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Inhibition of respiratory syncytial virus of subgroups A and B using deoxyribozyme DZ1133 in mice

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Abstract

Respiratory syncytial virus (RSV) commonly infects the upper and lower respiratory tracts. Currently, there is no effective treatment available. Deoxyribozymes are a potential therapeutic for RSV and their activity is based on the ability to bind and cleave complementary RNA sequences to inhibit protein expression. DZ1133 is a deoxyribozyme that targets the conserved genomic RNA sequence of the RSV nucleocapsid protein and has been shown to significantly inhibit various strains of RSV including subgroups A and B, standard A2 and CH18537 strains, and CQ381513, CQ381170, BJ01 and BJ04 strains. Treatment with DZ1133 decreased viral plaque formation in lungs of RSV-infected BALB/c mice. In addition, viral mRNA expression was reduced, airway inflammation was alleviated, and leukocyte counts were reduced in bronchoalveolar lavage fluid of RSV-infected mice. The antiviral effect of DZ1133 was dose-dependent (0.2–0.8 mg) and more efficient than antisense oligonucleotide inhibition of gene expression. However, levels of cytokines TNF-α, IFN-γ, IL-12, and IL-10 induced by RSV infection were not affected by DZ1133 treatment. Our data demonstrate that DZ1133 is a potential therapeutic agent against both subgroups A and B RSV infection *in vivo*.

Keywords: Respiratory syncytial virus; Deoxyribozyme; Antiviral; Mice

Human respiratory syncytial virus (RSV) is the leading viral cause of respiratory tract infections in infants and children worldwide. Nearly all children are infected with RSV by 2 years of age (Black, 2003). RSV re-infection occurs frequently and is associated with the development of wheezing and asthma (Holt and S1y, 2002; Openshaw et al., 2003; Kalina and Gershwin, 2004). Unfortunately, no effective treatment of RSV infection, beyond supportive measures, is currently available. However, several treatment strategies are under investigation. Ribavirin is the only therapeutic drug approved for RSV infection, but its effectiveness is controversial (Guerguerian et al., 1999; Long et al., 1997). RSV is a mutable RNA virus and has been classified into subgroups A and B. Epidemics of RSV are associated with numerous strains from both A and B subgroups (Guney et al., 2004), and the genotype has been found to vary from year to year (Kuroiwa et al., 2004). Given the frequency of RSV infection worldwide, novel and effective approaches for the treatment of RSV infection are urgently required.

Currently, targeting of the viral genome represents an attractive target for therapeutic intervention, particularly for non-segmented, negatively stranded RNA viruses such as RSV. Arresting virus replication by cleavage of the viral genome would abrogate viral amplification. Deoxyribozymes are synthetic, single-stranded DNA molecules with catalytic capabilities that can be selected from combinatorial libraries *in vitro* based on their ability to cleave a target RNA substrate. The most prominent deoxyribozyme to cleave RNA substrate named "10–23 deoxyribozyme" typically consists of a catalytic domain of 15 nucleotides and two binding arms of 6–12 nucleotides each. Deoxyribozymes generally bind to a complementary RNA sequence through Watson–Crick base pairing to cleave the RNA substrate between an unpaired purine (A or G) and a paired pyrimidine residue (C or U) (Santoro and Joyce, 1997). The first deoxyribozyme was reported in 1997 and has since been used as a model system with which to suppress protein expression from a variety of target genes (Achenbach et al., 2004; Sun et

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al., 2000; Schubert and Kurreck, 2004). Deoxyribozymes have also been successfully used *in vivo* (Zhang et al., 2004; Lowe et al., 2001; Peter et al., 2004; Isaka et al., 2004) and to treat human immunodeficiency virus, hepatitis C virus, and influenza virus (Chakraborti and Banerjea, 2003; Trepanier et al., 2006; Takahashia et al., 2004). Based on these data, deoxyribozymes have the potential to arrest or impede RSV replication through specific and effective inhibition of viral gene expression.

In our previous study we designed and tested deoxyribozymes targeted to RSV. Dexoyribozyme DZ1133, which targets the conserved genomic RNA sequence of RSV nucleocapsid (N) protein, was shown to actively inhibit RSV of subgroups A and B *in vitro* (Zhao et al., 2003; Xie et al., 2006). In this study, we investigate the antiviral activity of this deoxyribozyme against both subgroups A and B RSV in BALB/c mice to explore its effects on inflammation and the immune response in local airways.

1. Materials and methods

1.1. Cells and virus

The RSV standard A subgroup strain A2, B subgroup strain CH18537 (stored in our lab), and four isolated wildtype strains CQ381513, CQ381170 (from Chongqing Children's Hospital of China) and BJ01, BJ04 (from Beijing Children's Hospital of China provided by Prof. Kun-Ling Shen) were grown in Hep-2 cells and purified by centrifugation. Determination of viral titer by plaque forming assay was performed as described elsewhere (Jennifer and McKimm-Breschkin, 2004). The viral titers of the six strains ranged from 2.42×10^6 to 4.60×10^7 PFU/ml. The RSV protein N genomic RNA in all six RSV strains contained the conserved start signal: 5′-AUUUUGUCCC-3′, as determined by genetic sequencing. The isolated wildtype strains also had their subgroup identified as previously described (Xie et al., 2006). CQ381170 belonged to the B subgroup, while the other three isolated wildtype strains belonged to the A subgroup.

