In vivo Delivery of Catalytic RNase P Ribozyme as an Antiviral Agent

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Abstract

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Engineered M1GS ribozyme derived from RNA subunit of *E. coli* can be a very promising antiviral agent. The focus of this dissertation has been to develop a method for stable, safe and effective delivery of ribozyme into the viral infection sites of an animal and achieve antiviral effect. First, the M1GS expressing constructs which target the overlapping mRNA region of MCMV assembly protein (mAP) and M80 were successfully delivered into MCMV-infected CB17 SCID mice through a modified hydrodynamic transfection procedure and block viral pathogenesis. The expression of ribozymes was observed in the liver and spleen. Compared to the control groups, animals receiving the functional ribozyme construct exhibited a significant reduction of viral gene expression and infection. Viral titers in the spleens, livers, lungs, and salivary glands of the functional ribozyme-treated SCID mice at 21 days after infection were 200 to 2,000 fold lower than those in the control animals. Moreover, survival of the infected animals significantly improved upon receiving the functional ribozyme construct. This study successfully uses a hydrodynamic transfection method to deliver ribozymes into animal and demonstrates the feasibility of using M1GS ribozymes for inhibition of viral gene expression in animals. Second, using human cytomegalovirus (HCMV) infection of differentiated macrophages as the model, the study showed that *Salmonella* can efficiently deliver RNase P-based ribozyme sequence in human specific human cells, leading to substantial ribozyme expression and effective inhibition of viral infection. A functional M1GS ribozyme was constructed to target the overlapping mRNA region of the capsid scaffolding protein (CSP) and assemblin, which are essential for viral capsid formation. Substantial expression of ribozymes was observed in cells that were treated with attenuated *Salmonella* strains carrying the ribozyme sequence constructs. A reduction of 87 – 90% in viral CSP expression and a reduction of about 5,000 fold in viral growth were observed in cells that were treated with *Salmonella* carrying the sequence of the functional ribozyme but not with control groups. This study showed for the first time that ribozymes delivered via *Salmonella*-based vectors are highly active and specific.
in blocking viral infection in cultured cells. Third, a functional M1GS ribozyme that targets the overlapping mRNA region of MCMV M80.5 and protease was constructed. A novel attenuated strain of *Salmonella*, which exhibited efficient gene transfer activity and little virulence in mice, was constructed and used for delivery of anti-MCMV ribozyme *in vivo*. Oral inoculation of the attenuated *Salmonella* strain in mice efficiently delivered antiviral M1GS RNA into targeted organs, leading to substantial expression of ribozyme without causing significant adverse effects in the animals. Furthermore, the MCMV infected mice that were treated orally with *Salmonella* carrying the functional M1GS sequence displayed reduced viral gene expression, decreased viral titers and improved survival compared to the control group. This study provided the first direct evidence that oral delivery of M1GS RNA by *Salmonella*-based vectors effectively inhibits viral gene expression and replication in mice. Moreover, this study demonstrates the utility of *Salmonella*-mediated oral delivery of RNase P ribozyme for gene targeting applications *in vivo*. 
To my family
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CHAPTER I

Introduction
Human cytomegalovirus

Human cytomegalovirus (HCMV) or human herpesvirus 5 (HHV-5) belongs to the Beta-herpesvirinae genus of the Herpesviridae family. The Beta-herpesviruses subfamily includes HCMV (and its primate CMV relatives), muromegalovirus (murine cytomegalovirus, MCMV), roseolovirus and proboscivirus (1). All subgroup members share nonexclusive characteristics such as restricted host range, long reproductive cycle, slow growth in cell cultures, enlarged infected cells (cytomegalia), and latent infection in specific cells and tissues (1). As the prototype of the beta-herpesviruses subfamily, HCMV is well characterized. The first part of this chapter will give a brief introduction to human cytomegalovirus and discuss about this ubiquitous pathogen from four aspects: genome and virion structure, virus replication, virus infection in host and anti-viral therapy.

1. Viral genome and virion structure

Human cytomegalovirus has the largest genome not only in the Beta-herpesvirus subgroup, but also among all sequenced human herpesviruses such as herpes simplex virus 1 (HSV-1), varicella–zoster virus (VZV), Epstein–Barr virus (EBV), and Kaposi's sarcoma-associated herpesvirus (KSHV) (1). As the largest known human herpesvirus, HCMV contains a ~230 kb linear double-stranded DNA genome with the capacity of encoding over 220 open reading frames. HCMV genome belongs to the class E genome structure in which a unique long (UL) region and unique short (US) region are flanked by terminal and internal repeats (b and b' repeats; c and c' repeats). The b and b' repeats stand for terminal repeat long (TRL) and internal repeat long (IRL). While a and a' repeats are usually termed internal repeat short (IRS) and terminal repeat short (TRS) (2, 3). A directly repeated alpha sequence (300-600 bp) is found at the genome termini and functions during genome packaging and encapsidation (4, 5).

HCMV is the most structurally complex herpesvirus. The mature viral particle has a multilayer and roughly spherical structure of ~2000-3000A in diameter, which comprises a glycoprotein containing envelope, a proteinaceous tegument layer, and a viral DNA containing capsid. As showed in Figure-1.1A, the inner icosahedral capsid is composed of 5 viral capsid structural proteins. A ~230 kb long linear double-stranded DNA with a lytic origin of DNA replication (oriLyt)-associated RNA are packaged inside the capsid(6). The whole nucleocapsid is embedded within a matrix layer termed tegument which contains at least 27 multiple viral proteins as well as some cellular and viral RNA (7-9). The tegument proteins maybe involved in viral DNA releasing, regulation of viral gene expression and progeny virion maturation. The tegument layer is further surrounded by a host endoplasmic reticulum-Golgi intermediate compartment (ERGIC) derived lipid bilayer envelope which contains more than 20 viral glycoproteins. These proteins are necessary for viral attachment and penetration into the host cells (10-12). Besides the mature infectious virion, the
HCMV-infected cell cultures can produce another two types of viral particles: (i) non-infectious enveloped particles (NIEPs) which contains enveloped immature capsids that lack viral DNA, and (ii) dense bodies (DB) which are enveloped particles that lack nucleocapsid and viral DNA, but contain several abundant tegument proteins (1).

2. Viral replication

During natural infection, HCMV can replicate in many differentiated host cell types, such as epithelial cells, endothelial cells, smooth muscle cells, mesenchymal cells, hepatocytes, granulocytes and monocyte derived macrophages (13). Latent infection can be detected in macrophage progenitor cells in the bone marrow and in peripheral monocytes (13). Regardless of the cell types or the virus strains, during productive infection in permissive cells, three general classes of viral gene expression are performed coordinately and regulated cascade of transcriptional events. These 3 classes of viral genes are immediate-early gene (IE) (α), delayed-early gene (DE) (β) and late gene (L) (γ). Furthermore, these viral genes can be subdivided into several subclasses (1).

As showed in Figure-1.1B, the infectious virion initializes infection by either endocytosis or fusion with the cell membrane, which releases the nucleocapsid and some tegument proteins into the cytoplasm. The whole virus binding and penetration step is very rapid and efficient. After viral entry, the nucleocapsid is uncoated and transported across the cytoplasm. This step may be mediated on microtubules. After docking at the nuclear pores, the nucleocapsid injects of the viral DNA through nuclear pores into the nucleus, where replication and capsid assembly take place. Expression of the immediate-early genes immediately follows viral penetration and doesn’t rely on the expression of any other viral genes. Functional immediate-early gene products control the expression of delayed-early gene expression. The delayed-early genes can be divided into β1 and β2 subclasses which are different in timing and shut-off characteristics (1, 14-16). At last, the late genes get expressed and can be divided into γ1 and γ2 subclasses which are based on timing and dependence on viral DNA replication (14-16). The transcription of CMV protein-coding genes is directed by host transcription machinery which is regulated by viral transactivators. The translation of viral proteins is entirely directed by host. Once virus progeny is mature and starts to egress and release, infected cells continue to produce viral particles for several days. This step is controlled by the viral strain and the constellation of cell death suppressors encoded by the strain (1).

3. Viral infection in normal and in immunocompromised host

Human cytomegalovirus infection is ubiquitous all over the world. In developing countries, almost 100% adult are infected with this virus. In developed countries, for example US, it is known that over 50% of total population and nearly 90% of urban population are seropositive. Especially, 0.5-2.5% of newborns is
congenitally infected with cytomegalovirus. Transmission of cytomegalovirus may happen throughout lifetime and mainly via contact such as via saliva/urine/fomite, sexual contact, placental transfer, breast feeding, blood transfusion, solid-organ transplantation and haematopoietic stem-cell transplantation (1).

In the normal immunocompetent host, after primary infection, CMVs will establish and maintain persistent and latent life-long infection (1). The viral replication is strictly controlled by the host immune system. In this case, most acquired cytomegalovirus infections are clinically silent and rarely cause serious illness. In a very low frequency, it can result in a mononucleosis syndrome and other complications of primary infection such as arthralgia, arthritis, ulcerative colitis, pneumonitis, hepatitis, aseptic meningitis, and myocarditis (17).

