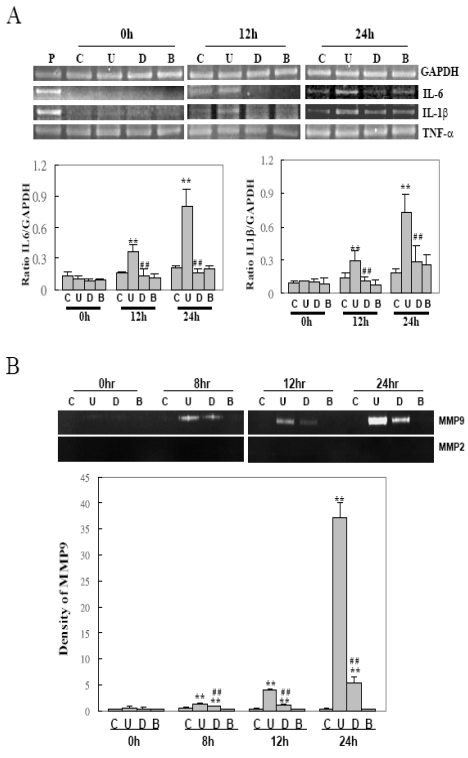

Fig. 2

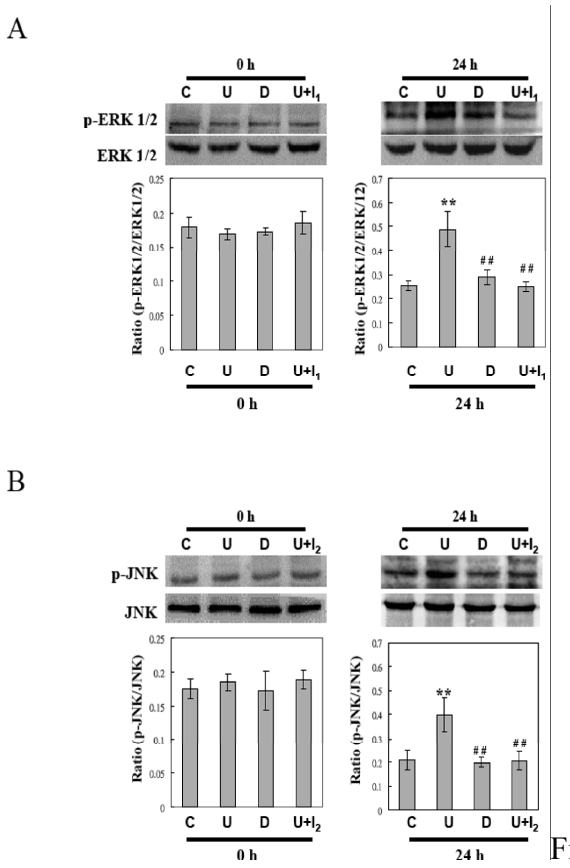

Fig. 3

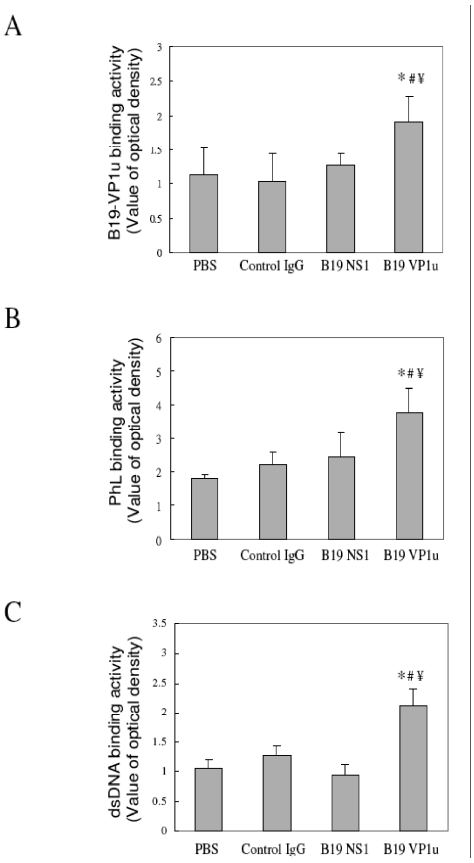

Fig. 4

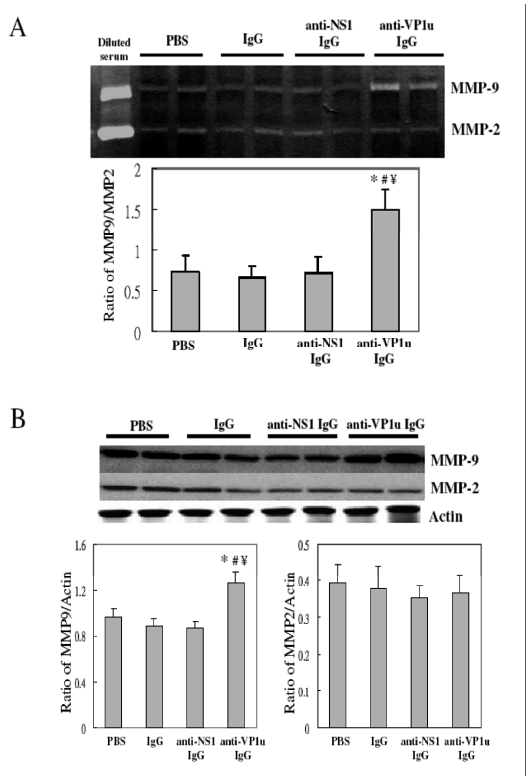

Fig.5

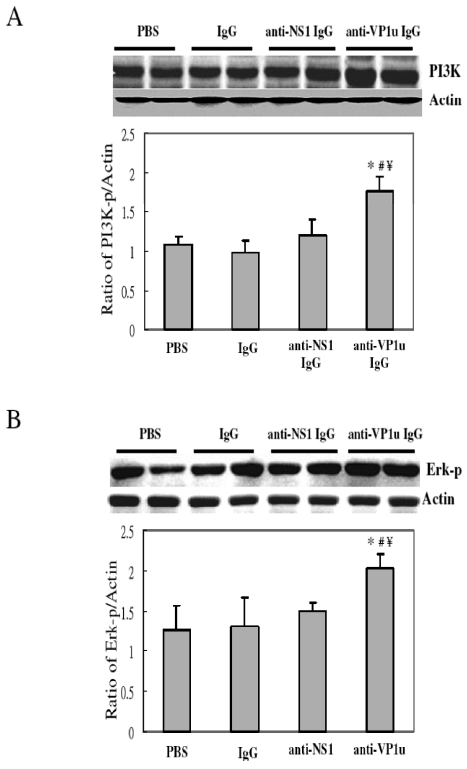

Fig. 6

Conclusion

Taken together, parvovirus B19-VP1u and its sPLA2 enzymatic activity are critical for eliciting macrophage responses associated with a variety of inflammatory processes. Our experimental results demonstrate the effects of sPLA2 activity in B19-VP1u proteins by increasing migration, phagocytosis, and inflammatory responses such as significant increases of MMP9 activity, IL-6 and IL-1β mRNA expression in macrophages [22]. Moreover, we also firstly demonstrated the aggravated APS-like syndromes in NZB/W F1 mice that were received anti-B19-VP1u IgG. Additionally, it could provide clues in understanding the roles of anti-B19-VP1u IgG in SLE and suggest possible therapeutic potential by inhibiting PI3K or ERK signaling pathway [23]. These studies may provide clues in understanding the role of B19-VP1u in the host response to B19 infection and B19-related diseases.