1.2. Oligonucleotides

The deoxyribozyme DZ1133 (5′-*TG*GGGCAAAGGCTAGCTACAACGAACAAAG*ATG*-3′) has a catalytic domain that is underlined. The antisense oligonucleotide, AS1133 (5′-*TG*GGGCAAATACAAAG*ATG*-3′), targets the same conserved sequence of RSV genomic RNA at the start signal of the N gene. The mutant deoxyribozyme, mutDZ1133 (5′-*TG*GGGCAAAGGCA*AGCTACAACGAACAAAG*ATG*-3′), contains a mutated nucleotide (T → A*) at the 4th position of the catalytic domain (underlined). Oligonucleotides were synthesized using an ABI 392 DNA/RNA synthesizer, modified with phosphorothioates (shown as italics in the provided sequences), and linked with 3′-Cholesteryl-TEG CPG (Glen Research, Sterling, VA). Oligonucleotides were purified by high performance liquid chromatography at the Academy of Military Medical Sciences of China. The ability of DZ1133 to cleave protein N RNA and mediate anti-RSV effects were previously demonstrated *in vitro* (Xie et al., 2006).

1.3. Animal protocol

Specific pathogen free, 8–12 week old, female BALB/c mice were purchased from the Academy of Medical Sciences of China and maintained in micro-isolator cages under pathogen free conditions in the animal research facility of Chongqing University of Medical Sciences. Mice were randomly grouped with 8 mice per group. Following anesthetization, mice were intranasally inoculated with 10^5 (RSV CH18537, CQ381513, CQ381170, BJ01 and BJ04) or 10^6 (RSV A2) PFU/50 μl virus (according to the maximal titer of the RSV strains in cell culture). Twenty-four and 48 h post-infection the mice were intranasally administered with 0.2–0.8 mg DZ1133, AS1133, or mutDZ1133 dissolved in 50 μl phosphate buffer solution (PBS). The dose and time of delivery were based on the media effective concentration (EC50) of DZ1133 determined in our previous cell culture experiments, and based on the intranasal delivery of antisense oligonucleotides against RSV in rats (Xie et al., 2006; Cramer, 2005). The intranasally delivered DZ1133 was labeled with fluorescein to assay its distribution in lung tissue and was found to be well distributed (data not shown). Infected control mice were treated with 50 μl PBS, and negative control mice were treated with 0.4 mg DZ1133 without prior RSV-infection. Uninfected mice received intranasal instillation of PBS served as blank control. Some mice were sacrificed on day 3 post-infection (the peak of RSV replication according to our preliminary experiments) for viral assays and collection of bronchoalveolar lavage fluid (BALF), and some mice were sacrificed on day 6 post-infection for pulmonary histopathology.

1.4. Plaque forming assay

Fresh lung samples were removed, weighed, and homogenized at 4 °C in 10 volumes of PBS containing 0.218 M sucrose, 25 mM hydroxyethyl piperazine ethanesulfonin acid, 4.8 mM sodium L-glutamate, 200 U/ml penicillin G and 200 μg/ml streptomycin (pH 7.4). Vero monolayers, 80% confluent in Costar 6-well plates, were used for the plaque assay. Thawed tissues were clarified, serially diluted, plated on Vero monolayers, and adsorbed for 2 h. The overlay mediums containing equal volumes of 1.5% agarose and 2% FBS 1640 culture medium were added to each well. After 5 days of incubation at 37 °C and addition of 5 mg/ml 3-(4,5-diphenyl thiazol-2-yl)-2,5-diphenyl tretrazolium bromide for the last 4 h, plaques were counted. The resulting titer was divided by the lung weight and reported as PFU per gram of lung.

1.5. RT-PCR of the RSV N gene mRNA

Total cellular RNA was isolated from frozen lung samples using Trizol reagent (GibcoBRL, LifeTechnologies) and 4 μl total RNA was reverse transcribed to cDNA by M-MLV reverse transcriptase enzyme (Promega, Co.) according to the manufacturer's instructions. Following cDNA synthesis, the PCR mixtures contained 2 μl cDNA, 2 U Hot start Taq™ DNA polymerase (Takara Biotech, Co.), 5 × Taq™ DNA polymerase buffer, 1.0 nM each dNTP, forward and

reverse primers (1 μM each) in 25 μl total volume. The primers used for the RSV N gene resulting in a 460 bp product were: 5′-CTGGTCTTACAGCCGTGAT-3′(F) and 5′-CCAGCAGCATTGCCTAATAC-3′(R). Primers for β-actin resulting in a 366 bp product were 5′-ACCAACTGGGACGACATGGAGAAGATC-3′(F) and 5′-GTAGCCACGCTCGGTCAGGATCTTCAT-3′(R). The PCR program consisted of the following steps: 94 °C for 5 min, amplification for 40 cycles (94 °C for 40 s, 46 °C for 40 s, 72 °C for 40 s) for the RSV N gene, and amplification for 25 cycles (94 °C for 40 s, 60 °C for 40 s, 72 °C for 40 s) for the β-actin gene, and the last extension cycle for 72 °C for 7 min. The amplified products were separated by electrophoresis on 1.5% agarose gels and standard curves were generated to show that PCR detection was in the linear range. The values for RSV N mRNA were normalized to β-actin mRNA levels.

