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 非結構蛋白之單一點突變對其抗原性及細胞凋亡之研究

計畫類別：個別型計畫
計畫編號：NSC93-2314-B-040-010-
執行期間：93 年 08 月 01 日至 94 年 07 月 31 日
執行單位：中山醫學大學免疫學研究所

計畫主持人：蔡嘉哲

計畫參與人員：徐再靜　張純華

報告類型：精簡報告

處理方式：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中　華　民　國 94 年 10 月 28 日

中文摘要
人類微小病毒 B19(B19)與自體免疫疾病之發生有密切關係，過去我們發現 B19 感染會導致抗嗜中性白血球細胞質抗體及抗牛心脂抗體之產生及在紅斑性狼瘡病人有 B19 的感染，另外也發現將 B19 非結構蛋白(NS1)轉染到 COS-7 細胞會導致細胞凋亡並經由粒線體路徑。Momoeda *et al* 在 1994 年曾發表 NS1 在第 334 胺基酸作單一突變(即從 lysine 變成 glutamate)，會導致 NS1 對細胞之毒性消失，這研究顯示 NS1 在調節細胞功能之重要性。而過去自體免疫疾病的研究已知道自體抗原之蛋白質或酵素功能位置往往就是重要的抗原決定位，因此我們將 NS1 之第 334 胺基酸作單一點突變後，轉殖 E coli.系統並分別表達 Wild type(原生型,NS1)和 Mutant type(突變型,NS1K334E)的蛋白質，再利用重組蛋白作為抗原和感染 B19 之病人血清進行西方墨點法分析，結果發現 NS1K334E 的突變並不會改變 NS1 在所有蛋白質的表現和純化時為低可溶的情形，以及並不會改變感染 B19 之病人血清的辨識。另外，將 NS1K334E 突變型轉染到 COS-7 細胞，分析細胞對細胞凋亡之影響。結果發現此一突變可使細胞凋亡情形降低。因此 K334E 突變點會影響 NS1 之細胞毒性，但並不影響 NS1 之抗原決定位。這些結果資料可以提供我們一些線索，以更加了解人類微小病毒 B19 感染及其非結構蛋白 NS1 對於抗原決定位及非紅血球系細胞之影響，並作為未來人類微小病毒 B19 疫苗及致病機轉相關研究的參考。

關鍵詞: 人類微小病毒 B19、非結構蛋白 NS1、單一突變、抗原決定位、細胞凋亡

Abstract
The association of human parvovirus B19 (B19) infection and autoimmune diseases has been suggested. Previously, we have found that B19 infection can induce the production of anti-neutrophil cytoplasmic antibody and anti-cardiolipin antibody, and B19 infection in systemic lupus erythematosus (SLE). In addition, we also found that B19 nonstructural protein (NS1) induces apoptosis through mitochondria cell death pathway in monkey epithelial COS-7 cells. These observations encouraged us to study more about the possible link between B19 infection and autoimmune diseases. Momoeda *et al* found that a putative nucleoside triphosphate-binding domain in the nonstructural protein of B19 is required for cytotoxicity. They found that the cytotoxicity of the B19 NS1 was abolished by a single mutation of amino acid within the NTP-binding domain at position 334. Recently, an important effort in autoimmunity research has been directed at defining the autoepitopes on protein antigen. It has also been known that autoepitopes are located at functional or active sites of the antigens. It's our aim to define if the autoepitope of NS1 is located at position 334.
An expression plasmid of pET-32a vector containing the B19 nonstructural protein NS1 or NS1 K334E was constructed and cloned into the E coli expression system, BL21(DE3) and produced recombinant proteins for analysis of autoepitopes of NS1. The mutant NS1 gene of mutation at 334 amino acid was constructed into a cytomegalovirus episomal vector, pEGFP-C1 and transfected it into monkey epithelial cells COS-7. The transfected cell lines

were analyzed for the mechanism of apoptosis. In our results, we found that the mutant cells showed decreased death in repeated experiments. The average percentages of COS-7 cells apoptosis were 18%, 6%, and 4% for cells transfected with the wild-type NS1, NS1 K334E, and the control vector, respectively. These results indicated that the NS1K334E mutant lost its ability to apoptosis and NS1 K334E did not affect the antigenicity. These findings may provide alternative information for further study in B19 infection and characterization of B19 NS1 protein in antigenisity and non-erythroid linkage cells, and these will be used as reference to further study in B19 recombinant vaccine and pathogenesis.