In immunocompromised or immunologically immature individuals, reduced or dysfunctional primary immune response and subsequent long term immunity cannot restrain viral replication after reactivation from latent infection. This will lead to uncontrolled viral replication and very serious diseases. In this population, HCMV is a very common opportunistic pathogen and can cause significant morbidity and mortality (1). Severe CMV infections can be detected in several populations, which include AIDS patients with very low CD4 cell counts, recipients of allogeneic stem cell transplants, recipients of solid organ transplants, patients receiving immunosuppressive chemotherapy for cancer or collagen vascular disease, and congenital immunodeficiencies (1). For example, congenital CMV infection of newborns is the leading cause with birth abnormalities, mental retardation, blindness, and deafness in newborns (1, 18). Moreover, CMV infection is responsible for retinitis in patients with HIV which is characterized by haemorrhagic retinal necrosis. This infection is one of the most common opportunistic diseases in patients with AIDS and is the leading cause of AIDS-related blindness (1, 19). For another example, when CMV establishes latent infection in bone marrow progenitor cells, it can cause significant morbidity and mortality in organ transplant recipients by disseminating to other organs from the bone marrow via leukocyte associated viremia (1). Similarly, CMV can cause severe infection in haematopoietic stem cell transplantation. Pneumonitis is the most serious manifestation of cytomegalovirus infection which usually results in 60-80% mortality without proper antiviral treatment (17).

4. Anti-CMV therapy and animal model

Current antiviral therapy against human cytomegalovirus infection includes intravenous immunoglobulin delivery and oral / intravenous drugs such as ganciclovir (GCV), foscarnet (FOS), cidofovir (CDV) and maribavir (MBV) (20). Both strategies have been shown to be effective in some cases; however, they have limitations during clinical therapy. The typical limitations include (i) low bioavailability, serious toxic side effects (e.g. neutropenia, anemia, thrombocytopenia, renal toxicity and electrolyte imbalances), (ii) inconsistent results and (iii) increasing incidence of
viral resistance to the drugs. All these limitations present a significant public health concern on cytomegalovirus infection and related antiviral therapy. Particularly, the emergence of drug-resistant strains of cytomegalovirus has posed an urgent need for the development of new drugs and treatment strategies. Consequently, it is of great public health importance that new drugs utilizing novel mechanism against human cytomegalovirus be developed to combat this virus.

Murium cytomegalovirus (MCMV) shares many similar features with its human counterpart, human cytomegalovirus. For example, MCMV also belongs to Beta-herpesvirinae subfamily and is characterized by strict species specificity, secretory glands and hematopoietic tissue tropism, and a slow growth and replication cycle. After acute primary infection, MCMV can establish life-long persistent and latent infection. As such, MCMV infecting mice provides a good animal model for studying CMV pathogenesis in vivo and thus has been extensively used for developing and screening novel antiviral agents (1, 21-23). For example, MCMV infection of the CB17 SCID mice which lack functional T and B lymphocytes represents an excellent animal model to study CMV pathogenesis in immunocompromised hosts. This is a good animal model for assessing the efficacy of novel antiviral therapy for blocking viral infection and virulence. Intraperitoneal infection of SCID mice with MCMV leads to a biphasic infection. Viruses initially infect and replicate inside the spleen and liver. Then the viruses are disseminated via leukocyte-associated viremia from the spleen and liver to other peripheral organs, such as the lungs and salivary glands (1, 21, 23). SCID mice are highly susceptible to MCMV and can succumb to as little as 10 plaque-forming units (pfu) of the virus, primarily because of liver damage and failure associated with viral lytic replication in the organ (1, 23, 24). Analysis of viral replication in these mice can be used for studying whether new therapeutic approaches block CMV opportunistic infection and prevent viral-associated diseases in immunocompromised hosts.

**M1GS ribozyme as an antiviral agent**

1. Nucleic acid-based gene targeting approaches

The ideas of developing nucleic acid-based drugs that can degrade target RNA, mRNA and viral RNA in particular was proposed back to year 1960s. Now the nucleic acid-based gene interference technologies have been well developed and represented a promising gene-targeting strategy for specific inhibition of target RNA sequences of choice (25-27). Antisense molecules, such as oligonucleotides (AS-OGN), small interfering RNAs (siRNA), ribozymes and DNazymes, have been thoroughly studied and some of them have been developed as gene therapy agent to efficiently and specifically block target RNA expression. Current stage of drug studies varies from pre-clinical trial to phase III clinical trial, and some even approved in the market and for clinical application (28). Without any doubts, Nucleic-acid-based gene
interference approaches represent not only very powerful tools in both basic and clinical research, but also promising therapeutic agents for human diseases.

In relation to antiviral infection, antisense molecules have been used as antiviral agents to inhibit viral gene expression and abolish viral replication. For example, one antiviral molecule Vitravene, which is developed based on AS-OGN strategy, has been approved for use against HCMV retinitis (29). More recently, siRNAs were proven to be effective in inducing endogenous RNase of the RNA-induced silencing complex (RISC) in the RNAi pathway to inhibit gene expression and growth of several human viruses (26, 30, 31). Furthermore, RNA enzymes as antisense catalytic RNA molecules are also being developed as promising gene target agents to specifically cleave viral RNA of choice. Both hammerhead and hairpin ribozymes have been shown to cleave viral mRNA sequences and inhibit viral replication in cells infected with human viruses (32, 33). Ribozymes have also been shown to cleave viral mRNA sequences and inhibit viral replication in human cells (34). Ribozymes are catalytic RNAs naturally occurred and expressed in a wide range of organisms. The second part of this chapter will focus on ribonuclease P (RNase P) and its derivative M1GS RNA, especially their antiviral application.

2. Ribonuclease P

Ribonuclease P (RNase P) is a ribonucleoprotein complex found in all organisms examined. In bacteria, RNase P enzymes consist of a single protein subunit and a single catalytic RNA unit. For example, RNase P found in *Escherichia coli* is composed of a basic 14 kDa C5 protein subunit and a 377 nucleotides-length M1 RNA subunit. Compared to their bacterial counterparts, eukaryotic RNase P enzymes are much more complex. As in human, RNase P enzymes have at least ten protein subunits (14-115 kDa) and one RNA subunit called H1 RNA (35, 36).

RNase P is responsible for the 5’ maturation of tRNA. This enzyme catalyzes a hydrolysis reaction to remove the 5’ leader sequence of precursor tRNA (ptRNA), which is responsible for the maturation of 5’ termini of all tRNAs (37, 38). A unique feature of RNase P is that it recognizes the structure, rather than the sequence, of the substrate, which allows the enzyme to hydrolyze different natural substrates in vivo and in vitro (Figure-1.2B). Accordingly, any complex of two RNA molecules that resembles a tRNA molecule can be recognized and cleaved by RNase P (39, 40). RNase P mediated inhibition of gene expression, an application based on this special feature, has been shown to be a novel and useful nucleic acid-based gene interference strategy for specific inhibition of mRNA sequences of choice (35, 41).

3. M1GS as a promising tool for antiviral therapy

As mentioned above, RNase P of *Escherichia coli* contains a catalytic RNA subunit (M1 RNA) (Figure-1.2A). In vitro assay showed that M1 RNA can cleave its
pre-tRNA substrate at high divalent ion concentration in the absence of C5 protein (42). Thus, M1RNA can be engineered to cleave tRNA-like substrates and other target RNAs, including specific mRNAs (37-39, 43). A sequence-specific ribozyme, M1GS, constructed by attaching to M1 RNA an additional small RNA (guide sequence [GS]) which contains a sequence complementary to a target mRNA and a 3’ proximal CCA (Figure-1.2C), is effective in blocking substrate mRNA expression (41, 44). It generally believed that the guide sequence complementary binds to its target mRNA and directs covalently attached M1RNA to the cleavage site and catalyze the hydrolytic reaction. Previous studies have demonstrated that M1GS RNA and RNase P are effective in cleaving both viral and cellular mRNAs and blocking their expression in cultured cells, including inhibition of gene expression of human influenza and herpes viruses (44-47).

Compared to other nucleic acid-based interference approaches such as antisense oligonucleotides and RNA interference (RNAi) (48, 49), M1GS-based strategy possesses several unique features which make M1GS catalytic RNA a promising gene targeting tool for treatment of viral diseases. First, M1 RNA is one of the most abundant and efficient catalytic RNAs found in nature. Unlike most other catalytic RNA molecules which undergo one round self-cleavage or transposition, RNase P catalyzes the cleavage reaction with multiple turnovers in the cell (50). Second, M1GS ribozymes can fold into a defined active conformation in the absence of its substrates, and catalyze the reaction by binding to the substrate which potentially results in the most efficient cleavage of its target mRNA (37, 51). Furthermore, well-folded M1GS RNAs may be less susceptible to degradation by intracellular RNases. Third, expression of M1GS RNAs for as long as one month does not lead to significant cytotoxicity in cultured human cells. Fourth, M1GS RNAs are highly specific. For example, for two substrates that share the first nine contiguous base pairs complementary to the guide sequence, a ribozymes can still cleave its substrate specifically instead of the similar substrate (52). Fifth, M1GS RNAs can be engineered to target and cleave any target sequence, including viral mRNA sequences. The low sequence requirements at the cleavage site provide M1GS ribozymes flexibility to be used against almost any target, including position-fixed target sites (52).

4. Optimization of efficient M1GS RNAs

In vitro selection is a process where enrichment of molecules with desired properties is accumulated via repetitious cycles of isolation and amplification (53-55). This technology has been used to enhance enzyme activity and develop new functional nucleic acids (56-59). Although many methods have been used to increase the activity of M1GS RNAs, in vitro selection method has been used to successfully generate novel M1GS RNA variants with high activity (60).
Basically, the mutant M1GS ribozyme pool was generated and the entire in vitro selection process was carried out through iterative cycles with six steps in each round (Figure-1.3) (50, 61). (1) The ribozyme molecules were annealed to a 5’ biotinylated substrate. (2) The annealed complexes were allowed to bind to a streptavidin column in the absence of divalent ions such as Mg\(^2+\). Unbound ribozymes were washed away. (3) Cleavage buffer containing divalent ion was added to the column to allow the M1GS RNA-mediated cleavage reaction to occur. (4) The M1GS RNAs that cleave the substrates were released from the column and loaded on a denaturing gel where they were recovered. (5) Through reverse transcription-PCR (RT-PCR), the cDNA copies of recovered M1GS RNAs were synthesized. (6) New M1GS RNA pool was generated by T7 RNA polymerase mediated in vitro transcription. The sequences isolated after several rounds of selection can be cloned and determined. Using this unique selection procedure, we have successfully generated M1GS variants with high efficiency in cleaving target mRNA in vitro and blocking target viral mRNA expression in cultured cells (60, 62, 63).

