

A CpG oligodeoxynucleotide inducing anti-coxsackie B3 virus activity in human peripheral blood mononuclear cells

Zhongyi Cong¹, Min Wan², Xiuli Wu², Li Wang¹, Xiaoping Hu², Fenglei Yang¹, Musheng Bao², Xuesong Zhang³, Jianzhu Chen⁴, Liying Wang² & Yongli Yu¹

¹Department of Immunology, College of Basic Medicine, Jilin University, Changchun, China; ²Department of Molecular Biology, College of Basic Medicine, Jilin University, Changchun, China; ³The Blood Center of Jilin Province, Changchun, China; and ⁴Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA

Correspondence: Yongli Yu, Department of Immunology, Jilin University, Changchun 130021, China. Tel.: +86 431 85166471; fax: +86 431 85166471; e-mail: ylyu@mail.jlu.edu.cn

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CpG ODN; coxsackie virus; IFN- α ; IFN- β ; IFN- ω ; IFN- γ .

Introduction

Coxsackie viruses are Enteroviruses belonging to the *Picornaviridae* family of single positive-stranded RNA viruses. Based on lesions observed in newborn mice, coxsackie viruses are classified into two large groups, with 24 coxsackie viruses in group A, and six in group B (Pulli *et al.*, 1995; Bolanaki *et al.*, 2005). Coxsackie A viruses affect skeletal muscle, while coxsackie B viruses cause pathological changes in several tissues, including the central nervous system, pancreas, liver and brown fat (Hyypia & Stanway, 1993). Coxsackie B viruses are the etiological agents of a wide spectrum of human diseases, including mild respiratory infection, aseptic meningitis, and fatal myocarditis. Outbreaks of coxsackie B virus infection occur annually throughout the world (Patel *et al.*, 2004), and coxsackie B3 virus (CVB3) is the most significant pathogen of acute and chronic myocarditis in humans (Liu & Mason, 2001; Patel *et al.*, 2004).

Myocarditis caused by CVB3 experiences three phases. In phase 1, the virus enters and proliferates in the myocardium and subsequently activates the host immune system. In rare

Abstract

Coxsackie B3 virus (CVB3) is the most significant pathogen causing myocarditis in humans, and antiviral therapy would be most effective in the early stages of the disease. Here we provide evidence that BW001, a C-type CpG oligodeoxynucleotide, induces anti-CVB3 activity in human peripheral blood mononuclear cells (PBMCs). In parallel, we have demonstrated that BW001 induces human PBMCs to express mRNAs of multiple types of interferon (IFN), including IFN- α , IFN- β , IFN- ω and IFN- γ , and to express mRNAs of at least 11 subtypes of IFN- α . The induced IFNs may contribute to the anti-CVB3 activity. The results suggest that BW001 could be developed into a medication with the potential to treat CVB3 infectious diseases by inducing natural mixed IFNs.

cases, if host immune activation continues unabated despite elimination of the virus, T cells may target the host's own tissue through molecular mimicry, initiating phase 2 of the disease. In this stage, cytokine activation and cross-reacting antibodies may further accelerate cardiac damage. The cumulative effect may lead to dilated cardiomyopathy, when the disease develops into phase 3 (Rezkalla *et al.*, 1996; Liu & Mason, 2001). In general, it has been considered that antiviral therapy would be most effective in phase 1 of the disease process (Levi & Alejos, 2001; Liu & Mason, 2001). Potentially effective antiviral agents include coxsackie receptor blockers (See & Tilles, 1992), nucleoside analogues (Kishimoto *et al.*, 1988; Heim *et al.*, 1997), immunoglobulin (McNamara *et al.*, 1997; Bozkurt *et al.*, 1999) and interferon (IFN) (Heim *et al.*, 1995).

In recent years, synthesized CpGs containing oligodeoxynucleotides (ODNs) have been demonstrated to be potent activators of antiviral immunity. It has been found that CpG ODN 2216 can inhibit *in vitro* replication of the hepatitis B virus and HIV type 1 (Gurney *et al.*, 2004; Schlaepfer *et al.*, 2004; Li *et al.*, 2006). Local administration of CpG ODN 1826 or 1018 inhibited the replication of herpes simplex

virus type 2 and reduced the incidence and severity of genital herpes in mouse models (Pyles *et al.*, 2002; Ashkar *et al.*, 2003; Harandi *et al.*, 2003). In addition, CpG ODN 1668 was effective in preventing and treating friend virus-induced leukemia in mice (Olbrich *et al.*, 2002, 2003), and CpG ODN 1826 protected senescence-accelerated mice from lethal influenza virus infection (Dong *et al.*, 2003). Moreover, our previous study showed that BW001, a novel C-type CpG ODN, displayed strong antisevere acute respiratory syndrome coronavirus and antivascular stomatitis virus activity *in vitro* (Bao *et al.*, 2006).