Key words: Human parvovirus B19、nonstructural protein (NS1)、a single mutation、epitope、apoptosis

Introduction

Human parvovirus B19 (B19) was discovered in 1975 [1] and has been associated with a variety of clinical manifestations, including rash, thrombocytopenia, leucopoenia, fetal wastage, hypocomplementemia, autoimmune hemolytic anemia, arthritis and vasculitis [2]. It is the causative agent in erythema infectiosum (EI). Additionally, B19 infection is associated with elevated levels of antinuclear antibody (ANA), anti-double stranded DNA antibody (anti-dsDNA), anti-neutrophil cytoplasmic antibodies (ANCA), and anti-cardiolipin antibodies (aCL) [3-4]. The association of B19 infection with autoimmune diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjogren's syndrome (SS), primary biliary cirrhosis (PBC), polymyositis (PM) and vasculitis has been suggested [5-6]. However, the mechanism by which these B19-associated diseases affect the patient is poorly understood. Although parvovirus B19 is known to have a limited tropism in human tissues [2], B19 DNA or antigen has been found in the heart, liver, spleen, kidney, testes, skin, cerebrospinal fluid and synovium of children and adults [7-8].

B19 is a small single-stranded DNA virus that contains 5596 nucleotides, and it has two large open reading frames. The genes on the left side of the genome encode the nonstructural protein (NS1) and those on the right side encode two capsid proteins (VP1 and VP2)[2]. NS1 has been reported to function as a transcription regulator by directly binding with the DNA sequence of the p6 promoter and with the Sp1/Sp3 transcription factors [9]. NS1 has also been shown to be involved in DNA replication, cell cycle arrest and the initiation of apoptosis in erythroid lineage cells [10-13] and non-erythroid lineage cells [14]. NS1 contains a nucleoside triphosphate (NTP) binding motif in the middle of the protein, and the K334E (lysine to glutamate at position 334) mutant with a disruption in the NTP-binding domain dramatically suppressed the cytotoxic activity of NS1 [15] and cell arrest [16]. These results suggest that the NTP-binding domain of NS1 is essential for the induction of apoptosis and

cell cycle arrest.

Cloning and expression of NS1 in prokaryotic [17-18] and eukaryotic [19-20] system have provides an opportunity to study some of the immunological properties of NS1. The occurrence of NS-1 specific IgG antibodies in the sera derived from patient with B19-associated arthritis or persistent B19-infection was first described in 1995 [17-18] and confirmed by Hemaurer et al [21]. Kerr and Cunniffe [19] also showed that NS1-specific antibodies are associated with chronic but not acute arthritis following B19 infection in adults. Recently, Heegarrd *et al* found that NS1 IgG is a common finding in most unselected recently and previously B19-infected individuals [22]. However, the antigenisity of NS1 mutant is still unclear. In this study, we examine whether the NTP-binding domain of the B19 nonstructural protein is required for its antigenisity.

Materials and methods

Study subjects

Thirth Serum samples from healthy immunocompetent individuals, patients with clinically suspected acute B19 infection and B19 persistently infected patients were tested for presence of B19 specific IgM and IgG antibodies by a commerial enzyme-linked immunoassay (ELISA). Persistent B19 infection was defined as B19 DNA positively by nested polymerase chain reaction (nested PCR) in serum samples for a time period of at least six months.

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 [6].