Summary of the thesis research

As with any gene therapy strategy, safety, stability and delivery of the antiviral agents remain a major concern. As previous in vitro studies showed, potentially, RNase P ribozyme can be a very promising antiviral agent. However, at current stage, a system for stable, safe and effective delivery of ribozymes into the viral infection sites of an animal host is lacking. The aim of this dissertation is to develop a practical delivery method for RNase P M1GS ribozyme and to study whether the method can effectively inhibit viral infection and replication in vivo.

First, as showed in Chapter II, the ribozyme expressing constructs which target the overlapping mRNA region of MCMV assembly protein (mAP) and M80 were delivered into MCMV-infected CB17 SCID mice through a modified hydrodynamic transfection procedure. Expression of ribozymes was observed in the liver and spleen. Compared to the control animals that did not receive any M1GS constructs or received the disabled ribozyme construct, animals receiving the functional ribozyme construct exhibited a significant reduction of viral gene expression and infection. Viral titers in the spleen, liver, lung, and salivary gland of the functional ribozyme-treated SCID mice at 21 days after infection were 200 to 2,000 fold lower than those in the control animals. Moreover, survival of the infected animals significantly improved upon receiving the functional ribozyme construct. This study successfully uses a hydrodynamic transfection method to deliver ribozymes into animal and demonstrates the feasibility of using M1GS ribozymes for inhibition of viral gene expression in animals. This modified intravenous injection method is useful to demonstrate the feasibility of delivering novel antiviral compounds into animals and to test their activity in vivo (64-66), however, it is not suitable for clinical applications. To achieve safe, stable and efficient delivering purpose, new system needs to be developed and tested.
Invasive bacteria, such as *Salmonella*, which possess the ability to enter human cells, are capable of transferring genetic material into host cells leading to efficient expression of transgenes (67). Recently, attenuated *Salmonella* strains have been shown to function as a carrier system for delivery of nucleic acid-based vaccines and antitumor small hairpin RNAs (shRNAs) for cancer therapy (68-71). How the genetic materials got transferred and expressed in the host cell is still unclear. It’s hypothesized that once the bacteria undergo intracellular lysis inside the *Salmonella*-containing vacuole, plasmid DNA is released and transferred into nucleus where it is expressed by cellular machinery. Figure-1.4 demonstrates a hypothesis how *Salmonella* bacteria enter human macrophages and transfer M1GS construct into the host cells and eventually achieve antiviral effect.

As a potential delivery system for ribozyme, *Salmonella*-based vectors exhibit several unique and attractive features. First, the *Salmonella*-based vector is of low cost and easy to prepare, store, and transport. Second, it is practical to administer the bacteria through the oral route which proven to be efficacy and acceptability (72-74). Third, it is possible to generate new attenuated mutants (75). Fourth, *Salmonella* strains have been widely used as vaccines both in human and veterinary medicine (67, 72). Finally, integration of bacteria-delivered DNA into host cell genome has not been reported (76), and no oncogenesis promotion of the bacterial infection has been known. Thus, the low-cost, safe, noninvasive administration and lifelong suitability of the therapy, combined with easy preparation of the bacterial carrier, supports the notion that *Salmonella* represents an attractive and promising gene delivery tool for gene therapy for human diseases, including those caused by viral infections.

As showed in Chapter III and IV, *Salmonella* can efficiently deliver RNase P-based ribozyme sequence in specific human and mouse cultured macrophage cells, leading to substantial ribozyme expression and effective inhibition of viral infection. In differentiated human macrophage, M1GS RNAs that target the overlapping mRNA region of HCMV capsid scaffolding protein (CSP) and assemblin were delivered by attenuated *salmonella* strain SL7207. A reduction of 87–90% in viral CSP expression and a reduction of about 5,000-fold in viral growth were observed in cells that were treated with *Salmonella* carrying the sequence of the functional ribozyme but not with those carrying the sequence of a control ribozyme that contained mutations abolishing the catalytic activity. To our knowledge, this study showed for the first time that ribozymes expressed following targeted gene transfer with *Salmonella*-based vectors are highly active and specific in blocking viral infection.

As showed in Chapter IV, in mouse macrophage, M1GS RNAs that target the overlapping mRNA region of M80.5 and protease were delivered by a novel attenuated *salmonella* strain SL101. An 80-85% reduction in the expression of M80.5/protease and a 2,500-fold reduction in viral growth were observed. Furthermore, oral inoculation of the attenuated *Salmonella* strain in mice efficiently delivered antiviral M1GS RNAs into targeted organs, leading to substantial
expression of ribozyme without causing significant adverse effects in the animals. The MCMV infected mice that were treated orally with *Salmonella* carrying the functional M1GS sequence displayed reduced viral gene expression, decreased viral titers and improved survival compared to the untreated mice or mice treated with *Salmonella* containing control ribozyme sequences. These results provide the first direct evidence that oral delivery of M1GS RNA by *Salmonella*-based vectors effectively inhibits viral gene expression and viral replication in mice. Moreover, this study demonstrates the utility of *Salmonella*-mediated oral delivery of RNase P ribozyme for gene targeting applications *in vivo*. 
Figure 1.1 (A) Schematic diagram illustrating CMV structure. (B) Summary of CMV viral replication pathway inside the host cells: (1) attachment and penetration, (2) entry and uncoating, (3) viral DNA release into nucleus, (4 & 5) viral early-gene expression (6) viral DNA synthesis, (7 & 8) viral late-gene expression and capsid assembly, (9) DNA encapsidation, (10) nucleus egress, (11) envelopment and transport, (12) viral release.
Figure 1.2 (A) The proposed secondary structure of RNA subunit (M1RNA) of RNase P from *E. coli* (77). (B) Schematic representation of natural substrates for RNase P. (C) A hybridized complex of a target RNA (e.g. viral mRNA) and M1GS RNA. The arrow shows the site of the cleavage by RNase P and M1 RNA.
Figure 1.3 Schematic representation of the in vitro selection for the generation of active M1GS RNA ribozyme variants that specifically and efficiently cleave a target mRNA. (1) annealing of M1GS RNA pool with a 5' biotinylated substrate. (2) binding the complex to streptavidin agarose column. (3) M1GS RNA-mediated cleavage of the substrate in the presence of Mg\(^{2+}\) buffer. (4) recovering M1 RNA with denaturing gel electrophoresis. (5) synthesis of cDNA copies of RNA molecules with RT-PCR. (6) in vitro transcription of the generated cDNAs with T7 RNA polymerase. The sequences isolated after several rounds of selection were cloned and sequenced.
Figure 1.4 Schematic representation of *Salmonella*-assisted gene therapy. *Salmonella* (rod) transformed with pU6-M1GS (oval inside *Salmonella*) infects a macrophage and resides in SCV (*Salmonella*-containing vacuole). After the bacteria undergo intracellular lysis, the plasmid contents are released and transferred into nucleus where they can be expressed by cellular machinery. M1GS RNAs will bind with and cleave the target viral mRNA to block the viral replication.
REFERENCES


CHAPTER II

Modified hydrodynamic transfection:

Effective inhibition of viral pathogenesis by RNase P-based ribozymes in animals
ABSTRACT

A functional RNase P ribozyme (M1GS RNA) was constructed to target the overlapping mRNA region of two murine cytomegalovirus (MCMV) capsid proteins essential for viral replication: the assembly protein (mAP) and M80. The customized ribozyme efficiently cleaved the target mRNA sequence in vitro. Moreover, 80% reduction in the expression of mAP and M80 and a 2,000-fold reduction in viral growth were observed in cells expressing the ribozyme. In contrast, there was no significant reduction in viral gene expression and growth in cells that either did not express the ribozyme or produced a “disabled” ribozyme carrying mutations that abolished its catalytic activity. When the ribozyme-expressing constructs were delivered into MCMV-infected SCID mice via a modified “hydrodynamic transfection” procedure, expression of ribozymes was observed in the liver and spleen. Compared to the control animals that did not receive any M1GS constructs or received the disabled ribozyme construct, animals receiving the functional ribozyme construct exhibited a significant reduction of viral gene expression and infection. Viral titers in the spleens, livers, lungs, and salivary glands of the functional ribozyme-treated SCID mice at 21 days after infection were 200 to 2,000 fold lower than those in the control animals. Moreover, survival of the infected animals significantly improved upon receiving the functional ribozyme construct. Our study examines the use of M1GS ribozymes for inhibition of gene expression in animals and demonstrates the utility of RNase P ribozymes for gene targeting applications in vivo.
INTRODUCTION

Human cytomegalovirus (CMV), a common opportunistic pathogen, causes significant morbidity and mortality in immunocompromised or immunologically immature individuals, including neonates, AIDS patients, and transplant recipients (1). The emergence of drug-resistant strains of CMV has posed a need for the development of new drugs and treatment strategies. Murine cytomegalovirus (MCMV) infection of mice resembles its human counterpart with respect to pathogenesis, thus providing an animal model for studying CMV infection in vivo and for screening novel agents and developing new antiviral approaches (1-4). For example, the CB17 SCID mice, which lack functional T and B lymphocytes, are highly susceptible to MCMV infection (5, 6). Analysis of viral replication in these mice can be used for studying whether new therapeutic approaches block CMV opportunistic infection and prevent viral-associated diseases in immunocompromised hosts.