In this study, we investigated the anti-CVB3 activity induced by BW001 in human peripheral blood mononuclear cells (PBMCs), and the types and subtypes of IFNs contributing to the activity.

Materials and methods

Oligodeoxynucleotides

Nuclease-resistant phosphorothioate-modified ODNs were synthesized by Sangon Biotech Company (Shanghai, China). The CpG ODNs used in this study were BW001 (5'-TCGTCGGGTGCGACGTCGCAGGGGGG-3'), 2216 (5'-GGgggacgatcgtcGGGGGg-3'), 2006 (5'-TCGTCGTTTTGTCGTTTTGTCGTT-3') and c274 (5'-TCGTCGAACGTTTCGATGAT-3') (Krug *et al.*, 2001; Marshall *et al.*, 2003). 2216, 2006 and c274 represent typical A-type, B-type and C-type human-cell-stimulating CpG ODNs, respectively. BW002 (5'-TGCTTGGGTGGCAGCTGCCAGGGGGG-3') is the non-CpG control of BW001, which is the same as BW001 except that the CG sequence is reversed. Capital and lowercase letters represent phosphorothioate and phosphodiester linkage, respectively. All ODNs were diluted in TE buffer (10 mmol L⁻¹ Tris, 1 mmol L⁻¹ EDTA, pH 7.0) using pyrogen-free reagents, and were tested for endotoxin using the Limulus amoebocyte lysate assay (Associates of Cape Cod, Inc.).

Cells and cell line

Human PBMCs were isolated from buffy coats (Blood Center of Jilin Province, China) by Ficoll – Hypaque density gradient centrifugation (Pharmacia) and washed three times with Iscove's modified Dulbecco's medium (GIBCO). The viability of the PBMCs was 95–99% as determined by trypan blue exclusion. Hela cells (human uterine cervix cancer cell line, from ATCC) were cultured at 37 °C in a 5% CO₂ humidified incubator and maintained in Iscove's modified Dulbecco's medium supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) and antibiotics (100 IU mL⁻¹ of penicillin and 100 IU mL⁻¹ of streptomycin).

CVB3 protection assay

CVB3 (from Dr Yaping Chang, Department of Immunology, Jilin University, Changchun, China) was multiplied in Hela cells. After titration by a cytopathic effects method, the virus was stored in aliquots at –70 °C until use. The antiviral activities in the culture supernatants of CpG ODN-treated human PBMCs were measured as described (Tokunaga *et al.*, 1992). Briefly, the supernatants of cultured PBMCs (2 × 10⁶ cells mL⁻¹) stimulated with BW001, 2216, 2006, c274 or BW002 (3 µg mL⁻¹) for 36 h were collected and stored in aliquots at –70 °C until use. The Hela cells were seeded into 96-well flat-bottomed plates (2 × 10⁴ cells well⁻¹) and cultured for 12 h to confluence. The cells were incubated with 100 µL of the 10-fold-diluted supernatants for 24 h and then challenged with 10 × TCID₅₀ (50% tissue-culture infectious doses) of CVB3 for a further 48 h. After staining with 0.5% crystal violet, the cytopathic effects were examined using a multi-well microtiter plate reader at A_{570 nm} and expressed as an OD value. The recombinant human IFN-α 2b used in this assay was purchased from Hansheng Pharmaceutical Corp. Ltd (Zhejiang, China).

Analysis of IFN-α induced by CpG ODN

Human PBMCs (2 × 10⁶ cells mL⁻¹) were treated with BW001 or BW002 (3 µg mL⁻¹) for 36 h and the supernatants were harvested. IFN-α-detecting enzyme-linked immunosorbent assay (ELISA) kits (Pierce) were used for detecting IFN-α in the supernatants according to the manufacturer's protocols. The IFN-α in the supernatant was quantitatively calculated based on the standard curve.