DNA purification and PCR amplification

DNA was extracted from serum by using QIA Amp blood kit (QIAagen, Hidden, Germany) as directed by the manufacturer. B19 DNA detection was performed using a previously described nested PCR assay amplifying a 322 bp fragment in the gene corresponding to the NS1 protein [6].

Plasmids and site-directed mutagenesis

The wild type NS1 expression plasmid, pQE40-NS1, was described previously [14]. The QuikChange XL site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to engineer a lysine to glutamate (K334E) mutation into wild-type NS1 expression vector, pQE40-NS1, with the following oligonucleotide primers: 5’-CCGCCAAGTACAG

GAGAAACAAACT-3’ (forward primer) and 5’-AGTTTGTTTCTCCTGTACTTGGCGG-3’ (reverse primer) for K334E. We named these constructs pQE40-NS1-K334E. Successful mutagenesis of the plasmids was verified by sequencing.

Sequencing

Reaction products (PCR) were purified by commerical kits and directed sequenced using Amplicycle sequencing kit (Perkin-Elmer). All reactions were according to the manufacturer’s instructions.

Expression and purification of recombinant proteins

The recombinant plasmid pQE40, which encodes human parvovirus B19-NS1, was kindly provided by Professor Susanne Modrow, Institute for Medical Microbiology, Universität Regensburg, Regensburg, Germany. The pQE40-NS1 and pQE40-NS1-K334E plasmids were transformed into wild type E.coli M15. Expression of the wild-type and mutant NS1 proteins were induced at 25℃ by addition of 1 mM IPTG for at least 3h of culture. Then harvest cell by centrifugation at 4000g for 20 minutes and resuspend pellet in 50mM NaPO4 pH8/0.25mM EDTA (50ml/L). Add lysozyme to a final concentration of 1mg/ml and stand sample on ice for 30 mins. Break cells by sonicator (W385, Heat Systems-ultrasonics, INC), and spin at 10,000g for 30 minutes. The cells were lysed in sodium phosphate buffer pH 8.0 containing 8M Urea followed by sonification. The sample was loaded onto a 2 ml Ni2+-NTA column (Amersham Pharmacia Biotech, Little Chalfont, UK) and washed with PES buffer (50 mM NaPO4 pH 8/ 0.25mM EDTA/100 mM NaCl). Protein then was eluted with 0.1M to 2 M imidazole gradient and dialyzed with PBS with 6M or lower concentrations of Urea. Eluted fractions were analyzed by SDS-PAGE and immunoblotting. The bacteria were harvested by centrifugation and checked for the presence of protein by SDS-PAGE followed by staining with Coomassie Brilliant Blue or by analysis on Western Blot using NS-1 specific polyclonal rabbit sera.

SDS-PAGE and Western blotting

Cells were lysed in an aliquot volume of whole-cell extraction buffer A (140 mM NaCl, 10 mM Tris, pH 7.5, 1.5 mM $MgCl_2$, 0.5% NP-40) and protease inhibitor cocktail (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) for 30 min on ice. Cell lysates were then microcentrifuged at 14,000 rpm for 10 min to remove the insoluble component. Thirty μg of protein from the supernatants were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were applied to the gel and separated at 100-150 V for 1.5 hr. The gel was then electrophoretically transferred to nitrocellulose, according to the method of Towbin et al. [23] The strips were washed twice with PBS-Tween for 1 hr and incubated with secondary antibody consisting of alkaline phosphatase conjugated goat anti-rabbit or mouse IgG antibodies. The substrate of nitroblue tetrazolium/5-bromo-4-chloro-3

indolyl phosphate (NBT/BCIP) was used to detect the reaction. Anti-B19 NS1 antibody was made by immunization of recombinant His-tag NS1. Anti-GFP antibody was purchased from Invitrogen.