Nucleic acid-based gene interference technologies represent promising gene-targeting strategies for specific inhibition of mRNA sequences of choice (7-9). For example, ribozymes have been shown to cleave viral mRNA sequences and inhibit viral replication in human cells (10). More recently, siRNAs were proven to be effective in inducing endogenous RNase of the RNA-induced silencing complex (RISC) in the RNAi pathway to inhibit gene expression and growth of several human viruses (8, 11, 12). Thus, nucleic-acid-based gene interference approaches can be used as a tool in both basic and clinical research, such as in studies of tumorigenesis and antiviral gene therapy.

RNase P is a ribonucleoprotein complex responsible for the 5′ maturation of tRNA (13, 14). Altman and colleagues (13) have previously shown that RNase P of Escherichia coli contains a catalytic RNA subunit (M1 RNA) that can be engineered to cleave tRNA substrates and other target RNAs, including specific mRNAs (13-16). A sequence-specific ribozyme, M1GS, constructed by attaching M1 RNA to a guide sequence complementary to a target mRNA (Figure-2.1A-B) is effective in blocking mRNA expression in cultured cells (17, 18). Unlike other nucleic-acid-based interference approaches, such as antisense oligonucleotides and RNAi (8, 9), M1GS-based strategy is unique because of the use of M1 RNA, one of the most efficient catalytic RNAs found in nature (13, 14). Previous studies have shown that M1GS RNA and RNase P are effective in cleaving both viral and cellular mRNAs and blocking their expression in cultured cells, including inhibition of gene expression of human influenza and herpes viruses (7, 17, 19, 20). However, it has not been reported whether M1GS RNAs also function to inhibit gene expression in vivo in animals.

Using MCMV infection of mice as the model, in this study we provide direct evidence that M1GS RNA is effective in inhibiting viral gene expression and blocking viral infection and pathogenesis, leading to improved survival of animals. M1GS ribozymes were constructed to target the region of the mRNA encoding MCMV...
capsid assembly protein (mAP). The mAP coding sequence completely overlaps with and is within the 3’ coding sequence of another viral capsid protein, M80 (21). Both mAP and M80 are essential for viral capsid formation and CMV replication (22, 23). We showed that the constructed ribozymes efficiently cleaved the target mRNA sequence in vitro and effectively inhibited mAP expression and viral growth in cultured cells. When constructs carrying M1GS expression cassettes were introduced to MCMV-infected SCID mice via a modified hydrodynamic transfection procedure (24-26), viral gene expression and growth in various organs of these animals were reduced and animal survival significantly improved. In addition to investigating the activity of M1GS ribozymes in animals, our study demonstrates the feasibility of developing effective RNase P ribozymes as a novel class of antiviral agents for treatment of viral diseases in vivo.
RESULTS

1. M1GS-mediated cleavage of the mAP mRNA sequence \textit{in vitro}

The mRNA (mAP mRNA) encoding the mAP and the M80 mRNA coterminate at the same 3’ polyadenylation site (21, 27). M1GS-mediated cleavage at the overlapping region of the M80 and mAP mRNAs is expected to block the expression of both mRNAs simultaneously and should lead to a significant reduction of MCMV growth. To choose a targeted region that is accessible to binding of ribozymes to achieve efficient cleavage, we used an \textit{in vivo} mapping approach with DMS (17, 28) to determine the accessibility of the region of the mAP mRNA in MCMV-infected cells. A position 88 nucleotides downstream from the mAP translational initiation codon (29) was chosen as the cleavage site for M1GS RNA. This site appears to be one of the regions most accessible to DMS modification and presumably to ribozyme binding (30).

Ribozyme M1-1 was constructed by covalently linking the 3’ terminus of M1 RNA with a guide sequence of 18 nucleotides that is complementary to the targeted mAP mRNA sequence. The control ribozyme, M1-2, was constructed in a similar way and derived from C102 RNA, a M1 mutant that contained several point mutations at the P4 domain and was at least 10^4-fold less active than M1 RNA in cleaving a pretRNA (31). M1-1 and M1-2 were synthesized \textit{in vitro} from DNA coding for these ribozymes and were incubated with substrate Map39, which contained the targeted mAP mRNA sequence of 39 nucleotides (Figure-2.1C). Cleavage of Map39 by M1GS yielded two cleavage products of 12 and 27 nucleotides, respectively (Figure-2.1C). Efficient cleavage of Map39 by M1-1 was observed (Figure-2.1D, lane 2), whereas cleavage by M1-2 was barely detected (Figure-2.1D, lane 3). The differential cleavage efficiencies observed for M1-1 and M1-2 may not be a result of their different binding affinities to the mAP mRNA sequence, because experiments with gel-shift assays indicated that the binding affinity of M1-2 to Map39 ($K_d = 0.30 \pm 0.04$ nM) is similar to that of M1-1 ($K_d = 0.34 \pm 0.05$ nM). Because M1-2 contains the same antisense guide sequence and similar affinity to Map39 as M1-1 but is catalytically inactive, this ribozyme can be used as a control for the antisense effect in our experiments in cultured cells.

2. Efficient expression of the ribozymes in cultured cells

The DNA sequences coding for M1-1 and M1-2 were cloned into retroviral vector LXSN and placed under the control of the small nuclear U6 RNA promoter, which has previously been shown to express M1GS RNA and other RNAs efficiently (17, 32, 33). To construct cell lines that express M1GS ribozymes, amphototropic packaging cells (PA317) (33) were transfected with LXSN-M1GS DNA to produce retroviral vectors that contained the genes for M1GS RNA. NIH 3T3 cells were then infected with these vectors, and cells expressing the ribozymes were cloned.
An additional cell line that expressed ribozyme M1-TK, a ribozyme targeting the mRNA for the thymidine kinase of HSV-1 (17), was also constructed. No cleavage of Map39 by M1-TK was observed in vitro (Figure-2.1D, lane 4). We used this cell line to determine whether M1GS RNA with an incorrect guide sequence could target mAP mRNA in tissue culture. The level of M1GS RNA in each cell clone was determined by Northern analysis, using the expression of mouse RNase P RNA (mP1 RNA) as the internal control (Figure-2.1E and F) (34, 35). We only used the cell lines that expressed similar levels of these ribozymes for further studies. The constructed lines and a control line in which cells were transfected with LXSN vector DNA alone were indistinguishable in terms of their growth and viability for ≤3 months (data not shown), suggesting that the expression of the ribozymes did not result in significant cytotoxicity.

3. Inhibition of MCMV gene expression and growth by M1GS ribozymes in cultured cells

To determine the efficacy of the ribozymes in inhibiting the expression of MCMV mAP and M80, cells were infected with MCMV at a multiplicity of infection (MOI) of 0.1 to 1. Total RNAs were isolated from the infected cells at 8–72 h after infection. The expression levels of mAP and M80 mRNAs were determined by Northern analyses. The level of the 7.2-kb-long viral transcript (7.2-kb RNA), whose expression is not regulated by mAP or M80 under the assay conditions (36), was used as an internal control for the quantitation of expression of mAP and M80 mRNAs (Figure-2.2A). A reduction of 80 ± 7% and 80 ± 8% (average of three experiments) in the expression level of M80 and mAP mRNA was observed in cells that expressed M1-1, respectively (Table-2.1). In contrast, a reduction of <10% in the expression level of these two mRNAs was observed in cells that expressed M1-2 or M1-TK. These results suggest that the significant reduction of mAP mRNA expression in cells that expressed M1-1 was because of the targeted cleavage by the ribozyme. The low level of inhibition found in cells that expressed M1-2 was presumably because of an antisense effect, because M1-2 exhibits similar binding affinity to the target sequence as M1-1 but is catalytically inactive.

The protein expression of mAP was determined by using Western analysis (Figure-2.2D) with the expression of actin as the internal control (Figure-2.2C). The results of three independent experiments are summarized in Table-2.1. A reduction of ≈84% in the level of mAP protein was observed in cells that expressed M1-1 RNA. In contrast, a reduction of <10% was found in cells that expressed M1-2 or M1-TK RNA. The low level of reduction in the expression level of mAP protein observed in cells that expressed M1-2 was probably because of the antisense effect of the guide sequence.

To determine whether the growth of MCMV is inhibited in the ribozyme-expressing cells, cells were infected by MCMV at an MOI of 1. Virus stocks were prepared from the infected cultures (cells and culture medium together) at 1-day
intervals through 5 days after infection, and their titers were determined. At 4 days after infection, a reduction of at least 2,000-fold in viral yield was observed in cells that expressed M1-1 (Figure-2.2E). No significant reduction was found in those that expressed M1-2 or M1-TK (Figure-2.2E). These results suggest that M1GS-mediated targeting of viral mAP mRNA effectively inhibits MCMV growth. There was no significant difference in the level of inhibition of viral AP gene expression and growth among different cloned cell lines that expressed M1-1.

4. Inhibition of MCMV infection and pathogenesis by M1GS ribozymes in animals

Immunodeficient animals, such as SCID mice, have been shown to be extremely susceptible to MCMV infection (1, 5, 6). To determine the effect of M1GS ribozymes on the replication and infection of MCMV in vivo, we applied hydrodynamic transfection (24-26) of plasmid LXSN-M1GS DNA to SCID mice that had been i.p. infected 24 h earlier with MCMV. To further allow sustained expression of M1GSs in these animals, a similar hydrodynamic transfection procedure was repeated every 72 h. Transfection efficiency was assessed by detecting the expression of M1GS RNAs in the tissues (Figure-2.3A and B) and the expression of GFP, whose coding sequence is included in the LXSN vector (data not shown). Consistent with previous hydrodynamic transfection reports (24-26), the transfection appeared to be efficient as substantial amount of M1GS (Figure-2.3B) and GFP-positive cells were found in the livers and spleens of the animals.