Reverse transcriptase PCR detection of IFN mRNA

Human PBMCs (1 × 10⁷ cells mL⁻¹) were treated with BW001 (3 µg mL⁻¹) for 12 h and then lysed for total RNA extraction using the acid phenol-guanidinium thiocyanate-chloroform method as described in Sambrook & Russell (2001). The total RNA of 100 ng was reverse transcribed into cDNA in a reaction with 50 pmol of oligo (dT)₁₈ primer, 10 U of reverse transcriptase XL (AMV), 1 mmol L⁻¹ dNTPs and 20 U RNase inhibitor and deionized H₂O up to 20 µL. The reaction was maintained at room temperature for 10 min and then at 42 °C for 1 h. The reaction mixtures were heated to 95 °C for 5 min to denature the RNA-cDNA hybrids and quick-chilled on ice, and 2 µL of the mixture was used for PCR. Negative control reactions containing all the reagents except the reverse transcriptase were performed in parallel. The PCR was performed with 1 U Taq DNA polymerase and a pair of the primers for IFN-α, IFN-β, IFN-ω, IFN-γ or β-actin as an internal control. The primers for IFN-α were consensus primers designed for amplifying at

Table 1. Sequences of the primers used in RT-PCR for amplifying interferons

Primer	Sequence	Length of yield fragments (bp)
IFN- α		182
Sense	5'-taccagcaXctgaatgac-3'*	
Antisense	5'-ctcatgatttctgctctg-3'	
IFN- β		452
Sense	5'-tggcaattgaatggagg-3'	
Antisense	5'-aggctaggagatcttcag-3'	
IFN- ω		798
Sense	5'-ataaagccaggagcatcc-3'	
Antisense	5'-atcttaggagctatgcc-3'	
IFN- γ		643
Sense	5'-attggctgttactgccag-3'	
Antisense	5'-aagtgagacagtcacagg-3'	
β -Actin		308
Sense	5'-acaccttctacaatgagc-3'	
Antisense	5'-catgaggtagtcagtcag-3'	

*X = a + g.

IFN, interferon.

least 13 IFN- α subtypes, including IFN- α 1, IFN- α 2, IFN- α 4, IFN- α 5, IFN- α 6, IFN- α 7, IFN- α 8, IFN- α 10, IFN- α 13, IFN- α 14, IFN- α 16, IFN- α 17 and IFN- α 21. The sequences of primers and the length of yielded cDNA fragments are showed in Table 1. All primers were synthesized by Sangon Biotech Company (Shanghai, China). The cycle profile of the PCR was as follows: 45 s at 94 °C for denaturation, 45 s at 58 °C for annealing, and 1 min at 72 °C for primer extension for 25 cycles, and, finally, a 10-min extension at 72 °C. PCR products were analysed on a 2% agarose gel in 1 \times TAE, stained with ethidium bromide and visualized under UV light.

Constructing and screening the IFN- α cDNA library

The PCR products amplified with IFN- α consensus primer were purified from 2% agarose gel by freezing and thawing the gel, and then inserted into pMD-18T plasmid vector (TaKaRa, Dalian, China) by DNA ligation reaction. The

ligation mixture was transformed into *Escherichia coli* JM109 (TaKaRa, Dalian, China) to construct an IFN- α cDNA library. The JM109 cells from the library were inoculated onto a 1.5% agar (Sanland International Inc) Luria-Bertani (LB) plate containing 1% tryptone (OXOID, UK), 0.5% yeast extract (OXOID), 1% NaCl and 50 $\mu\text{g mL}^{-1}$ of ampicillin. After incubation at 37 °C for 12 h, the colonies were randomly picked up. The plasmids were isolated from the colonies and were identified by enzyme digestion with EcoRI and HindIII (TaKaRa, Dalian, China). The inserts in the plasmid from 50 clones were sequenced by the Sangon Biotech Company (Shanghai, China). After conducting a BLAST search of all the sequences on the NCBI website, the percentages of clones carrying cDNAs representing various subtypes of IFN- α were calculated.

Statistical analysis

Data are shown as means \pm SD. The statistical significance of differences was determined using the paired two-tailed Student's *t*-test. Differences were considered statistically significant for $P < 0.05$.