Cell culture and Transfection

The NS1-K334E will also constructed into pEGFP-C1 mammalian expression vector and transfect it to COS-7 cells. COS-7 cells were originally obtained from American type culture collection (ATCC) and were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (GIBCO-BRL) at 37℃ and 5% CO_2 incubator. Cells were grown to 70% confluent in 100 mm culture plates before transfection. The transfection reaction was performed by using Lipofectamine plus reagents (Invitrogene, California, USA) with 2 μg of each plasmid, pEGFP-C1 or the pEGFP-NS1-K334E constructant according to the manufacture's instruction. The cells were then cultured in serum-free DMEM for 12 hr at 37℃ in 5% CO_2 incubator and subsequent in DMEM with 10% FBS. Expressions of EGFP-NS1 K334E proteins were examined by using fluorescence microscopy, Western blot analysis, and flow cytometric analysis described as following.

Flow cytometric analysis

The procedures for flow cytometric analysis were the same as used previously [14]. In brief, cells (~2 x 10^6) were fixed in 75% alcohol for 12-16 hr at 4℃, followed by RNase (1 mg/mL) treatment at room temperature for 30 min. Cells were stained with propidium iodide (PI, 10 μg/mL) for 30 min before cell cycle analysis with a flow cytometer (FACScan, Becton Dickinson, Bedford, MA).

Results and Conclusion

The plasmids pQE40-NS1 and pQE40 NS1-K334E were constructed and expressed the entire NS1 ORF with a 6x His tag attached to the C terminus. High levels of expression of the resulting 77kDa fusion protein were obtained in *E.coli* (Fig 1A and 1B, lane 1 and 3) following induction with IPTG (Fig 1A and 1B, lane 2 and 4). The identify of the protein was proven by Western Blot analysis with NS1-specific rabbit antiserum (Fig 1B). Introduction of a mutation in the triphosphate-binding domain, possibly diminishing the cytotoxic effects of NS1, did not improve overall expression or purification. The induced fusion proteins of both NS1 and NS1-K334E were purified by Ni2+-NTA column and were used for antigens to investigate the presence of NS1 and NS1-K334E specific antibodies in sera of patients with acute or past parvovirus B19 infection. Thirty serum samples were tested for their reactivities with the NS1 and NS1-K334E fusion proteins. According to the results of Western Blot analysis, the sera could divided into three groups of reaction patterns (Table 1 and Fig 2). First, serum samples from individuals with acute B19 virus infection showed the presence of

pecific antibodies to the NS1 and NS K334E proteins. Second, samples obtained from persons with incidence of past B19 infection showed no-reactivity in this assay. The first group consisted of sera derived from patients with acute parvovirus B19 infection contained both IgM and IgG to VP1 and VP2, whereas sera from individuals with past infection contained only IgG to the structural proteins.

The mutant NS1 gene of mutation at 334 amino acid was constructed into a cytomegalovirus episomal vector, pEGFP-C1 and transfected it into monkey epithelial cells COS-7. The transfected cell lines were analyzed for the mechanism of apoptosis. In our results, we found that the mutant cells showed decreased death in repeated experiments. The average percentages of COS-7 cells apoptosis were 18%, 6%, and 4% for cells transfected with the wild-type NS1, NS1 K334E, and the control vector, respectively.

These results indicated that the NS1K334E mutant lost its ability to apoptosis and NS1 K334E did not affect the antigenicity. These findings may provide alternative information for further study in B19 infection and characterization of B19 NS1 protein in antigenisity and non-erythroid linkage cells, and these will be used as reference to further study in B19 recombinant vaccine and pathogenesis.