Three sets of experiments were carried out to study the effect of M1GS on viral virulence and infection in vivo. First, the survival rate of the animals that received the M1-1-expressing plasmid was determined and compared to animals that received only PBS or PBS with the M1-2- or M1-TK-containing constructs. All noninfected animals that received M1GS-LXSN constructs via the hydrodynamic transfection procedure remained alive and exhibited no adverse signs for at least 90 days as compared with untreated noninfected animals (data not shown). Transfection of the M1-2 or M1-TK constructs had no effect on the survival of infected animals compared with those that received PBS only, because all of the mice died within 26–27 days after infection (Figure-2.4). In contrast, in infected mice expressing M1-1 life span improved significantly, because no animals died until 40 days after infection (Figure-2.4).

To further study the effect of M1GS expression in MCMV infection of these immunodeficient animals, viral replication in various organs of the animals was studied during a 21-day infection period before the onset of mortality of the infected animals. In these experiments, SCID mice were infected i.p. with $1 \times 10^4$ pfu MCMV and then injected with different plasmids via tail veins using a hydrodynamic transfection procedure. The transfection procedure was repeated every 72 h. At 1, 3, 7, 10, 14, and 21 days after infection, animals were killed and the spleens, livers, lungs, and salivary glands were harvested (5). The viral titers in each of the organs
from animals receiving either M1-2 or M1-TK were similar to those in animals that received only PBS (Figure-2.5A–D). In contrast, the titers in animals receiving M1-1 were consistently lower at every time point examined. At 21 days after infection, the viral titers in the spleens, livers, lungs, and salivary glands of the M1-1-treated animals were lower than those from animals receiving no M1GS constructs by 200-, 400-, 1,000- and 2,000-fold, respectively (Figure-2.5A–D). These results suggest that the expression of M1-1 inhibits viral infection in vivo, including in the spleen and liver.

Viral gene expression in the tissues was also examined. At 14 days after infection, the expression of viral M80 and mAP mRNA, as well as the mAP protein, was readily detectable in the livers and spleens of mice receiving control M1-2 and M1-TK constructs, whereas no appreciable expression was detected at this time point in M1-1-treated mice (Figure-2.3). More importantly, no difference could be detected between mice that received no M1GS constructs and those that received the control constructs M1-2 or M1-TK (Figure-2.3). These results are consistent with the notion that MCMV infection is inhibited in animals receiving the M1-1-expressing plasmid.
DISCUSSION

Nucleic-acid-based gene interference strategies, such as antisense oligonucleotides, ribozymes or DNAzymes, and RNAi represent powerful research tools and promising therapeutic agents against human diseases (7, 8, 11). For example, siRNAs are effective in inducing RNAi to inhibit gene expression in vitro and in vivo (8, 12, 25). Each of these approaches has its own advantages and limitations in terms of targeting efficacy, sequence specificity, toxicity, and delivery efficiency in vivo. The M1GS-based technology represents an attractive approach for gene inactivation because it generates catalytic and irreversible cleavage of the target RNA by using M1 RNA, a highly active RNA enzyme found in nature (13, 14). These properties, as well as the simple design of the guide sequence, make M1GS an attractive gene-targeting agent that can be generally used for antiviral and other in vivo applications. For M1GS ribozyme to be successful as a therapeutic tool for practical applications, the enzyme has to be highly active and stable in tissues and effective in blocking gene expression in vivo.

In the current study, we have designed a M1GS RNA targeting the overlapping region of MCMV mAP and M80 mRNA. The ribozyme cleaved the target mRNAs efficiently in vitro and, furthermore, reduced their expression levels by 80–85% and inhibited viral growth by 2,000-fold in cells that expressed the ribozyme. In contrast, a reduction of <10% in the levels of MAP expression and viral growth was observed in cells that expressed M1-2 or M1-TK. When MCMV-infected SCID mice were injected with plasmids carrying different M1GS-LXSN DNA via a hydrodynamic transfection procedure, the expression of M1GS RNAs was detected in several tissues, including the spleen and liver. All animals that received PBS only or PBS with the M1-2 or M1-TK constructs died within 27 days after infection, whereas those receiving M1-1 remained alive until 40 days after infection. In contrast, all noninfected animals that received M1GS-LXSN constructs via a hydrodynamic transfection procedure remained alive and exhibited no adverse signs for at least 90 days, as compared with untreated noninfected animals (data not shown). Furthermore, viral titers found in the spleens, livers, lungs, and salivary glands of the animals receiving the M1-1 construct were significantly lower than those in animals that received PBS only or PBS with the M1-2 or M1-TK constructs. M1-TK targets an unrelated mRNA, and M1-2 is catalytically inactive and contains the identical guide sequence to M1-1. Thus, the observed reduction in viral gene expression and inhibition of viral growth in the M1-1-expressing cells and in animals is primarily attributed to the specific targeted cleavage by the ribozyme, as opposed to the antisense effect of the guide sequence.

Our results also provide direct evidence that suggests that MGS RNAs are active and specific in vivo in animals. First, M1GS RNAs were readily detected in cultured cells and in the livers of the animals. Second, there are no significant differences in terms of growth and viability of the constructed ribozyme-expressing cell lines and the parental cells for up to 3 months. Noninfected animals that received
M1GS-LXSN constructs via a hydrodynamic transfection procedure exhibited no adverse signs for 90 days when compared with animals receiving only PBS (data not shown). These results suggest that the ribozymes do not significantly interfere with cellular gene expression and that expression of the ribozymes does not exhibit significant cytotoxicity both in vitro and in vivo. Third, M1GS RNA only inhibits the expression of mAP/M80. No reductions in the expression levels of other viral genes examined (e.g., mie1, M99, and m155) were observed in M1GS-expressing cells (Table-2.1). Fourth, the ribozymes appear to be active in cleaving the target mAP mRNA in vivo in animals. Reduced mAP expression, decreased viral titers, and increased survival were found in animals that received M1-1-expressing constructs but not in animals that received PBS only or PBS with the M1-2 or M1-TK constructs. These results suggest that M1GS-mediated cleavage of its target mAP mRNA in vivo is effective and specific, resulting in a decreased expression of mAP and inhibition of viral growth, leading to blocking viral infection and increasing survival of infected animals.

Human CMV is a member of the herpes virus family, which includes other different viruses such as HSV and EBV (1). This virus causes significant morbidity and mortality in immunoimmature or immunodeficient individuals, such as organ transplant recipients. MCMV infection of SCID mice represents an excellent animal model to study CMV pathogenesis in immunocompromised hosts and to assess efficacy of novel antivirals for blocking viral infection and virulence. I.p. infection of SCID mice leads to a biphasic infection, initially with viral infection and replication in the spleen and liver, followed by dissemination of the virus via leukocyte-associated viremia from the spleen and liver to other peripheral organs, such as the lungs and salivary glands (1, 2, 4). SCID mice are highly susceptible to MCMV and can succumb to as little as 10 pfu of the virus, primarily because of liver damage and failure associated with viral lytic replication in the organ (1, 4, 6). Hydrodynamic transfection procedure via tail vein injection, which was used in our study, has been shown to deliver plasmid DNA efficiently and rapidly to the liver and spleen and to allow efficient expression of the transfected genes in these organs (24-26). Our results indicate substantial expression of M1GS RNAs in the liver and spleen. Furthermore, viral gene expression in the liver was found to be substantially reduced in M1-1-expressing plasmid-treated mice. During a 21-day time course infection, viral titers in the spleens, livers, lungs, and salivary glands of the animals receiving the M1-1 construct were lower than those in animals that received PBS only or PBS with the M1-2 or M1-TK constructs. These results suggest that the delivery of M1GS-LXSN DNAs and the subsequent expression of M1GS RNA in the spleen and liver resulted in the inhibition of viral initial infection in these two organs, leading to an overall diminished systemic infection and viral dissemination in other organs, including the lungs and salivary glands. The improved survival of animals receiving M1-1 is probably because of the lower viral load found in the livers of these animals. This notion is consistent with the observation that a high level of viral lytic replication and production usually leads to severe damage of hepatic tissues and liver failure.
and contributes significantly to MCMV virulence and killing of SCID mice (1, 2, 5). Thus, our results suggest that inhibiting viral infection in the spleen and liver may significantly block viral systemic infection in other organs and increase host survival.

To successfully use M1GS for clinical applications, the ribozymes need to be delivered specifically into target tissues and cell types \textit{in vivo}. The hydrodynamic transfection method is useful to demonstrate the feasibility of delivering novel transgenes and compounds into animals and to test their activity \textit{in vivo} (24-26). However, it is not suitable for clinical applications. CMV establishes latent infection in bone marrow progenitor cells and causes significant morbidity and mortality in organ transplant recipients by disseminating to other organs from the bone marrow via leukocyte-associated viremia (1, 3, 4). Further studies should examine developing a clinically relevant bone marrow transplantation protocol to deliver DNA sequence coding for M1GS into the bone marrow progenitor cells (eg, CD34+ cells) and whether M1GS RNA can be expressed and inhibit CMV infection in these cells, leading to the blockage of viral infection in leukocytes and viral dissemination in blood. These studies, in combination with further engineering of M1GS RNAs to increase their cleavage activity and specificity, will significantly facilitate the development of more active and effective RNase P ribozymes for gene-targeting applications, including therapy of human CMV infection.
MATERIALS AND METHODS

Antibodies

The antirabbit polyclonal antibodies against MCMV assembly protein (mAP) were kindly provided by Annette Meyer (Parke-Davis Pharmaceutical Research Institute, Ann Arbor, MI) and John Wu (Promab, Inc., Albany, CA). The monoclonal antibody against mouse actin was purchased from Sigma.