1.6. Pulmonary histopathology

Lungs were inflated via a tracheal cannula at 20 cm of pressure with 4% paraformaldehyde and removed *en bloc* from the thorax. Lungs were dehydrated, embedded in paraffin, and cut to 5 μm thick sections. Tissue sections were stained with hematoxylin and eosin (H/E) for routine morphological studies. Histopathology was scored according to a previously described method (Cimolai et al., 1992).

1.7. Bronchoalveolar lavage

Mice were sacrificed and the trachea was immediately opened by incision of the cricothyroid membrane to flush the lungs twice with 0.5 ml ice-cold sterile PBS. Total leukocytes in bronchoalveolar lavage fluid (BALF) were measured using a hemocytometer. Differential cell counts were performed by counting 200 leukocytes on cytocentrifuged preparations stained with Wright based on conventional morphologic criteria of macrophages, lymphocytes, neutrophils and eosinophils.

1.8. ELISA for cytokines

Supernatants of cytokines in BALF were tested for TNF-α (Diaclone, France, Cat. no. 860.040), IFN-γ (Diaclone, France, Cat. no. 860.050), IL-12 (Biosource, USA, Cat. no. KMC0121), and IL-10 (Biosource, USA, Cat. no. KMC0101) using sandwich ELISA kits. The assays were performed according to manufacturers' instructions. Optical densities were read at 450 nm, and the concentrations of cytokines in each sample were determined from the standard curves.

1.9. Statistical analysis

Data were given as the mean ± S.D. and analyzed with SPSS for Windows Version 10.0 (SPSS Inc.). The analysis of variance between groups (ANOVA) was used to compare mean values between normally distributed data. A two-sided P-value of <0.05 was considered to be statistically significant.

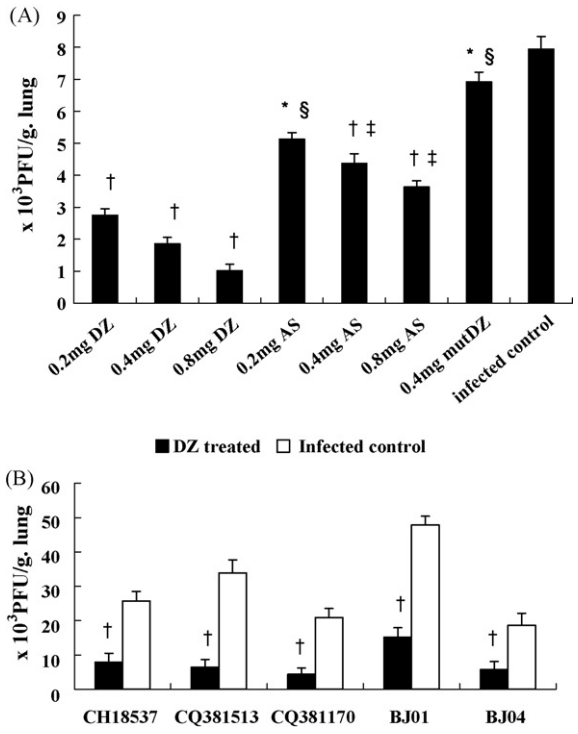

Fig. 1. Inhibitory effects of DZ1133, AS1133, and mutDZ1133 on viral titer of lung tissue in RSV-infected mice. BALB/c mice were infected with RSV standard A2 strain (10^6 PFU), CH18537 strain, and isolated wildtype strains CQ81513, CQ381170, BJ01 and BJ04 (10^5 PFU), then intranasally treated with 0.2–0.8 mg DZ1133, AS1133, and mutDZ1133 24 and 48 h post-inoculation. On day 3 post-infection mice were sacrificed to detect the viral titer of lung tissue by plaque forming assay. (A). Effects of DZ1133, AS1133, and mutDZ1133 on viral titer of lung tissue in RSV A2 strain infected mice. (B). Effects of 0.4 mg DZ1133 on viral titer of lung tissue in RSV standard CH18537 strain and isolated wildtype strains CQ81513, CQ381170, BJ01 and BJ04 infected mice. The data were showed as mean ± S.D. ($n = 8$/group). *$P < 0.05$ vs. infected control mice; †$P < 0.01$ vs. infected control mice; ‡$P < 0.05$ vs. 0.2 mg DZ treated mice; §$P < 0.01$ vs. 0.2 mg DZ1133 treated mice.