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 非結構蛋白之單一點突變 B19 NS1 K334E 對其抗原性及細胞凋亡之研究致病機轉, 達預期目標 80%, 此一模式建立可以更詳盡了解 NS1 protein 對 B19 感染及其非結構蛋白 NS1 對於抗原決定位及非紅血球系細胞之影響，並作為未來人類微小病毒 B19 疫苗及致病機轉相關研究的參考。

References:

1. Cossart YE, Field AM, Cant B, Widdows D. Parvovirus-like particles in human sera. Lancet 1975; 1: 72-73.
2. Young NS, Brown KE. Mechanisms of disease: Parvovirus B19 N Engl J Med 2004; 350:586-97.
3. Chou, CTN., Hsu TC, Chen RM, Lin LI, and Tsay GJ. Parvovirus B19 infection associated with the production of anti-neutrophil cytoplasmic antibody (ANCA) and anticardiolipin antibody (aCL). Lupus 2000; 9: 551-554.
4. Von Landenberg P, Lehmann HW, Knoll A, Dorsch S, Modrow S. Antiphospholipid antibodies in pediatric and adult patients with rheumatic disease are associated with parvovirus B19 infection. Arthritis Rheum 2003; 48: 1939-1947.
5. Lehmann HW, von Landenberg P, Modrow S. Parvovirus B19 infection and autoimmune disease. Autoimmun Rev 2003; 2: 218-23.
6. Hsu TC, Tsay GJ. Human parvovirus B19 infection in patients with systemic lupus

erythematosus. Rheumatology (Oxford). 2001; 40: 152-157.
7. Soderlund-Venermo M, Hokynar K, Nieminen J, Rautakorpi H, Hedman K. Persistence of human parvovirus B19 in human tissues. Pathol Biol (Paris) 2002; 50: 307-316.
8. Hokynar K, Soderlund-Venermo M, Pesonen M *et al*. A new parvovirus genotype persistent in human skin. Virology 2002; 302: 224-228.
9. Raab U, Beckenlehner K, Lowin T, Niller HH, Doyle S, Modrow S. NS1 protein of parvovirus B19 interacts directly with DNA sequences of the p6 promoter and with the cellular transcription factors Sp1/Sp3. Virology 2002, 293: 86-93.
10. Ozawa K, Ayub J, Kajigaya S, Shimada T, Young N. The gene encoding the nonstructural protein of B19 (human) parvovirus may be lethal in transfected cells. J Virol 1988; 62: 2884-2889.
11. Morita E, Sugamura K. Human parvovirus B19-induced cell cycle arrest and apoptosis. Springer Semin Immunopathol 2002; 24:187-99.
12. Morita E, Nakashima A, Asao H, Sato H, Sugamura K. Human parvovirus B19 nonstructural protein (NS1) induces cell cycle arrest at G(1) phase. J Virol 2003, 77: 2915-2921.
13. Yaegashi N, Niinuma T, Chisaka H *et al*. Parvovirus B19 infection induces apoptosis of erythroid cells in vitro and in vivo. J Infect 1999; 39: 68-76.
14. Hsu TC, Wu WJ, Chen MC, Tsay GJ. Human parvovirus B19 non-structural protein (NS1) induces apoptosis through mitochondria cell death pathway in COS-7 cells. Scand J Infect Dis 2004; 36:570-7.
15. Momoeda M, Wong S, Kawase M, Young NS, Kajigaya S. A putative nucleoside triphosphate-binding domain in the nonstructural protein of B19 parvovirus is required for cytotoxicity. J Virol. 1994; 68(12):8443-6.
16. Nakashima A, Morita E, Saito S, Sugamura K. Human parvovirus B19 nonstructural protein transactivates the p21/WAF1 through Sp1. Virol 2004;329:493-504.
17. von Poblotzki A, Gilger A, Lang B, Wolf H, Modrow S. Antibodies to parovirus B19 NS-1 protein in infected individuals. J Gen Virol 1995; 76:519-527.
18. von Poblotzki A, Hemauer A, Gilger A, puchhammer-Stockl E, Heinz FX, Pont J, Laczika K, Wolf H, Modrow S. Antibodies to the nonstructural protein of parvovirus B19 in persistently infected patients: implications for pathogenesis. J Infect Dis 1995;172:1356-1359.
19. Kerr JR, Cunniffe VS. Antibodies to parvovirus B19 nonstructural protein are associated with chronic but not acute arthritis following B 19 infection. Rheumatology (Oxford). 2000;39:903-908.
20. Ennis O, Corcoran A, Kavanagh K, Mahon BP, Doyle S. Baculovirus expression of parvovirus B19 (B19V) NS1:utility in confirming recent infection. J Clin Virol. 2001;22:55-60.
21. Hemauer A, Gigler A, Searle K, Beckenlehner K, Raab U, Broliden K, Wolf H, Enders G,