Viruses and cells

The Smith strain of MCMV (American Type Culture Collection) was grown in NIH 3T3 cells (CRL 1658; American Type Culture Collection) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Nu-Serum (Collaborative Research), as described previously (5).

Ribozyme and substrates

The DNA sequence that encodes substrate Map39 was constructed by PCR using pGEM3zf(+) as a template and oligonucleotides AF25 (5′-GGAATTCTAATACGACTCACTATAG-3′) and smAP (5′-CGGGATCCGCCCCGACTGAGGCTAGACCGTGTTGACTCATCCTATAGTGAGTGC-GTATTATATA-3′) as 5′ and 3′ primers, respectively.

Plasmid pFL117 and pC102 contain the DNA sequence coding for M1 RNA and mutant C102 driven by the T7 RNA polymerase promoter (20, 31). Mutant ribozyme C102 contains several point mutations (e.g., A347C348 → C347U348, C353C354C355G356 → G353G354A355U356) at the catalytic domain (P4 helix) (31). The DNA sequences that encode ribozymes M1-1 and M1-2 were constructed by PCR using the DNA sequences of the M1 and C102 ribozymes as the templates and oligonucleotides AF25 and M1mAP (5′-CCCGCTCGAGAAAAATGGTGCGTCTACCTCAGTCGAGTGC-GTATTATGTG-3′) as 5′ and 3′ primers, respectively.

M1GS RNAs and the Map39 mRNA substrate were synthesized in vitro by T7 RNA polymerase (Promega) following the manufacturer’s recommendations and further purified on 8% polyacrylamide gels containing 8 M of urea.

In vitro cleavage and binding studies

The M1GS RNA were mixed with the [32P]-labeled mRNA substrate. The cleavage reactions were carried out at 37°C in a volume of 10 μl for 40 min in buffer A [50-mM Tris (pH 7.5), 100-mM NH4Cl, and 100-mM MgCl2] (20). Cleavage products were separated in denaturing gels and quantitated with a STORM840 PhosphorImager (Molecular Dynamics). The procedures to measure the equilibrium
dissociation constants ($K_d$) of the M1GS-Map39 complexes were modified from Pyle et al. (37) and have been described previously (20). The values of $K_d$ obtained were the average of three experiments.

Expression of ribozymes in constructed cell lines

NIH 3T3 cells expressing different ribozymes were constructed, and the expression of these ribozymes was assayed using Northern analyses, following procedures described previously (5, 20).

The protocols for the construction of NIH 3T3 cells expressing different ribozymes were modified from Miller and Rosman (33) and have been described previously (17). In brief, amphotropic PA317 cells were transfected with retroviral vector DNA (LXSN-M1–1 and LXSN-M1–2) with the aid of a mammalian transfection kit purchased from GIBCO/BRL. Forty-eight hours after transfection, culture supernatants that contained retroviral vectors were collected and used to infect NIH 3T3 cells. At 48–72 h after infection, cells were incubated in culture medium that contained 500-μg/ml neomycin. Cells were subsequently selected in the presence of neomycin for 2 weeks, and neomycin-resistant cells were cloned.

For Northern analyses of the expression of the ribozymes, RNA fractions from M1GS-expressing cells were isolated, separated in a 2% agarose gel that contained formaldehyde, transferred to a nitrocellulose membrane, hybridized with the [$^{32}$P]-radiolabeled DNA probe that contained the DNA sequence coding for M1 RNA, and finally analyzed with a STORM840 PhosphorImager (20). The radiolabeled DNA probe used to detect M1GS RNAs was synthesized from plasmid pFL117 by using a random primed labeling kit (Boehringer Manheim).

Viral infection and assays for viral mRNA and protein expression

T-25 flasks of cells (≈10$^6$ cells) were either mock-infected or infected with MCMV, as described previously (5, 20). The MOI is specified in Results. The infected cells were incubated for 8–72 h. The RNA and protein samples were isolated from infected cells, and the expression of specific mRNAs and proteins were assayed by Northern and Western analyses, respectively, following procedures described previously (5, 20).

The RNA and protein samples were isolated as described previously (20). The RNA fractions were separated in 1% agarose gels that contained formaldehyde, transferred to a nitrocellulose membrane, hybridized with the [$^{32}$P]-radiolabeled DNA probes that contained the MCMV DNA sequence or the DNA sequence coding for mouse mP1 RNA, and analyzed with a STORM840 PhosphorImager. The DNA probes used to detect M1GS RNAs, mouse mP1 RNA, MCMV 7.2kb RNA transcript, and M80 and mAP mRNA were synthesized from plasmids pFL117, pmP1 RNA, pM7.2KB, and pM80, respectively.
For Western analyses, the polypeptides from cell lysates were separated on SDS/9% polyacrylamide gels cross-linked with N,N'-methylenebisacylamide, transferred electrically to nitrocellulose membranes, and stained using the antibodies against MCMV proteins and mouse actin in the presence of a chemiluminescent substrate (Amersham), and the stained membranes were analyzed with a STORM840 phosphorimager. Quantitation was performed in the linear range of RNA and protein detection.

**Viral infection and assays for viral growth**

To determine the level of the inhibition of viral growth, 5 × 10^5 cells were either mock infected or infected with MCMV at an MOI of 1. The infection was carried out by incubating cells with DMEM in the absence or presence of viruses at 37°C for 90 min and then with fresh DMEM for different periods of time (5). The cells and medium were harvested at 1, 2, 3, 4, and 5 days after infection, and viral stocks were prepared by adding 10% skim milk followed by sonication.

The titers of the viral stocks were determined by performing plaque assays (5) and the values obtained were the average of triplicate experiments. Plaque assays were performed in NIH 3T3 cells plated overnight to 60%–75% confluence in six-well cluster plates (Costar, Corning). Tenfold serial dilutions of virus samples in 1 ml of DMEM were inoculated onto each well of NIH 3T3 cells. After 90 minutes of incubation at 37°C in a 5% CO2 incubator, the cells were washed with DMEM, then overlaid with DMEM containing 1% low melt agarose (Sigma). Viral plaques were counted after 4–6 days under an inverted microscope. Each sample was titered in triplicate, and the titer of the sample was expressed as the average of the three values. Viral titers were recorded as pfu/ml of organ homogenate. The limit of virus detection in the organ homogenates was 10 pfu/ml of the sonicated mixture. Those samples that were negative at a 10-1 dilution were designated a titer value of 10 (10^1) pfu/ml.

**Infection and hydrodynamic transfection of animals**

CB17 SCID mice were obtained from Jackson Laboratory (Bay Harbor, Maine). 4-6 weeks old male mice were selected for further study. Animals were intraperitoneally infected with 1 × 10^4 pfu of MCMV (Smith strain). At 24 hours after infection, different plasmids DNAs were delivered to these animals using a modified hydrodynamic transfection method (24-26). In these experiments, the animal was first restrained in a rodent restraint and exposed the tail completely. Then the animal was anesthetized by short term exposure to isoflurane. Animal's tail was cleaned with alcohol and then warmed with heat source, such as gauze wet with warm autoclaved water. This procedure can make the tail vein visible and easy for injection. Rapidly inject 1-2 mL PBS with or without 20 μg of pLXSN-U6-M1-1 DNA into one tail vein of the animal within 5-10 seconds by using a syringe with hypodermic needles of 24–27 gauge size. The amount of injection volume is based on the animal blood volume.
and cardiac capacity. We showed that for four-to six-week-old CB17 SCID mice, a 1-2 mL injected volume (20 µg plasmid) is sufficient to reach the hydrostatic pressure required for a high level of gene expression. Syringe with hypodermic needles of 24-27 gauge size was used in injection. If the needle is placed properly, the injection process will be very smooth. Otherwise, a “tuber” will appear in the tail and injection has to be repeated from another part of the vein. The hydrodynamic transfection procedures were repeated every 72 hours. The transfection efficiency was evaluated by detecting the expression of M1GS RNAs in the tissues using northern analyses.

The transfection efficiency was evaluated by detecting the expression of M1GS RNAs in the tissues (e.g., livers) using Northern analyses and examining the GFP staining of the transfected cells in the tissues using fluorescence microscopy. The GFP-expressing cassette sequence was included in the LXSN vector sequence. Consistent with previous hydrodynamic transfection reports (24-26), we regularly achieved a transfection efficiency of 60% in the livers and 30% in the spleens.

Analysis of viral virulence, growth, and gene expression in SCID mice

The animals were observed twice daily, the mortality of the infected animals was monitored for at least 41 days after infection, and the survival rates were determined. To study viral growth and gene expression in mice, CB17 SCID mice were infected i.p. with $1 \times 10^4$ pfu of each virus. The infected animals (at least three animals per group) were sacrificed at 1, 3, 7, 10, 14, and 21 days after inoculation. The salivary glands, lungs, spleens, and livers were harvested and sonicated as a 10% (wt/vol) suspension in a 1:1 mixture of DMEM and 10% skim milk. Viral titers of the samples were determined using plaque assays as described below.

Plaque assays were performed in NIH 3T3 cells plated overnight to 60%–75% confluence in six-well cluster plates (Costar, Corning). Tenfold serial dilutions of virus samples in 1 ml of DMEM were inoculated onto each well of NIH 3T3 cells. After 90 minutes of incubation at 37°C in a 5% CO2 incubator, the cells were washed with DMEM, then overlaid with DMEM containing 1% low melt agarose (Sigma). Viral plaques were counted after 4–6 days under an inverted microscope. Each sample was titered in triplicate, and the titer of the sample was expressed as the average of the three values. Viral titers were recorded as pfu/ml of organ homogenate. The limit of virus detection in the organ homogenates was 10 pfu/ml of the sonicated mixture. Those samples that were negative at a $10^{-1}$ dilution were designated a titer value of $10 (10^1)$ pfu/ml.