2. Results

2.1. Inhibitory effect of DZ113 on the viral yield of RSV subgroups A and B

To investigate the antiviral capacity of DZ1133, it was compared with another anti-RSV reagent, AS1133. Three days post-infection, plaque-forming assays were performed to determine the viral titer of collected lung tissues. Administration of DZ1133 reduced the viral titers of lung tissue compared to RSV A2 strain-infected mice. The inhibitory effect of DZ1133 increased with its dose within the range of 0.2–0.8 mg and was more significant than the inhibitory effect of AS1133. Treatment with 0.4 mg mutDZ1133 on the RSV A2 strain was less than that of 0.4 mg AS133 (Fig. 1A). In mice infected with various RSV strains belonging to subgroup A or B, treatment with 0.4 mg DZ1133 also resulted in decreased viral titers (Fig. 1B).

2.2. Inhibition of subgroups A and B RSV viral replication

Expression of RSV N gene mRNA was quantitated by RT-PCR to measure the effects of DZ1133 activity on expression

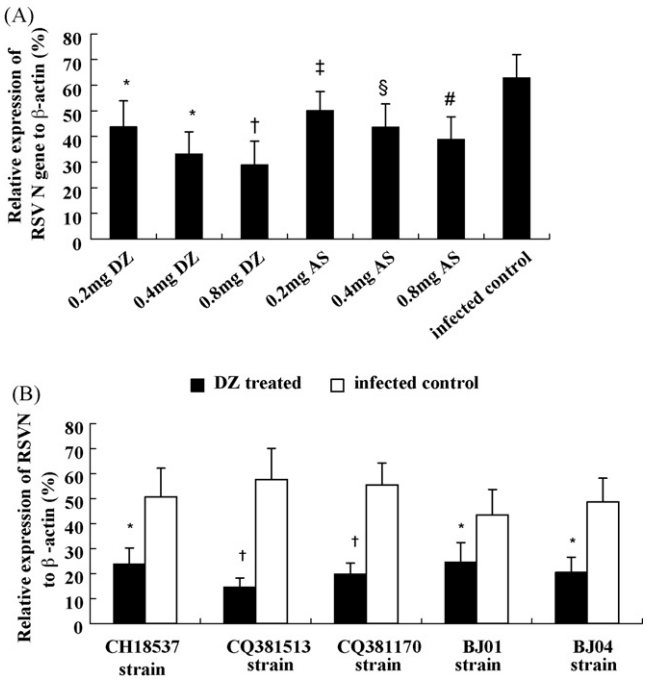

Fig. 2. Inhibitory effects of DZ1133 and AS1133 on mRNA expression of the RSV nucleocapsid N gene in RSV-infected mice. BALB/c mice were infected with RSV standard A2 strain (10^6 PFU), CH18537 strain, and isolated wild-type strains CQ81513, CQ381170, BJ01 and BJ04 (10^5 PFU), then intranasally treated with 0.2–0.8 mg DZ1133 or AS1133 24 and 48 h post-inoculation. Mice were sacrificed on day 3 post-inoculation to isolate total cellular RNA of lung issue and amplify RSV N gene mRNA by RT-PCR. (A) Relative expression of viral N gene mRNA against β-actin in RSV A2 strain infected mice. (B) Relative expression of viral N gene mRNA against β-actin in RSV strains CH18537, CQ81513, CQ381170, BJ01 and BJ04 infected mice. The data were showed as mean ± S.D. ($n = 8$/group). *$P < 0.05$ vs. respective infected control mice; †$P < 0.01$ vs. respective infected control mice; ‡$P < 0.05$, vs. 0.2 mg DZ treated mice; §$P < 0.05$ vs. 0.4 mg DZ treated mice; #$P < 0.05$ vs. 0.8 mg DZ1133 treated mice.

of the nucleocapsid protein. Treatments of 0.2, 0.4, or 0.8 mg of DZ1133 decreased N gene mRNA expression in the RSV A2 strain by 30.51, 47.38% ($P < 0.05$), and 53.97% ($P < 0.01$), respectively, compared to the infected control sample. Furthermore, the inhibitory effect of DZ1133 increased with dose. There was a higher inhibitory effect by DZ1133 on RSV A2 strain mRNA expression than that of AS1133 at the corresponding dosage. MutDZ1133, in contrast, showed no significant effect on RSV A2 strain mRNA expression ($P > 0.05$) (Fig. 2A). Assays of other RSV standard and isolated wildtype strains of subgroups A and B, including CH18537, CQ381513, CQ381170, BJ01, and BJ04 strains, showed significant inhibitory effects with DZ1133 treatment (0.4 mg) resulting in a 43.57–74.83% decrease in viral mRNA expression compared with controls (Fig. 2B).

