

行政院國家科學委員會專題研究計畫 成果報告

探討人類微小病毒 B19(B19)結構蛋白獨立區域(VP1 unique region)在 B19 感染與自體免疫疾病中所扮演的角色之研究

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中文摘要

病毒與自體免疫疾病,在近年來的研究已被證實具有相當密切之關係。最近,許多研究報告指出人類微小病毒 B19 (B19)感染相似於全身性紅斑性狼瘡(SLE)或類風濕關節炎(RA)等,此外 B19 感染病人也有自體抗體表現增加的情形。過去我們實驗室的一些研究發現 B19 感染會造成自體抗體的產生及與自體免疫疾病有密切之關係。此外,也發現 B19 感染對於非紅血球系細胞(COS-7)也會產生細胞凋亡(主要是經由粒線體路徑)的情形及促進 IL6 分泌。但至目前為止,人類微小病毒 B19 在這些自體免疫疾病發展的角色仍不清楚。人類微小病毒 B19 是屬於小病毒(Parvoviridae)且無套膜之單股 DNA 病毒,基因約為 5600 bases。主要由兩個殼體蛋白(結構蛋白)[VP1 (781 amino acids [aa]) 和 VP2 (554 aa)] 和非結構蛋白 NS-1 所組成。這兩個殼體蛋白除了 VP1 比 VP2 在 N 端多了 227 amino acids 外,而這多出的序列已被命名為結構蛋白獨立區域[VP1 unique region (VP1u)]。先前許多文獻研究指出,大部分的中和性抗原決定位是位在 VP1u。在最近更有文獻指出在 VP1u 上有 phospholipase A2 之 motif,並推論這區域和自體免疫的產生有著密切的關係。因此,我們為了了解人類微小病毒 B19 感染和自體免疫疾病之間的關係,特別是 B19 結構蛋白的 VP1u 蛋白在 B19 感染和自體免疫之間的角色,我們將 B19 之結構蛋白 VP1u (wild type)和 VP1u-D175A (mutant)構築到 pET32a 表達載體,純化 VP1u 和 VP1u-D175A 蛋白質,和 B19 感染病人血清作用並分析相關抗原抗體的表現。另外我們也將利用純化後的專一性 VP1u (wild type)和 VP1u-D175A (mutant)免疫兔子並得到血清。結果發現在 112 位的 B19 感染病人中屬於 B19 診斷型式為 DNA+/IgM+/IgG+ 的病人血清有 57%的血清樣本也會和牛心脂抗原(CL)及 β 2GPI 反應,而這些病人血清在經過 VP1u 抗原先作用後,再和牛心脂抗原(CL)及 β 2GPI 反應,結果發現確實也抑制了血清和牛心脂抗原(CL)及 β 2GPI 的結合反應。這些結果讓我們更進一步了解到在急性感染 B19 的病人所產生的抗牛心脂和抗 β 2GPI 蛋白質自體抗體和 VP1u 是有關係的。而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上扮演重要的角色。

關鍵詞：人類微小病毒 B19 (B19)、結構蛋白獨立區域(VP1u)、牛心脂抗原(CL)、 β 2GPI、抗磷脂質抗體

Abstract

The association of human parvovirus B19 infection and autoimmune diseases with autoantibodies has been strongly suggested in these years, especially in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). Previously, we have found that B19 infection may be linked to the induction of autoimmune response and B19 nonstructural protein (NS1) induces apoptosis through mitochondria cell death pathway in COS-7 cells. However, the role of B19 mediated autoimmune diseases has not been clarified. B19 is a small single-stranded DNA virus containing 5596 nucleotides, which has two large open reading frames. The B19 encodes two structural proteins, the major capsid (VP2) and minor capsid (VP1) proteins. VP1 has an identical amino-acid sequence to VP2 protein, and

exception for an additional 227 amino acids at its amino terminus (VP1 unique region, VP1u). According to homology studies, a phospholipase A2 motif is present in the amino acid sequence of the VP1 unique region. A very recent study shows the association of parvovirus B19 infection with the presence of anti-phospholipid antibodies. Thus, these observations encouraged us to study more about the possible link between B19 infection and autoimmune diseases. Sera from 102 clinically suspected cases of B19 infection were analyzed. Sera from patients with the diagnostic pattern DNA⁺/IgM⁺/IgG⁺ had a high frequency (57%) for recognition of CL and β 2GPI. Furthermore, adsorption experiments were performed by adding purified B19-VP1u, which partially suppressed the reactivity of anti-B19VP1u to CL and β 2GPI. Thus, serum from patients with acute B19 infection have a high frequency in recognition of CL and β 2GPI, and the phospholipase domain observed in the B19-VP1u may have contributed to the production of aPL. These findings may provide a clue for understanding the roles of B19-VP1u in B19 infection and aPL production.