In gene expression experiments, tissues were homogenized and total RNA and proteins were then isolated following described procedures (5). The expression of M1GS RNA and viral mRNA was determined using Northern analyses, whereas the expression of viral proteins was assayed using Western analysis.
Figure 2.1 Characterization of M1GS ribozyme in vitro and in cultured cells. (A and B) Schematic representation of a natural substrate (ptRNA) (A) and a complex formed between a M1GS RNA and its mRNA substrate (B). The site of cleavage by RNase P or M1 RNA is marked with a filled arrow. (C) Schematic representation of the substrate used in the study. The targeted sequence that binds to the guide sequence of the ribozymes is highlighted. (D) Cleavage of substrate Map39 by M1GS RNA. Map39 (20 nM) was incubated alone (lane 1), with 5 nM of M1-1 (lane 2), M1-2 (lane 3), or M1-TK (lane 4). Reactions were carried out for 40 min in buffer A [50-mM Tris·HCl (pH 7.5), 100-mM NH₄Cl, 100-mM MgCl₂] at 37°C. (E and F) Northern analyses of the expression of M1GS ribozymes in parental NIH 3T3 cells (−, lanes 5 and 8) or cloned cell lines that expressed M1-1 (lanes 6 and 9) and M1-2 (lanes 7 and 10). Equal amounts of RNA sample (25 μg) were separated on 2% agarose gels that contained formaldehyde, transferred to nitrocellulose membranes, and hybridized to a [³²P]-radiolabeled probe that contained the DNA sequence coding for M1 RNA (lanes 5–7) (E) or mP1 RNA (lanes 8–10) (F), the RNA subunit of mouse RNase P (34, 35). The hybridized products corresponding to the full-length retroviral transcripts (~6kb), transcribed from the LTR promoter, are at the top of the gel and are not shown.
Figure 2.2 M1GS-mediated inhibition of MCMV infection in cultured cells. (A–D) Expression levels of MCMV mRNAs (A and B) and proteins (C and D). The $5 \times 10^5$ cells were either mock-infected (lanes 1, 5, 9, and 13) or infected with MCMV (lanes 2–4, 6–8, 10–12, and 14–16) and were harvested at 8–72 h after infection. Northern and Western analyses were carried out using RNA (A and B) or protein samples (C and D) isolated from parental NIH 3T3 cells (–, lanes 1, 2, 5, 6, 9, 10, 13, and 14) and cell lines that expressed M1-2 (lanes 3, 7, 11, and 15) and M1-1 (lanes 4, 8, 12, and 16). Equal amounts of each RNA sample (25 $\mu$g) were separated on agarose gels that contained formaldehyde, transferred to nitrocellulose membranes, and hybridized to [$^{32}$P]-radiolabeled probes that contained the sequence of the MCMV 7.2-kb transcript (lanes 1–4) and mAP mRNA (lanes 5–8). The hybridized products corresponding to the 7.2-kb RNA, M80, and mAP mRNAs were $\approx$7.2, 2.1, and 1.1 kb, respectively (1, 23, 36). For Western analyses, equal amounts of protein samples (40 $\mu$g) isolated from cells were separated in SDS-polyacrylamide gels. The membranes were stained with the antibodies against mouse actin (C) and MCMV mAP (D) in the presence of a chemiluminescent substrate. (E) Growth of MCMV in NIH 3T3 cells and ribozyme-expressing cell lines. The $5 \times 10^5$ cells were infected with MCMV at an MOI of 1. Virus stocks were prepared from the infected cells at 1-day intervals through 5 days after infection. These values are the means of triplicate experiments, and the standard deviation is indicated by the error bars.
Table 2.1 Levels of inhibition of viral gene expression in cells that expressed M1-1, M1-2, and M1-TK, as compared to that in the parental NIH 3T3 cells that did not express a ribozyme (NIH 3T3)

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Viral gene class</th>
<th>Ribozymes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NIH 3T3, %</td>
<td>M1-TK, %</td>
</tr>
<tr>
<td>mie1 mRNA</td>
<td>α</td>
<td>0</td>
</tr>
<tr>
<td>M155 mRNA</td>
<td>γ</td>
<td>0</td>
</tr>
<tr>
<td>mAP mRNA</td>
<td>γ</td>
<td>0</td>
</tr>
<tr>
<td>M80 mRNA</td>
<td>γ</td>
<td>0</td>
</tr>
<tr>
<td>M112 protein</td>
<td>β, γ</td>
<td>0</td>
</tr>
<tr>
<td>M99 protein</td>
<td>γ</td>
<td>0</td>
</tr>
<tr>
<td>mAP protein</td>
<td>γ</td>
<td>0</td>
</tr>
</tbody>
</table>

The values shown are the means of triplicate experiments, and the values of standard deviation that were <5% are not shown.
Figure 2.3 Expression levels of M1GS RNA (A), viral mRNAs (B and C), and proteins (D and E). The livers were isolated from animals that were either mock-infected (lanes 7, 11, and 15) or infected with MCMV (lanes 1–6, 8–10, 12–14, and 16–18) and were harvested at 14 days after infection. Northern and Western analyses were carried out using RNA (A–C) or protein samples (D and E) isolated from the livers of the animals that received PBS only (-, lanes 1, 4, 7, 8, 11, 12, 15, and 16) or PBS with M1-2 (lanes 2, 5, 9, 13, and 17) and M1-1-expressing constructs (lanes 3, 6, 10, 14, and 18). Equal amounts of each RNA sample (50 μg) were separated on agarose gels that contained formaldehyde, transferred to nitrocellulose membranes, and hybridized to [32P]-radiolabeled probes that contained the sequence of the mP1 (lanes 1–3), M1 RNA (lanes 4–6), and mAP mRNA (lanes 7–10). For Western analyses, equal amounts of protein samples (80 μg) were separated in SDS-polyacrylamide gels. The membranes were stained with the antibodies against mouse actin (D) and MCMV mAP (E).
Figure 2.4 Mortality of the SCID mice infected with the Smith strain, followed by hydrodynamic transfection of PBS alone and PBS with different M1GS-LXSN DNA (M1-1, M1-2, and M1-TK). CB17 SCID mice (five animals per group) were infected i.p. with $1 \times 10^4$ pfu MCMV 24 hours before hydrodynamic transfection. The transfection procedure was repeated every 72 hours. Mortality of mice was monitored for at least 41 days after infection, and survival rates were determined.
Figure 2.5 Titters of MCMV in the spleens (A), livers (B), lungs (C), and salivary glands (D) of the infected SCID mice. CB17 SCID mice were infected i.p. with $1 \times 10^4$ pfu MCMV, followed by hydrodynamic transfection of PBS alone and PBS with different M1GS-LXSN DNA (M1-1, M1-2, and M1-TK) 24 hours later. The transfection procedure was repeated every 72 hours. At 1, 3, 7, 10, 14, and 21 days after infection, the animals (three mice per group) were killed. The salivary glands, lungs, spleens, and livers were collected and sonicated. The viral titters in the tissue homogenates were determined by standard plaque assays in NIH 3T3 cells. The limit of detection was 10 pfu/ml of the tissue homogenate. The viral titters represent the average obtained from triplicate experiments. The error bars indicate the standard deviation. Error bars that are not evident indicate that the standard deviation was less than or equal to the height of the symbols.
REFERENCES


CHAPTER III

*Salmonella*-mediated gene therapy (1):

Delivery of M1GS ribozymes by *Salmonella* effectively inhibits HCMV infection in human cells
ABSTRACT

A fundamental challenge in gene therapy is to develop approaches for delivering nucleic acid-based gene interfering agents, such as small interfering RNAs and ribozymes, to the appropriate cells in a way that is tissue/cell specific, efficient, and safe. Using human cytomegalovirus (HCMV) infection of differentiated macrophages as the model, the study showed that Salmonella can efficiently deliver RNase P-based ribozyme sequence in specific human cells, leading to substantial ribozyme expression and effective inhibition of viral infection. A functional RNase P ribozyme (M1GS RNA) that targets the overlapping mRNA region of two HCMV capsid proteins, the capsid scaffolding protein (CSP) and assemblin, was constructed. Substantial expression of ribozymes was observed in human differentiated macrophages that were treated with attenuated Salmonella strains carrying the ribozyme sequence constructs. A reduction of 87–90% in viral CSP expression and a reduction of about 5,000-fold in viral growth were observed in cells that were treated with Salmonella carrying the sequence of the functional ribozyme but not with those carrying the sequence of a control ribozyme that contained mutations abolishing the catalytic activity. This study showed for the first time that ribozymes expressed following targeted gene transfer with Salmonella-based vectors are highly active and specific in blocking viral infection. Moreover, these results demonstrate the feasibility to develop Salmonella-mediated gene transfer of RNase P ribozymes as an effective approach for gene-targeting applications.
INTRODUCTION

Human cytomegalovirus (HCMV), a common opportunistic pathogen, causes significant morbidity and mortality in immunocompromised or immunologically immature individuals, including neonates, AIDS patients and transplant recipients (1). Macrophages and their progenitor cells, such as monocytic cells, represent the major reservoirs for HCMV. HCMV can establish latent infection in undifferentiated monocytic cells but engage in productive and lytic replication in terminally differentiated macrophages, leading to viral pathogenesis. Thus, blocking HCMV infection and replication in macrophages is central in treating and preventing HCMV-associated diseases.