2.3. Effect of DZ1133 on pulmonary histopathology induced by RSV A2 strain

Pulmonary histopathology was examined by H/E stain from day 6 post-infection mice to examine the effects of DZ1133 on inflammation induced by RSV A2 strain infection. While the control animals presented a clear alveolus without inflammatory infiltration around bronchioles or vessels, RSV-infected mice appeared to have swelling of bronchiolar epithelia, dilation of alveolus, and extensive infiltration with an excess of lymphocytes and macrophages surrounding both bronchioles and pulmonary blood vessels. In the sections with severe lesions, part of the bronchiolar and alveolar epithelia were necrosed and desquamated into the cavities, and erythrocytes had leaked from the vessels. Treatment with DZ1133 and AS1133 ameliorated the histopathology of the RSV A2 strain-infected mice as reflected by the histopathological scores. Additionally, DZ1133 showed more significant effects on decreasing the histopathological scores of infected mice than AS1133 at the dosage of 0.4 and 0.8 mg (Fig. 3).

To determine whether DZ1133 induces inflammatory changes, histological inflammatory scores were compared between the blank control and the negative control treated with 0.4 mg DZ1133. There was no significant difference between the scores of these two groups ($P > 0.05$) (Fig. 3).

2.4. Effect of DZ1133 on leukocytes in BALF of RSV A2 strain infected mice

To determine the effect of DZ1133 on inflammatory cells in the local airways of RSV A2 strain-infected mice, the leukocytes in BALF were counted. Leukocytes numbers were dramatically increased, and a predominance of lymphocytes, as opposed to the preponderant macrophage in BALF of the blank control, were observed ($P < 0.01$). Treatment with 0.4 mg of DZ1133 reduced total leukocytes in BALF of RSV A2 strain-infected mice ($P < 0.05$), but did not effect the percentage of lymphocytes ($P > 0.05$). On the other hand, neither total leukocytes, nor the subset percentages, were significantly different between the blank control and the negative control ($P > 0.05$), indicating that DZ1133 itself does not increase the inflammatory cells in local airways (Fig. 4).

2.5. Effect of DZ1133 on cytokines in BALF of RSV A2 strain-infected mice

The concentrations of TNF-α, IL-12, IFN-γ, and IL-10 in BALF were tested by ELISA to explore the immunologic effect of DZ1133 on RSV A2 strain-infected and un-infected mice. RSV infection alone induced secretions of TNF-α, IL-12, IFN-γ, and IL-10 compared to controls. Treatment with DZ1133 did not decrease secretions of these cytokines during RSV infection, and there was no difference in TNF-α, IL-12, IFN-γ, and IL-10 levels between infected control mice and DZ1133-treated mice ($P > 0.05$). Additionally, cytokine levels of both the negative control and blank control were compared to determine the effect of DZ1133 on un-infected mice, and there was no difference in cytokine production between these two groups ($P < 0.05$) (Fig. 5).

3. Discussion

In the present study, the potent antiviral activity of the deoxyribozyme DZ1133 against both subgroups A and B of RSV