Results

BW001 induces strong anti-CVB3 activity in human PBMCs

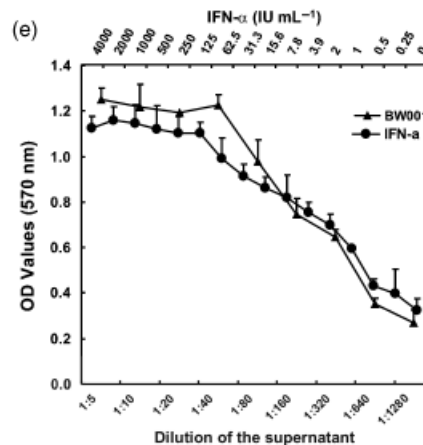
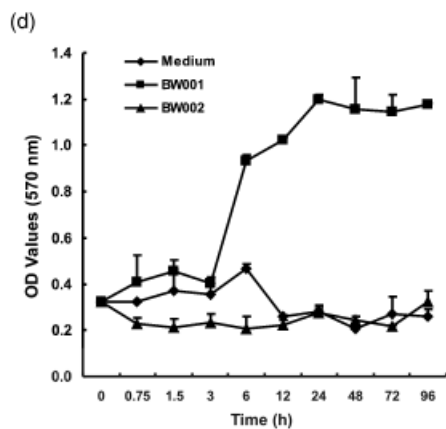
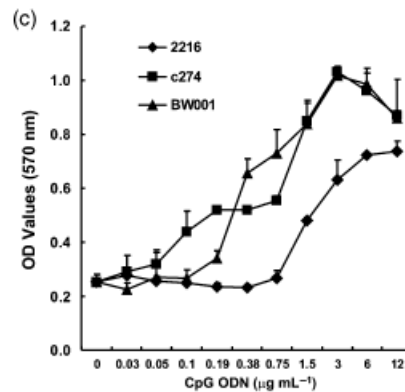
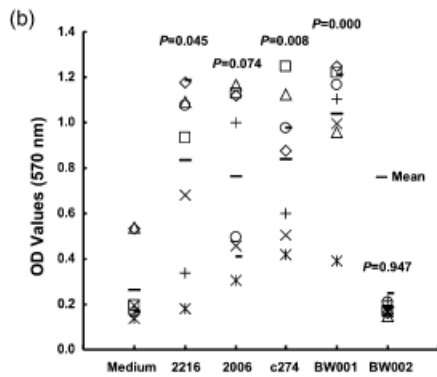
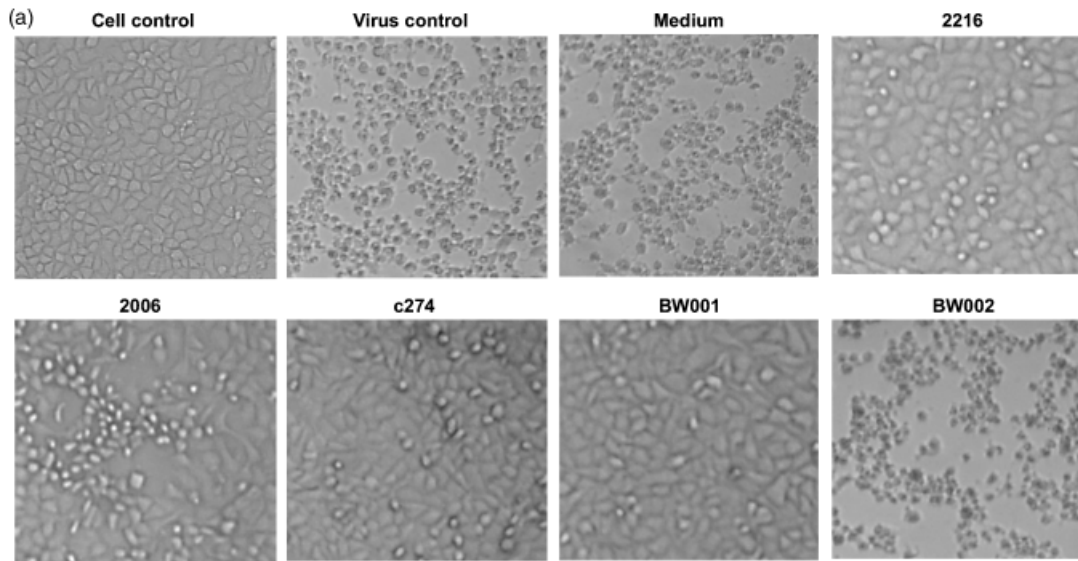
To study the anti-CVB3 activity induced by BW001, we cultured human PBMCs from one healthy donor with CpG ODN including BW001, BW002, 2216, 2006 and c274. The collected supernatants were used to protect the HeLa cells attacked by CVB3. Observations (Fig. 1a) under a light microscope showed that the supernatant induced with BW001 protected the cells from infection, but that the supernatant induced with BW002, a control ODN of BW001, failed to protect the cells. The supernatants induced with c274, a prototype of C-type CpG ODN, 2216, a prototype of A-type CpG ODN, and 2006, a prototype of B-type CpG ODN, also effectively protected the cells (Krug