Key words: Human parvovirus B19 (B19) 、 VP1 unique region (VP1u) 、 cardiolipin (CL) 、 β 2 glycoprotein I (β 2GPI) 、 anti-phospholipid antibody (aPL)

Introduction

Human parvovirus B19 (B19) is the only known human pathogen in the large *Parvoviridae* family [1-2]. B19 consists of a small non-enveloped particle enclosing a single-stranded linear 5.6-kb DNA genome. The icosahedral capsid consists of two structural proteins, VP1 (83 kDa) and VP2 (58kDa), which are identical except for 227 amino acids at the amino-terminal end of the VP1-protein, the so-called VP1-unique region (VP1u) [3-5]. Over 95% of capsid proteins are VP2, whereas VP1 accounts for < 5%. Although VP2 proteins predominate in the capsid, VP1 is critical to eliciting an appropriate immune response in humans and animals [6-8]. An antiserum produced by immunizing rabbits with a fusion protein containing the entire unique region sequence of VP1 neutralized virus activity [9]. When rabbits were inoculated with 11 overlapping fusion proteins spanning the entire VP1 capsid protein including VP2, the VP1 unique and VP1-VP2 junction regions had the strongest neutralizing epitopes [10]. Recently, Zuffi *et al* determined that amino acids 60-100 of the VP1u with a known neutralizing activity induce prolonged immune responses in humans [11]. Most neutralizing epitopes in the VP1u are linear in contrast to those in VP2 that are likely conformational. [12-13].

B19 infection has been associated with production various autoantibodies, including anti-nuclear antibody (ANA), anti-neutrophil cytoplasmic antibody (ANCA), anticardiolipin antibody (aCL), and anti-phospholipid antibody (aPL) [14-17]. Moreover, B19 infection and anti-phospholipid syndrome (APS) also show congruence in the presentation of symptoms [14-17]. The classical APS is characterized by the presence of aPL which bind target

phospholipid molecules, mainly through beta2-glycoprotein I (β 2GPI), and are associated with recurrent fetal loss and thromboembolic phenomena. Recently, the outside location and phospholipase domain [18-20] of B19 has been linked to B19-VP1u. However, the definite relationship between B19 infection, B19-VP1u, and aPL production remains unclear.

Materials and methods

Sera

Serum samples from 102 patients with clinical suspicion of parvovirus B19 infection were collected between March 2004 and December 2005 and tested in the Division of Rheumatology, Department of internal medicine, Chung Shan Medical University affiliated Hospital, Taichung, Taiwan. The clotted blood samples were centrifuged at 1250 rpm for 10 min and the sera were collected and stored at -70°C until examination. All subjects were the presence of specific antibodies against parvovirus B19 structural proteins were detected by B19 IgM/IgG ELISA kits (IBL, Hamburg, Germany) and B19 DNA were done by nested PCR as described below. All the study participants provided written informed consent.

B19 antibodies detection

The B19 IgM antibody (Parvovirus B19-IgM, IBL, HAMBURG) and B19 IgG antibody (Parvovirus B19-IgG, IBL, HAMBURG) were analyzed by enzyme-linked immunosorbent assay (ELISA) against B19 VP2 and VP1 structural protein according to the manufacturer's instructions.

DNA extraction and PCR amplification

DNA was extracted from serum by using QIA Amp blood kit (QIAagen, Hidden, Germany) as directed by the manufacturer. In the first round of PCR amplification, 0.2 μM of nucleotide primers corresponding to nucleotide (nt) 2381-2400 (B19SI) and nt 2781-2800 (B19ASI) (5'-CCTTTTCTGTGCTAACCTGC-3' and 5'-CCCAGG CTTG TGTAAGTCTT-3', respectively) were used. 2 μl of each sample were used in a 50 μl reaction containing 5 μl of 10x buffer (500 mM Tris-HCl pH 8.7, 50 mM NH_4Cl , 20 mM MgCl_2 , 400 mM KCl, 1% Triton X-100), 4 μl of 25mM dNTP, 2.5U of *Taq* DNA polymerase (Takara, Tokyo, Japan) and 36 μl sterilized water. After an initial denaturation step of 5 min at 94°C , thirty-eight cycles were performed at 94°C for 45 seconds, 54°C for 45 seconds, and 72°C for 1 min. After the first round amplification, 2 μl of the first PCR product was added to the second round PCR mixture containing 2 μM of each oligonucleotide primer corresponding to nucleotide 2429-2448 (B19SII) and nucleotide 2730-2751 (B19ASII) (5'-AAAGCTTTG TAGATTATGAG-3' and 5'-GGTTCTGCATGACTGCTATGG-3'). Then 30 cycles of amplification were performed using the described cycling parameters. B19 positive and

negative reference controls [27] were also included in each PCR reaction. The nested PCR

was used as it eliminates non-specific background and thus gives a clearer final product. Because of high sensitivity of the nested PCR reaction, stringent precautions were taken to avoid the risk of false-positive results.