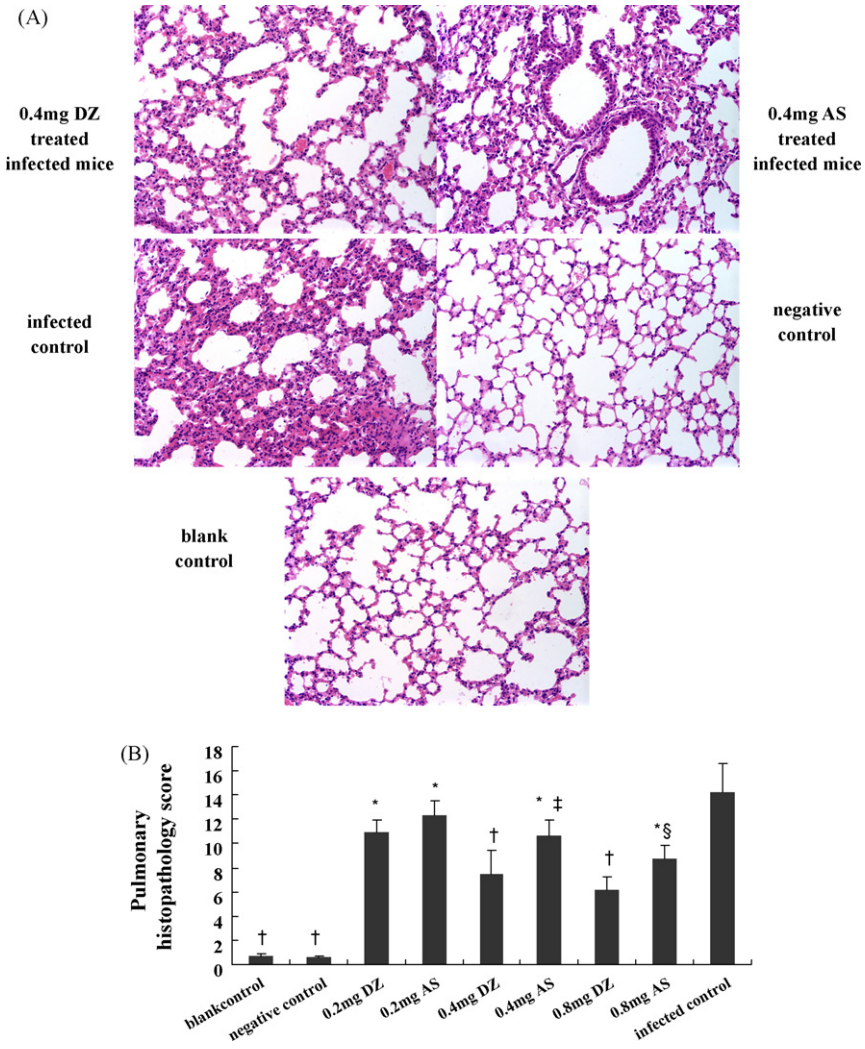

Fig. 3. Effects of DZ1133 and AS1133 on pulmonary histopathology in mice. BALB/c mice were infected with 10^6 PFU of RSV standard A2 strain, then intranasally treated with 0.2–0.8 mg DZ1133 or AS1133 24 and 48 h post-inoculation. On day 6 mice were sacrificed to prepare pulmonary sections for H/E stain. Uninfected mice received instillation of PBS served as blank controls, and the mice treated by 0.4 mg DZ1133 without infection served as negative controls. (A) Pulmonary photomicrographs of 0.4 mg DZ1133 treated mice, 0.4 mg AS1133 treated mice, infected control mice and negative control mice, original magnification ×250. (B) Pulmonary histopathological scores of the control and treated mice, data were shown as the mean ± S.D. ($n = 8$/group). *$P < 0.05$ vs. infected control mice; †$P < 0.01$ vs. infected control mice; ‡$P < 0.05$ vs. 0.4 mg DZ treated mice; §$P < 0.05$ vs. 0.4 mg DZ133 treated mice.

was demonstrated *in vivo*, and shown to inhibit viral replication better than antisense oligonucleotides. Furthermore, DZ1133 ameliorated airway inflammation induced by RSV infection.

Deoxyribozymes provide high sequence-specificity, relative ease and low cost of synthesis, high stability, as well as flexible and direct action in targeting the desired gene (Achenbach et al., 2004; Sun et al., 2000; Schubert and Kurreck, 2004). In this study, DZ1133 was intranasally administrated at 24 and 48 h post-RSV infection based on pharmacokinetic assays in BALB/c mice (data not shown). Local delivery to the target tissue offers significant advantages over systemic delivery. For example, high local concentrations at the target organ avoids drug dilution that occurs with systemic delivery, thereby allowing a lower dose to be administered which can decrease toxicity complications (Ali et al., 2001; Bitko and Barik, 2007). To increase cellular uptake, DZ1133 was linked with cholesterol at the 3′ terminus. This modification also helped prevent 3′ to 5′ exonuclease digestion.

The targeting specificity of deoxyribozymes is determined by the sequence of the binding arms. DZ1133 binds and cleaves the genomic RNA sequence of the RSV nucleocapsid N protein. The N protein plays an important role in the RSV life cycle, particularly in the process of encapsidation of the genome RNA into an RNase-resistant nucleocapsid, as a component of the viral polymerase complex for transcription and replication, and in the interactions of matrix proteins during virus assembly (Grosfeld et al., 1995). Administration of DZ1133 resulted in reduced viral titers and mRNA expression of the RSV A2 strain *in vivo*, and in a dose-dependent manner. A dose of 0.4 mg of DZ1133 showed notable antiviral activity in the A2 strain, as well as in other RSV strains.

The ideal target for a dexoyribozyme as an anti-RSV agent would be a highly conserved site that is maintained in all subtypes of the highly mutable virus. However, RSV protein and nucleic acid sequences vary considerably between RSV sub-