Fig. 1. BW001 induces human peripheral blood mononuclear cells (PBMCs) to produce strong anti-coxsackie B3 virus (CVB3) activity. (a) Photomicrographs of HeLa cells protected by BW001-induced supernatant from CVB3 infection. PBMCs were treated with or without various oligodeoxynucleotides (ODNs) ($3 \mu\text{g mL}^{-1}$) for 36 h, and the supernatants were used to treat HeLa cells for 24 h. The cells were attacked by $10 \times$ TCID₅₀ (50% tissue-culture infectious doses) of CVB3 for 48 h and then photographed (magnification $\times 100$). (b) Anti-CVB3 activities of the supernatants from the PBMCs of eight healthy donors. The anti-CVB3 activity in the supernatant (10-fold dilution) was tested by a virus protection assay and represented as an OD value. Each symbol represents the anti-CVB3 activity of one supernatant. Each horizontal line represents the median value of anti-CVB3 activity. 'P': ODN vs. medium. (c) Dose effect of BW001 in inducing anti-CVB3 activity. The human PBMCs were cultured with various doses of CpG ODN for 36 h and the supernatants were assay for their anti-CVB3 activity. Each symbol represents the mean OD value \pm SD of anti-CVB3 activity. (d) Kinetics of BW001-induced anti-CVB3 activity. The human PBMCs were cultured with ODNs at $3 \mu\text{g mL}^{-1}$ for 96 h, and the supernatants were collected at various time points for assaying of their anti-CVB3 activities. (e) Comparison of anti-CVB3 activities between BW001-induced supernatants and recombinant human interferon (IFN)- α . PBMCs were cultured with $3 \mu\text{g mL}^{-1}$ of BW001 for 36 h and the supernatants were collected to determine their anti-CVB3 activities in parallel with the determination of the activity of recombinant human IFN- α . 'IU' represents the international unit of IFN- α . Data from one representative experiment of three are shown.

et al., 2001; Marshall *et al.*, 2003).

To approach a statistical evaluation, the human PBMCs from eight healthy donors were incubated with various ODNs ($3 \mu\text{g mL}^{-1}$) including BW001, BW002, c274, 2216 and 2006. The supernatants were assayed for their anti-

CVB3 activity, which was expressed as an OD value in a bioassay. The results (Fig. 1b) showed that, compared with medium control and BW002, BW001, c274 and 2216 could significantly stimulate the PBMCs to produce anti-CVB3 substances that protect Hela cells from CVB3 infection



($P=0.000$, 0.008 and 0.045 respectively), whereas 2006 failed to induce a significant protection ($P=0.074$). Together, these data indicate that BW001 and other A-type or C-type CpG ODNs can effectively induce an anti-CVB3 reaction in human PBMCs.

We next investigated the dose effect and the kinetics of BW001 in inducing anti-CVB3 activity. In the dose-effect assay, human PBMCs were stimulated with various amounts of BW001, 2216 and c274, and the supernatants were collected and assayed for their anti-CVB3 activities. The results (Fig. 1c) showed that BW001 at $0.38 \mu\text{g mL}^{-1}$ induced evident protection on Hela cells. The protection was increasingly evident when the dose of BW001 was increased from 0.38 to $3 \mu\text{g mL}^{-1}$, and reached a peak at $3 \mu\text{g mL}^{-1}$. The anti-CVB3 activity induced by c274 displayed a similar profile. In contrast, the anti-CVB3 activity induced by 2216 was inferior to that induced by BW001 or c274. In the kinetics assay, human PBMCs were cultured with BW001 or BW002 at $3 \mu\text{g mL}^{-1}$ and the supernatants were collected at various time points for testing. The results (Fig. 1d) showed that apparent activity was induced at 6 h, and that peak activity occurred at 24 h and was maintained until 96 h. In comparison, BW002-stimulated supernatants displayed no anti-CVB3 activity.

To conduct further analysis, we tested the anti-CVB3 activities induced by BW001 and the anti-CVB3 activities of recombinant human IFN- α in parallel. The supernatants (diluted from 1:5 to 1:1280) of cultured human PBMCs and the IFN- α at various concentrations were tested for their anti-CVB3 activities. As shown in Fig. 1e, the profiles of the anti-CVB activities induced by BW001 and by IFN- α were similar. Both had a plateau and then decreased gradually. Based on the intersection of two curves, we could deduce that 160-fold-diluted supernatant showed equal anti-CVB3 activity to that of 7.8 IU mL^{-1} of IFN- α .