Plasmids and site-directed mutagenesis

A 681-bp DNA fragment encompassing nucleotides 2444-3144 of the B19 genome (plasmid pYT104-C) was amplified by the polymerase chain reaction using primers 5'-CCGAATTCATGAGTAAAAAAGTGGCAAATGGTGGG-3' (forward) and 5'-GCGTCGACGCTTGGGTATTTTTCTGAGGCG-3' (reverse), which were introduced a *Bgl II* site at the 5' end and a *Sal I* site at the 3' end for cloning into pET-32a. The amplification was performed in a 50 µl reaction volume containing 10x reaction buffer (Promega, Madison, Wisconsin, USA), 1.5 µM of MgCl₂, 200 µM of dNTPs, 1µM of each primer and 2.5 units of Taq DNA polymerase (Promega, Madison, Wisconsin, USA) using a Perkin-Elmer Gene Amp PCR system 2400 (Perkin-Elmer, USA). After an initial denaturation step of 5 min at 94°C, thirty cycles were performed at 94°C for 45 seconds, 54°C for 45 seconds, and 72 °C for 1 min. The amplification PCR products were subjected to electrophoresis on a 1% agarose gel. The ligatant is called pET32a-VP1u. The QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to engineer an aspartate to alanine (D175A) mutation into the wild-type VP1-unique region expression vector, pET32a-VP1u, with the following oligonucleotide primers: 5'-CTCATTGGACTGTAGCAGCTGAAGAGC-3' (forward primer) and 5'-GCTCTTCAGCTGCTACAGTCCAA TGAG-3' (reverse primer) for D175A. The PCR reaction was performed according to manufactures' instructions. This construct is named as pET32a-VP1uD175A and verified by sequencing forwardly and reversely. The plasmids was then transformed into *Escherichia Coli* BL21-DE3 competent cells (Invitrogen, Carlsbad, CA, USA) by chilling on ice for 30 minutes and bathing in 42°C water-bath for 30 seconds.

Preparation of recombinant human B19 VP1 unique protein

E. coli (BL21-DE3) clones containing VP1u and VP1u-D175A cDNA in pET-32a expression vector (Novagene, Cambridge, MA) were grown overnight in one liter L-Broth containing 100ug/ml ampicillin at 37°C with shaking. When the OD 600 reached 0.7-0.9, protein expression was induced by addition of IPTG to a concentration of 1 mM and incubated for another 3 hr. The cells were harvested by centrifugation at 4000 g for 20 min and resuspended in 20 ml sonication buffer (50 mM NaPO₄ pH 8/0.25 mM EDTA). Lysozyme was added to a final concentration of 1mg/ml and kept on ice for 30 min. The cells were sonicated (W385, Heat systems-ultrasonic, INC) for a total of 30 min at 5 min intervals, centrifuged 10,000 g for 30 min. The pellet was dissolved with 10 ml buffer B for 1 hr at room temperature, and centrifuge lysate at 10,000 g for 30 min at room temperature to pellet the cellular debris. The supernatant was loaded onto a Ni-NTA spin column (Qiagen, Chatsworth, CA, USA) and

washed and eluted with Buffer and analyzed by SDS-PAGE for further use. Since the B19VP1u and B19VP1uD175A cDNA are constructed into pET-32a expression vector, the expressed recombinant proteins will contain S-tag and His-tag that represent the molecular weight of nearly 47Kda.

Rabbit antisera

For generation of antisera directed against the B19-VP1u and B19-VP1uD175A, two female New Zealand White rabbits were immunized subcutaneous in the neck region with 0.5 mg of purified recombinant B19-VP1u and B19-VP1uD175A in Freund's complete adjuvant followed by injection at two-week intervals with 0.25 mg of B19-VP1u and B19-VP1uD175A protein in Freund's incomplete adjuvant. All sera reacted specially with the B19-VP1u and B19-VP1uD175A in immunoblotting analysis.

Anti-dsDNA, Anti-cyclic citrullinated peptide (CCP), anti-cardiolipin (aCL), and anti-β2GPI antibodies

We used direct antigen-specific ELISA kits to detect anti-dsDNA, anti-CCP, aCL and anti-β2GPI antibodies (INOVA Diagnostics, Inc. San Diego, CA, USA) as the manufacturer's instructions.