Modrow S. Seroprevalence of parvovirus B19 NS-1 specific IgG in B19-infected and uninfected individuals and in infected pregnant women. J Med Virol. 2000;60:48-55.
22. Heegaard ED, Rasksen CJ, Christensen J. Detection of parvovirus B19 NS1-specific antibodies by ELISA and Western Blotting employing recombinant NS1 protein as antigen. J Med Virol 2002;67:375-383.
23. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 1979; 76: 4350-4354.

Fig 1.

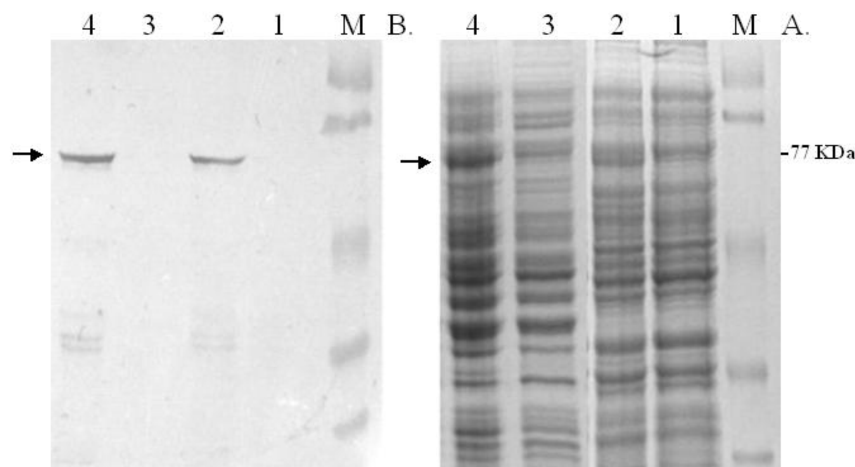

Fig 2.

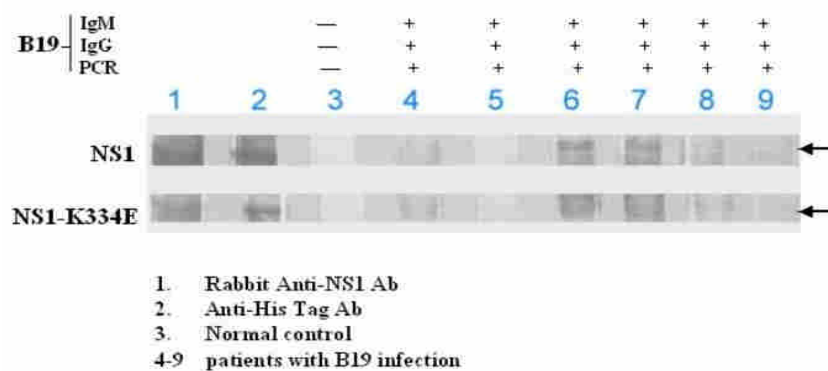

Table 1. Occurrence of antibodies to parvovirus B19 structural (VP1/VP2) and

nonstructural (NS1) proteins from thirty study subjects

Patient	VP1/2	VP1/2	B19	NS1	NS1K334E
	IgM	IgG	DNA	IgG	IgG
Acute B19 infection	6	6	5	4	4
Convalescent B19 infection	0	16	0	0	0
Without B19 infection	0	8	0	0	0