BW001 induces human PBMCs to produce multiple types of IFN and most subtypes of IFN- α

To investigate the mechanisms involved in the anti-CVB3 activity induced by BW001, we assessed the expression of various types of IFNs and subtypes of IFNs induced by BW001. On the basis of the result shown in Fig. 1d, we isolated total mRNA from human PBMCs incubated with BW001 for 12 h. At this time point, the anti-CVB3 activity was obviously induced. Using reverse transcriptase (RT)-PCR, the IFN- α , IFN- β , IFN- ω and IFN- γ mRNAs in the total mRNA were analysed. β -actin-specific mRNA was used as an internal control. The results showed that the BW001-stimulated human PBMCs expressed mRNAs of IFN- α , IFN- β , IFN- ω and IFN- γ (Fig. 2a), and that higher levels of mRNAs of IFN- α and IFN- β were induced than those of IFN- ω and IFN- γ . These results imply that the anti-CVB3

activity induced by BW001 may correlate with the expressions of IFN- α , IFN- β , IFN- ω and IFN- γ . There is increasing evidence that IFN- α is a major factor in conferring antiviral activity to the human body, and we therefore evaluated the IFN- α produced by BW001-stimulated human PBMCs from a number of healthy donors. The PBMCs from six donors were treated with $3 \mu\text{g mL}^{-1}$ BW001 or BW002 for 36 h, and the supernatants were then harvested for IFN- α detection with an ELISA kit. The results showed that BW001 could induce all six donors' PBMCs to produce IFN- α , and the levels of IFN- α ranged from 80 to 200 pg mL^{-1} . The control ODN BW002 did not induce IFN- α (Fig. 2b). The data confirm that BW001 can activate human PBMCs to secrete high levels of IFN- α . To clarify the subtypes of IFN- α that are induced by BW001 further, we tried to analyse the mRNA expression profile of 13 subtypes of IFN- α . Because of the amino acid homology among the subtypes of IFN- α , it is difficult to distinguish them by antibody-based methods such as ELISA. Furthermore, the absence of introns in the genes of IFN- α subtypes and the similarity in the size of genes of IFN- α subtypes make it impossible to discriminate the subtypes of IFN- α by RT-PCR. To conduct the analysis, total mRNAs were isolated from PBMCs from a healthy donor whose PBMCs were treated with BW001 produced 160 pg mL^{-1} of IFN- α . The total IFN- α -specific cDNAs were amplified with IFN- α consensus primers in a RT-PCR and then were used to construct a cDNA library. The inserts of fifty clones in the library were randomly sequenced. After numerating, we found that the clones carrying IFN- α 1 and IFN- α 13 cDNAs comprised 24% of the clones in the library; IFN- α 2, 14%; IFN- α 7, 14%; IFN- α 5, 10%; IFN- α 16, 10%; IFN- α 21, 10%; IFN- α 8, 8%; IFN- α 4, 4%; IFN- α 10, 4% and IFN- α 17, 2%; and that clones positive for IFN- α 6 and IFN- α 14 were not detected (Fig. 2c). The data demonstrate that BW001 can stimulate human PBMCs to express at least 11 subtypes of IFN- α mRNAs, suggesting that BW001 has the potential to stimulate the production of most subtypes of IFN- α .

Discussion

The results of this study demonstrate that *in vitro* incubation of human PBMCs with BW001 induces anti-CVB3 activity. The activity was parallel with the expression of IFN- β , IFN- ω , IFN- γ , and 11 IFN- α subtypes. The data suggest that BW001 may have considerable therapeutic potential for the treatment of CVB3 infection by inducing mixed and natural IFNs.

CVB3 is the most significant pathogen of acute and chronic myocarditis in humans (Liu & Mason, 2001; Patel *et al.*, 2004), and acute myocarditis is characterized by the rapid development of life-threatening congestive heart failure and arrhythmias. In the later stages of the disease, an excessive immune response might aggravate the myocyte