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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 結構蛋白獨立區域及抗人類微小病毒 B19 結構蛋白獨立區域抗體誘發自體免疫之分子機制。結果讓我們更進一步了解到人類微小病毒 B19 結構蛋白獨立區域及抗 B19-VP1u 抗體與抗磷脂質症候群的密切關係，達預期目標 90%。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 感染時病人先天免疫防禦及對自體免疫疾病鼠之影響。這些研究結果已被 *J Biomed Sci* (SCI)雜誌接受刊登 [22-23]。

出席國際學術會議心得報告

計畫編號	97-2314-B-040-009
計畫名稱	人類微小病毒B19結構蛋白獨立區域及抗人類微小病毒B19結構蛋白獨立區域抗體誘發自體免疫之分子機制
出國人員姓名 服務機關及職稱	徐再靜副教授 中山醫學大學免疫學研究所
會議時間地點	2008.10.26~2008.10.30 澳洲(Australia)柏斯(Perth)-弗里曼圖 Esplande Hotel
會議名稱	第十一屆先天免疫國際研討會(Natural Immunity 2008，NK2008)
發表論文題目	Effects of human parvovirus B19 VP1 unique region protein on macrophage responses

會議經過

2008 年 10 月 25 日搭乘新加坡航空班機由台北經新加坡轉機於 10 月 26 日清晨抵達澳洲 柏斯，再經約 1 小時車程抵達會議地點弗里曼圖。經短暫休息後於 10 月 26 日下午至會場 Esplande Hotel 辦理報到並取得相關會議資料及參與開幕晚宴。而這個會議最主要是集合世界各地基礎與臨床的先天免疫學研究專家共同為先天免疫學研究進行相關領域整合及交流而使得先天免疫學的研究進展能更迅速，尤其在天然殺手細胞(Natural Killer cells)。會議議程 10 月 27 日-10 月 30 日分為幾個主軸做廣泛及深入的研究分析，包括十二大主題: (1) Bice Perussia Lecture (2) Development & education (I) (3) Infection (4) Recognition & activation (5) Clinical application (6) Migration & Signaling (7) Regulation & Interactions (8) Development & education (II) (9) Cancer (10) Recognition & activation(II) (11) Dendritic cells (12) Infection(II)。各國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，大家仍把握時間在場內場外進行討論，把握難得的機會和世界各地的免疫學者互相交流。本會議約有將近三百名學者參與。

與會心得

我所發表的壁報論文在壁報間展示，主要和先天免疫及感染有關。在這次會議上也認識了不少世界級免疫大師，尤其是 Natural Immunity 和 European Journal of Immunology 等知名免疫學雜誌主編，此外也包括來自歐美(哈佛、劍橋)、亞洲(日本、韓國、台灣)、澳洲等名校知名國際大學教授、博士後研究、博士生、資深研究助理等優秀研究人員，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。這些年先天免疫研究在 NK 的新發現主要藉由探討 polymorphism 和 C57BL/6 老鼠之所以可抵抗病毒感染主要在於啟動 multiple NKC-encoded NK cell receptors,像 NKG2D, Ly49H and Ly49D，而 Ly49H 又影響 CD8 T cells 反應為主軸。因此接續探討此 receptor 與接受細胞間的訊息路徑，將有助於未來疫苗發展，並延及感染、腫瘤方面研究。另外在生技產品上也有新的研發，像快速利用單管分離血液中的 NK 細胞及利用 MACS 磁株分析儀分離 NK 細胞等，也於此次會議中首度發表並介紹使用及應用。因此此次澳洲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。

Effects of human parvovirus B19 VP1 unique region protein on macrophage responses

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Activity of secreted phospholipase A (sPLA2) has been implicated in a wide range of cellular responses. However, little is known about the function of human parvovirus B19-VP1 unique region (VP1u) with sPLA2 activity on macrophage. In the present study, we report that migration, phagocytosis, IL-6, IL-1b mRNA, and MMP9 activity are significantly increased in RAW264.7 cells by B19-VP1u protein with sPLA2 activity, but not by B19-VP1uD175A protein that is mutated and lacks sPLA2 activity. Additionally, significant increases of phosphorylated ERK1/2 and JNK proteins were detected in macrophages that were treated with B19-VP1u protein, but not when they were treated with B19-VP1uD175A protein. Taken together, our experimental results suggest that B19-VP1u with sPLA2 activity affects production of IL-6, IL-1β mRNA, and MMP9 activity, possibly through the involvement of ERK1/2 and JNK signaling pathways. These findings could provide clues in understanding the role of B19-VP1u and its sPLA2 enzymatic activity in B19 infection and B19-related diseases.