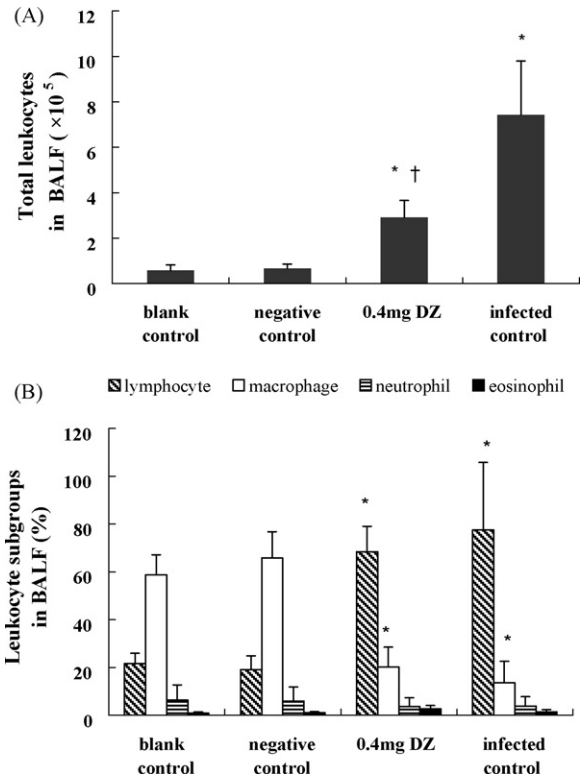

Fig. 4. Effects of DZ1133 on leukocyte counts in BALF of mice. BALB/c mice were infected with 10^6 PFU of RSV standard A2 strain, then intranasally treated with 0.4 mg DZ1133 24 and 48 h post-inoculation. On day 3 mice were sacrificed, 0.5 ml ice-cold sterile PBS was flushed into the lung two times to collect the BALF samples. Uninfected mice receiving instillation of PBS served as blank controls, and the mice treated with 0.4 mg DZ1133 without infection served as negative controls. (A) Effect of DZ1133 on the total leukocyte count in BALF of RSV A2 strain infected mice. (B) Effect of DZ1133 on the percentages of leukocytic subsets in BALF of RSV A2 strain infected mice. Differential cell counts were based on conventional morphologic criteria of macrophages, lymphocytes, neutrophils and eosinophils. The data were showed as mean ± S.D. ($n = 8$/group). *$P < 0.01$ vs. blank control mice; †$P < 0.05$ vs. infected control mice.

groups. The RSV N protein is the most conserved among all 11 known viral proteins, with 86% identity in nucleic acid sequence and 96% amino acid identity between subgroups (Sullender, 2000; Chen et al., 2000; Collins et al., 1990a,b). The conserved start signal for the RSV N gene is considered to be the target of the DZ1133. The RSV A2 strain displays the most virulence and highest titer than the other five strains used in this study. Therefore, 10^6 PFU of the RSV A2 strain were used, and only 10^5 PFU were given of RSV CH18537, CQ381513, CQ381170, BJ01, and BJ04 strains in the challenge of BALB/c mice. DZ1133 showed significant antiviral effects against subgroups A and B types of RSV, as well as against standard A2 and CH18537 strains, against the CQ381513 and CQ381170 strains isolated in the south of China, and against the BJ01 and BJ04 strains isolated in the north of China. These studies demonstrated that DZ1133 could protect against both divergent RSV prototype strains and against isolated wildtype strains. Although only six strains of RSV were investigated in this study, future studies could address the activity of DZ1133 in additional RSV strains.

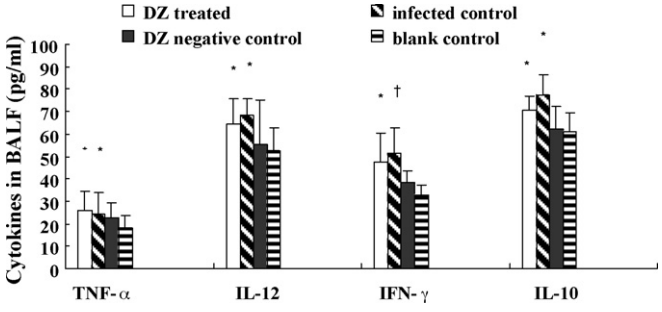

Fig. 5. Effects of DZ1133 on cytokines in BALF of mice. BALB/c mice were infected with 10^6 PFU of RSV standard A2 strain, then intranasally treated with 0.4 mg DZ1133 24 and 48 h post-inoculation. On day 3 mice were sacrificed to collect BALF for cytokine assays by ELISA. Uninfected mice receiving instillation of PBS served as blank controls, and mice treated with 0.4 mg DZ without RSV infection served as negative controls. Data were shown as the mean ± S.D. ($n = 8$/group). *$P < 0.05$ vs. blank control mice; †$P < 0.01$ vs. blank control mice.

In recent years, several antisense approaches of silencing specific genes have been developed: antisense RNA, antisense oligodeoxynucleotides (AS-ODNs), ribozymes, deoxyribozymes, and small interfering RNA (siRNA). An advantage of using deoxyribozymes is their stability that derives from their DNA-based structure. Therefore, deoxyribozymes would be more stable than RNA-based antisense RNA, ribozyme, or siRNA derivatives. Secondly, deoxyribozymes can function independently, whereas antisense RNA, AS-ODNs, and siRNA rely on cellular mechanisms to achieve silencing (Breaker, 2000). Other researchers have reported AS-ODNs that inhibit RSV replication *in vitro* and *in vivo* (Cirion et al., 1997; Player et al., 1998; Barnard et al., 1999; Leaman et al., 2002). We compared the antiviral activities of AS-ODNs and deoxyribozymes targeting the same RNA sequence in our mouse model. DZ1133 was shown to be more potent than the AS-ODN AS1133 at the same dose. Our results are comparable to the findings of Toyoda et al. (2000) that showed that the inhibitory effect of deoxyribozymes on an influenza virus are stronger than those of AS-ODNs *in vitro*. Therefore, the inhibitory effect of deoxyribozymes would appear to depend on the catalytic domain, rather than the binding arms that have the same sequence as AS-ODNs. MutDZ1133 contained a mutation at the 4th position of the catalytic domain and lost most of its biological activity. Nucleotides at the borders of the catalytic domain have been shown to be directly involved in the formation of the catalytic center of a deoxyribozyme. Substitutions at the conserved positions of 1, 2, 4, 6, and 14 of the catalytic domain will induce complete loss of catalytic activity of a deoxyribozyme (Zaborowska et al., 2002). Surprisingly, mutDZ1133 showed a much weaker ability to inhibit viral plaque formation and RSV mRNA expression than AS1133, despite the prediction that the mutant deoxyribozyme would be capable of inducing a conventional antisense effect based on the presence of its binding arms that would mimic AS1133. We hypothesize that the 15 nucleotides of the inactivated catalytic domain prevent the mutant deoxyribozyme from binding the target RNA, meanwhile the non-pairing, intervening sequence makes the RNA target a poor substrate for RNase H which is important for the biological activity of antisense oligonucleotides (Liu et al., 2001).