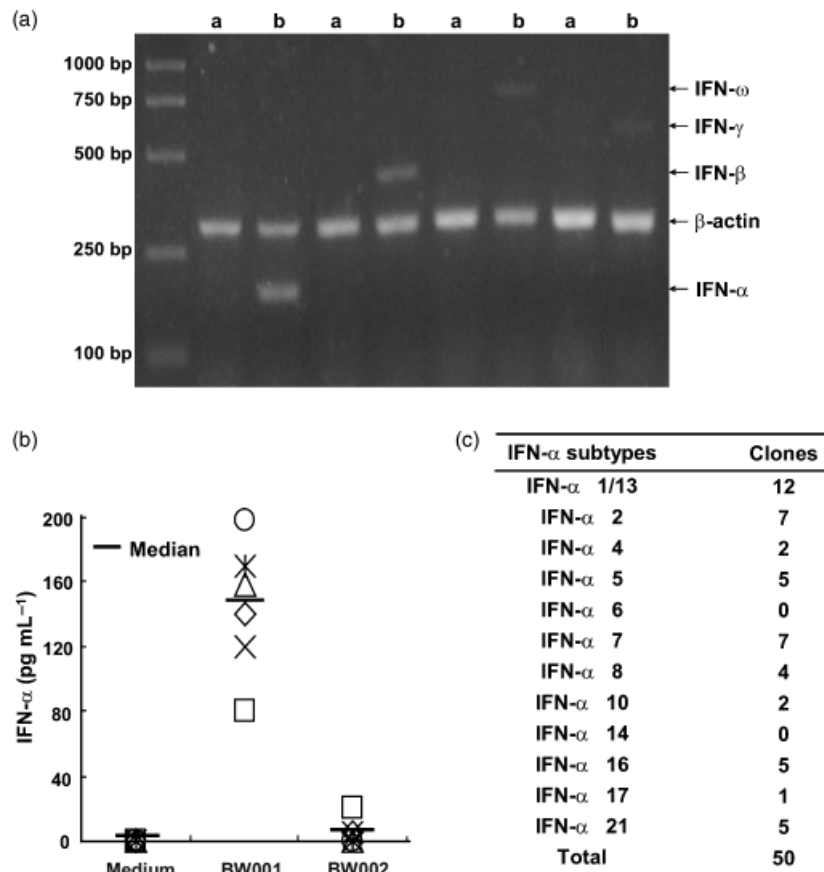


Fig. 2. BW001 induced human PBMCs to express multiple types of IFNs and most subtypes of IFN- α . (a) Detection of various IFNs from human PBMCs by RT-PCR. Human PBMCs were cultured in medium (lane a) or treated with BW001 (lane b) for 12 h and then harvested for total RNA isolation. The primers for amplifying the various IFNs were indicated after the arrows. The results showed that IFN- α , IFN- β , IFN- ω and IFN- γ can all be induced by BW001-treated human PBMCs. (b) IFN- α produced from human PBMCs stimulated by BW001. Human PBMCs from six blood donors were cultured in the medium or in medium containing BW001 or BW002 ($3 \mu\text{g mL}^{-1}$) for 36 h, and the supernatants were harvested for IFN- α detection with an ELISA kit. Each symbol represents the average level of IFN- α produced from the human PBMCs from one donor, and the horizontal line represents the median of the level of IFN- α from six donors. Data from one representative experiment of three are shown. (c) IFN- α subtypes expressed by BW001-stimulated human PBMCs. Human PBMCs (donor 056315) were stimulated with BW001 for 12 h and then harvested for isolation of total RNA. The total cDNAs were synthesized using an oligo (dT)₁₈ primer and then used as templates for PCR amplification of IFN- α s with IFN- α consensus primers. The IFN- α cDNA library was constructed by inserting the PCR products into pMD-18T plasmids. The inserts were randomly sequenced and the IFN- α subtypes were determined from the NCBI website. The inserts of 50 clones were sequenced, and the clones representing different IFN- α subtypes were counted. The results show that most subtypes of IFN- α can be expressed by BW001-stimulated human PBMCs.

destruction. Experiments in mice have shown that antiviral treatment consisting of IFN, immunoglobulins and nucleoside analogues in the early stages of the disease could limit its development (Levi & Alejos, 2001; Liu & Mason, 2001). The finding that BW001 induced significant anti-CVB3 activity in human PBMCs in the 6 h after administration implies that BW001 could be used as an alternative medication for the early treatment of CVB3 infection.

The *in vitro* induction of anti-CVB3 activity in human PBMCs is probably the result of IFN production. Upon invasion by viruses, the cells of the human body can produce various type I IFNs, including IFN- α , IFN- β and IFN- ω , to

limit the virus infection. In the human immune system, plasmacytoid dendritic cells expressing Toll-like receptor 9 are responsive target cells of CpG ODN with respect to antiviral reactions (Krug *et al.*, 2001). After the challenge of viruses, plasmacytoid dendritic cells are capable of secreting very large amounts of IFN- α/β , at levels up to a thousand times more than are typically produced by most cells (Siegal *et al.*, 1999). The results presented here imply that a significant control of CVB3 infection might be achieved *in vivo* by the injection of BW001, which will induce type I IFN. IFN has been reported to inhibit the replication of viruses, activate natural killer cells (Pestka *et al.*, 2004), kill

virus-infected cells, and facilitate the generation of cytotoxic T lymphocytes that conduct further powerful killing of virus-infected cells.