ELISA

Five hundred ng of B19-VP1u or B19-VP1uD175A recombinant protein in 100ul PBS (1mM) was used and immobilized on the surface of each sample well of 96-well plates. ELISA was performed according to the method of Rubin *et al* [21]. The serum samples were pretreated with RF-absorbent (IBL, HAMBURG) to avoid false-positive IgM results due to the presence of RF and to prevent competition by IgG antibodies. For absorption experiments, 0.5μg VP1u or B19-VP1uD175A recombinant protein were coated on the well and the sera were pre-incubated with 5μg (1mM) VP1u recombinant protein for one hour at 37°C before ELISA was performed.

Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using 12.5% acrylamide slab gel with 5% acrylamide stacking gel, was performed according to the method of Laemmli [22]. Rabbit antiserum against B19-VP1u or B19-VP1uD175A were diluted with 5% nonfat dry milk in PBS and reacted with the nitrocellulose strips and incubated for 1.5 hr at room temperature. The strips were washed twice with PBS-Tween for 1hr and adding secondary antibody consisting of alkaline phosphatase conjugated goat anti-human or rabbit IgG antibodies. The substrate NBT/BCIP (nitroblue tetrazolium/ 5-bromo-4-chloro-3 indolyl phosphate) was used to detect antigen-antibody complexes.

Results and Conclusion

Experimental results indicated that no difference exists in recognition of B19-VP1u and B19-VP1uD175A proteins by anti-B19VP1u IgG and IgM in serum samples from patients with the B19 diagnostic patterns DNA⁺/IgM⁻/IgG⁺, DNA⁻/IgM⁺/IgG⁺, DNA⁺/IgM⁺/IgG⁺ and DNA⁻/IgM⁻/IgG⁺. Furthermore, a high frequency (57%) in recognition of CL and β 2GPI by anti-B19-VP1u was observed in serum from patients with the diagnostic pattern DNA⁺/IgM⁺/IgG⁺. The inhibition of binding by purified B19-VP1u and B19-VP1uD175A in serum from patients with the B19 diagnostic pattern DNA⁺/IgM⁺/IgG⁺ was 59 to 77%, and 64% to 82%, respectively. However, the inhibition of binding to CL and β 2GPI by absorption with 5 ug (1mM) of B19-VP1u was 41 to 51% and 37 to 55%, respectively. It indicated that B19VP1u protein competes and influences the binding of antibodies in serum from patients with the B19 diagnostic pattern DNA⁺/IgM⁺/IgG⁺ to both CL and β 2-GPI. In this study, no difference existed in recognition of B19-VP1u and B19-VP1uD175A proteins by anti-B19-VP1u IgG and IgM in serum from patients with B19 diagnostic patterns DNA⁺/IgM⁻/IgG⁺, DNA⁻/IgM⁺/IgG⁺, DNA⁺/IgM⁺/IgG⁺ and DNA⁻/IgM⁻/IgG⁺. Thus, acute phase of B19 infection could be an important stage in autoantibody production in B19-infected subjects, especially aPL antibodies. However, molecular mimicry between the B19 pathogen and the β 2GPI molecule may also present and trigger an immune response, and the definite epitopes cause of production of autoantibodies or APS requires further studies. However, the structure modification and generation of immunogenic epitopes on β 2-GPI in patients with B19 infection are still unclear. Structure simulation and prediction of conformational cryptic epitopes of both β 2-GPI and B19 VP1u will be crucial for further clarifying the relationships among induction of anti- β 2-GPI and aCL autoantibodies by B19 VP1u. In conclusion, our experimental results indicate that serum from patients with acute B19 infection have a high frequency in recognition of CL and β 2GPI, and the phospholipase domain observed in the B19-VP1u may have contributed to the production of aPL. However, these findings may provide a clue for understanding the roles of B19-VP1u in B19 infection, aPL production, and B19-related APS syndrome.

計畫結果自評部分

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在急性感染 B19 的病人所產生的抗牛心脂和抗 β 2GPI 蛋白質自體抗體和 VP1u 是有關係的，達預期目標 80%。此外在動物實驗和細胞實驗方面，目前也在繼續分析。這些初步的線索將可提供我們未來對於人類微小病毒 B19 -VP1u 在人類微小病毒 B19 感染時病人抗磷脂質抗體產生上扮演重要角色深入研究的依據。而部分研究結果已投稿並被 *Clinica Chimica Acta* (SCI)雜誌接受刊登。

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