The efficiency of DZ1133 *in vivo* was not predicted from its previous tests *in vitro* (Xie et al., 2006), and was also mod-

est compared to that of reported siRNA targeting RSV (Bitko et al., 2005; Barik and Bitko, 2006). Similarly, the anti-RSV compound, ribavirin, showed potent anti-RSV effects in cell culture experiments and in animal models, but failed in the clinic (Guerguerian et al., 1999; Long et al., 1997). These results seem to indicate that reducing viral replication in an established infection is not sufficient to reverse disease. The limited biological activity of deoxyribozymes *in vivo* may also be due to nucleolytic degradation of deoxyribozymes in body fluids by various enzymes (Achenbach et al., 2004; Sun et al., 2000; Schubert and Kurreck, 2004; Dass, 2004). Despite the modification of DZ1133 with phosphorothioates to prolong the half-life of deoxyribozymes *in vivo*, the antiviral activity of DZ1133 *in vivo* was unsatisfactory. However, phosphorothioate modification of deoxyribozymes may reduce binding affinity to the target RNA, and induce unwanted side effects and toxicities from non-specific binding (Wang et al., 2002; Phillips and Zhang, 2000; Beigelman et al., 1995). Further studies will be needed to improve deoxyribozyme biostability, increase target affinity, reduce toxicity, and to investigate the effectiveness of using other target sequences of RSV.

Virus-induced histological inflammation and immune dysregulation are critical aspects of the pathogenesis of RSV and the targets of therapy (McNamara and Smyth, 2002). We explored the effect of DZ1133 on local inflammation and the immune response in response to RSV infection. Treatment with DZ1133 decreased the pulmonary histological inflammatory scores of mice challenged with RSV in a dose dependent manner. Administration of 0.4 mg of DZ1133 reduced pulmonary histological inflammatory scores of RSV A2 strain infected mice by 47.36%, and the total leukocyte count in BALF by 60.83%, indicating the deoxyribozyme treatment ameliorated airway inflammation induced by RSV infection. However, there were no significant differences in the percentages of macrophage, lymphocyte, neutrophil or eosinophil subpopulations between the infected control and the 0.4 mg DZ1133 treated mice. Additionally, the local secretions of TNF-α, IL-12, IFN-γ, and IL-10 in BALF were not influenced by DZ1133 compared with RSV-infected control mice. DZ1133 inhibits RSV replication and reduces inflammatory infiltration, but has limited effect on the type of inflammation and immunological dysfunction of the local airway. Immunocytes have been shown to be activated in the early stages of RSV infection and to continue overproduction of TNF-α, IL-12, IFN-γ and IL-10 (Braciale, 2005; Rutigliano and Graham, 2004; Wang et al., 2004). Immune dysregulation, once elicited by RSV infection, is not reversed following deoxyribozyme treatment. RSV-induced pathology is hypothesized to be the result of direct viral cytopathic effects and the inflammatory response to infection. Thus, the most efficient treatment for acute RSV infection should combine anti-viral and anti-inflammatory therapy (Blanco et al., 2005; Openshaw, 2005). There is no difference in IFN-γ secretion between RSV-infected control mice and DZ1133-treated mice, so the antiviral activity of DZ1133 may depend on the direct inhibition of viral gene expression rather than the indirect effect of IFN-γ production, which is an important antiviral cytokine in innate and adaptive immune responses (Durbin et al., 2002). The negative control mice dripped with DZ1133 and the blank control mice dripped with PBS had no significant differences in histopathological scores, leukocyte counts, or TNF-α, IL-12, IFN-γ, and IL-10 levels in BALF, indicating the safety of intranasally delivered deoxyribozyme.

In summary, the deoxyribozyme DZ1133 targeting the genomic RNA sequence of the nucleocapsid N gene inhibits viral replication of both subgroups A and B of RSV standard strains and local isolated strains, and alleviates inflammation induced by RSV infection without visible side-effects *in vivo*. Although DZ1133 mediates limited inhibition of anti-RSV and anti-inflammation effects, it is more efficient than antisense oligonucleotide targeting of RSV. Deoxyribozymes still represent a potential anti-RSV therapeutic agent with improved efficacy for clinical applications.

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