In humans, there are at least 13 subtypes of IFN- α (Maeda *et al.*, 1980; Pestka *et al.*, 1987; Pestka, 2000). It is generally believed that the transcription of the various IFN- α species is modulated through multiple signalling pathways depending on the virus and types of cells infected. It has been found that a distinct virus may induce a characteristic profile of IFN production. For example, Newcastle disease virus preferentially induces the IFN- α 8 subtype in infected cells (Barnes *et al.*, 2001); hepatitis C virus induces increased IFN- α 5 in PBMCs, Sendai virus causes human peripheral blood leucocytes to express at least nine IFN- α species, including IFN- α 1, IFN- α 2, IFN- α 4, IFN- α 7, IFN- α 8, IFN- α 10, IFN- α 14, IFN- α 17 and IFN- α 21 (Nyman *et al.*, 1998). On the other hand, different IFN- α subtypes seem to exhibit different profiles of antiviral activity (Cajean-Feroldi *et al.*, 2004). For example, the antivascular stomatitis virus effects *in vitro* are due to the existence of IFN- α 1, IFN- α 21, IFN- α 16, IFN- α 5, IFN- α 4, IFN- α 6, IFN- α 14, IFN- α 8, IFN- α 7, IFN- α 17 and IFN- α 10 (Schanen *et al.*, 2006). Recombinant human IFN- α 1, IFN- α 2, IFN- α 5, IFN- α 8 and IFN- α 10 could inhibit the replication of hepatitis C virus. Among these, IFN- α 8 was the most and IFN- α 1 was the least effective (Koyama *et al.*, 2005). Our results that BW001 induced 11 IFN- α subtypes from the human IFN- α gene portfolio suggest that the anti-CVB3 activity induced by BW001 is correlated with the production of mixed IFN- α in a balanced ratio.

In addition to type I IFN, IFN- γ may also contribute to the anti-CVB3 activity induced by BW001. The induced IFN- γ , together with the induced IFN- α , activates natural killer cells to kill CVB3-infected cells in the early stages of the infection. In the ongoing immune response, the induced IFN- γ promotes virus-infected cells to express major histocompatibility complex proteins that will facilitate the recognition of cell surface-bound viral antigens and the cytolysis of virus-infected cells by cytotoxic T lymphocytes.

The property of inducing naturally mixed types of IFN and IFN- α subtypes may make BW001 a promising agent to be developed for the treatment of CVB3 infection and other virus-associated diseases. In comparison with the most widely used recombinant IFN, BW001 may have several advantages in the treatment of virus infections. The first is that BW001 induces mixed IFNs, whereas the recombinant IFN currently used is usually a single type of IFN. For instance, recombinant IFN- α 2b is used to treat hepatitis C, hepatitis B, genital warts, Kaposi's sarcoma, hairy cell leukemia, chronic myelogenous leukemia, malignant melanoma, advanced hepatocellular carcinoma, and Erdheim-Chester disease (Samuel, 2001; Braithe *et al.*, 2005; Kurokohchi *et al.*, 2005; Sarin *et al.*, 2005; Ward & Kugelmas,

2005); and IFN- β is used for the treatment of relapsing forms of multiple sclerosis (Banwell *et al.*, 2006; Derwenskus & Lublin, 2006). Obviously, the synergistic action of mixed IFNs is more effective than a single type of IFN. The second advantage is that BW001, with a phosphorothioate backbone, is more resistant to endogenous nucleases and therefore has a longer half-life both *in vitro* and *in vivo* (Sands *et al.*, 1994; Yu *et al.*, 2002). For comparison, in the human body, the half-life of a regular recombinant IFN- α is 3–4 h and that of the long-term recombinant IFN- α carrying polyethylene glycol is 35 h (Glue *et al.*, 2000). The third advantage is that BW001 induces endogenous IFNs with no immunogenicity in the human body. Repeated use of recombinant IFNs was reported to induce IFN-neutralizing antibodies that will hinder continuous therapy (Oberge & Alm, 1989; Lok *et al.*, 1990). It is noteworthy that naturally occurring IFNs have been proved to have a higher antiviral activity than their recombinant counterparts *in vitro* (Heim *et al.*, 1995; Dahl *et al.*, 2004).

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