Nucleic acid-based gene interference technologies, including ribozymes and small interfering RNAs, represent promising gene-targeting strategies for specific inhibition of mRNA sequences of choice (2). Altman and colleagues have previously shown that RNase P of Escherichia coli contains a catalytic RNA subunit (M1 RNA) that can be engineered to cleave tRNA substrates and other target RNAs, including specific mRNAs (3, 4). A sequence-specific ribozyme, M1GS, constructed by attaching to M1 RNA a guide sequence (GS) complementary to a target mRNA (Fig. 1 A and B), is effective in blocking mRNA expression in cultured cells (4, 5). M1GS-based strategy represents a unique nucleic acid-based interference approach because of the use of M1 RNA, one of the most efficient catalytic RNAs found in nature (3). Previous studies have shown that M1GS RNAs and RNase P are effective in cleaving both viral and cellular mRNAs and blocking their expression in cultured cells, including inhibition of gene expression of human influenza and herpes viruses (5-7).

A fundamental challenge in gene-targeting therapy is to develop approaches for delivering genetic material to the appropriate cells of a patient in a way that is tissue/cell specific, efficient, and safe (8, 9). Many of the vectors currently in use are based on attenuated or modified viruses, or synthetic vectors in which complexes of DNA, proteins, and/or lipids are formed in particles. Like all other nucleic acid-based interference approaches, delivery and expression of M1GS sequences in specific cell types and tissues is central in developing this technology for gene-targeting applications.

Invasive bacteria, such as Salmonella, which possess the ability to enter human cells, are capable of transferring genetic material to host cells, leading to efficient expression of the transferred genes (9). Attenuated Salmonella strains have recently been shown to function as a carrier system for delivery of nucleic acid-based vaccines and antitumor small hairpin RNAs (shRNAs) for cancer therapy (10-13). In these studies, plasmid constructs, which contained the transgenes under the control of a eukaryotic expression promoter, were introduced to the attenuated bacterial strains. These attenuated strains can target specific cells, such as dendritic cells,
macrophages, and epithelial cells, and survive within the target cells to a limited extent. How the plasmid DNA from a bacterial vector is transferred to the host is not completely understood. It is generally believed that once bacteria undergo lysis intracellularly, the plasmid content is released, leading to expression of the encoded transgenes in the construct by cellular machinery (Figure-3.1) (11, 14).

Macrophages represent the major in vivo reservoir for *Salmonella* following their systemic dissemination (15) and, therefore, are considered an optimal target for a *Salmonella*-based gene therapy. Using HCMV infection of differentiated macrophages as the model, we in this study provide direct evidence that *Salmonella* can efficiently deliver the M1GS sequence into human differentiated macrophages, leading to substantial expression of the ribozymes. M1GS ribozymes were constructed to target the region of the mRNA encoding the HCMV capsid scaffolding protein (CSP). CSP completely overlaps with and is within the 3’ coding sequence of another viral capsid protein, assemblin (1). Both CSP and assemblin are essential for HCMV capsid formation and replication (1). Our results showed that targeted gene delivery of RNase P ribozyme by *Salmonella* to HCMV-infected cells resulted in effective inhibition of viral gene expression and replication. Furthermore, these results demonstrate the feasibility of developing *Salmonella*-based vectors for delivering RNase P ribozymes for treatment of viral diseases.
RESULTS

1. In vitro cleavage of HCMV mRNA sequence by M1GS ribozyme

Using DMS, we employed an in vivo mapping approach (5) to determine the accessibility of the region of the CSP mRNA in HCMV-infected cells, and we chose a highly accessible region as the cleavage site for M1GS RNA. A functional ribozyme, M1-C1, was constructed by covalently linking the 3’ terminus of an engineered M1GS ribozyme, V57, with a guide sequence of 18 nucleotides that is complementary to the targeted mRNA sequence. V57 is a M1 RNA variant (G190 → U190 and A258 → C258) generated from an in vitro selection procedure, and ribozymes derived from this variant are among the most active M1GS RNAs in cleaving the CSP mRNA and the thymidine kinase (TK) mRNA of herpes simplex virus 1 (HSV-1) (Figure-3.2) (16). A control ribozyme, M1-C2, was derived from M1-C1 by introducing several point mutations (A347C348 → C347U348 and C353C354C355G356 → G353G354A355U356) at the catalytic P4 domain. These mutations reduced the activity of M1 RNA in cleaving a pre-tRNA by at least 10,000-fold (7). M1-C2 is therefore expected to be catalytically inactive.

Incubation of a substrate containing the CSP mRNA sequence with functional ribozyme M1-C1 yielded efficient cleavage (Figure-3.2D, lane 2). In contrast, cleavage by M1-C2 was barely detected (Figure-3.2D, lane 3). Gel-shift assays indicate that the binding affinity of M1-C2 to the CSP mRNA substrate, measured as the dissociation constant (K_d), is similar to that of M1-C1 (Figure-3.2C). Since M1-C2 contains the same antisense guide sequence and exhibits similar affinity to the CSP mRNA substrate as M1-C1 but is catalytically inactive, this ribozyme was used as a control for the antisense effect in our experiments (see below).

2. Efficient gene delivery for the expression of ribozymes in macrophages by Salmonella

The DNA sequence coding for M1-C1 and M1-C2 were cloned into vector pU6, which contains a GFP expression cassette, and placed under the control of the small nuclear U6 RNA promoter. This promoter, which is transcribed by RNA polymerase III, has previously been shown to express M1GS RNA and other RNAs steadily (5, 17). To determine whether Salmonella-mediated delivery for expression of a M1GS RNA with an incorrect guide sequence could affect the CSP mRNA, the DNA sequence for ribozyme M1-TK, which was derived from V57 and targeted the HSV-1 TK mRNA (16), was also cloned into vector pU6. No cleavage of the CSP mRNA substrate by M1-TK was observed in vitro (Figure-3.2D, lane 4). The DNAs of constructs containing the M1GS sequence were transformed into auxotrophic Salmonella strain SL7207, which is attenuated in virulence and pathogenesis in vivo and has been shown to function efficiently as a gene delivery carrier for the expression of several transgenes in mammalian cells (12, 13).
Four sets of experiments were carried out to characterize *Salmonella* carrying the plasmids with the ribozyme sequences. First, growth analyses of *Salmonella* were performed. There was no significant difference in the growth kinetics of *Salmonella* carrying no constructs or constructs pU6-M1-C1, pU6-M1-C2, pU6-M1-TK, and the pU6 empty vector in LB broth (Figure-3.3A), indicating that the presence of the ribozyme sequence does not result in an impaired viability of the bacterial carrier. Second, Northern analysis indicated that neither the GFP nor the M1GS RNA transcript was detected in *Salmonella* carrying different ribozyme constructs. Furthermore, no GFP signal was observed when *Salmonella* was examined under a fluorescence microscope. These results suggested that M1GS RNA, which was under the control of the U6 expression cassette, was not expressed in the *Salmonella* vector. Third, to determine whether *Salmonella* can efficiently deliver the M1GS sequences into human cells, differentiated macrophage THP-1 cells were infected with *Salmonella* SL7207 carrying pU6-M1-C1, pU6-M1-C2, pU6-M1-TK, and the pU6 empty vector. At 24 hours postinfection, more than 80% of cells were GFP positive, indicating efficient gene transfer mediated by *Salmonella*. Fourth, to examine the ribozyme expression after *Salmonella*-mediated gene transfer, total RNAs were isolated from *Salmonella*-infected cells at 24 hours postinfection. The levels of M1GS RNAs (Figure-3.3B) were assayed with Northern analyses, using H1 RNA [the RNA subunit of human RNase P (3)] as the internal control (Figure-3.3C). Similar levels of ribozymes were found when cells were infected at the same multiplicity of infection (MOI) with *Salmonella* carrying different ribozyme sequences. The M1GS RNAs appeared to be exclusively expressed in the nuclei as they were detected only in the nuclear but not the cytoplasmic RNA fractions. This is consistent with previous observations that the transcripts expressed by the U6 promoter are primarily localized in the nuclei (7, 17).

3. Inhibition of HCMV gene expression by ribozymes delivered via *Salmonella*-based vector

To determine the effect of the M1GS sequence delivered by *Salmonella* on HCMV gene expression, differentiated THP-1 cells were first treated with *Salmonella* carrying the pU6-M1GS plasmids. The *Salmonella*-containing cells were then isolated by FACS analysis based on GFP expression, and infected with HCMV at a multiplicity of infection (MOI) of 0.05–1. Total RNAs were isolated from the infected cells at 8–72 hours postinfection. The expression levels of CSP and assemblin mRNAs were determined by Northern analyses. The level of the 5-kb long viral immediate-early transcript (5-kb RNA), whose expression is not regulated by assemblin and CSP under the assay conditions (1), was used as an internal control for the quantitation of expression of the target mRNAs (Figure-3.4A). A reduction of about 90 ± 8% and 90 ± 9% (average of three experiments) in the expression levels of CSP and assemblin mRNA was observed in cells that expressed M1-C1, respectively (Figure-3.4B and Table-3.1). In contrast, a reduction of less than 10% in the expression levels of these two mRNAs was observed in cells that were treated
with *Salmonella* carrying the M1-C2 or M1-TK sequences. The CSP protein levels in cells that were treated with *Salmonella* carrying the M1-C1 sequence-containing plasmid were also reduced. Proteins were isolated from cells at 24–72 hours postinfection, separated in SDS-polyacrylamide gels, and transferred to identical membranes. One of the membranes was stained with an anti-CSP antibody, and another membrane was stained with a monoclonal antibody against human actin (Figure-3.4C and D). The latter serves as an internal control for the quantitation of CSP protein expression. A reduction of about 87% in the protein level of CSP (from three independent experiments) was observed in cells treated with *Salmonella* carrying the M1-C1 sequence, while a reduction of less than 10% was found in cells that were treated with *Salmonella* carrying the plasmids expressing the M1-C2 or M1-TK RNAs. The low level of inhibition found in cells treated with *Salmonella* carrying the M1-C2 sequence was presumably due to an antisense effect because M1-C2 exhibited similar binding affinity to the target sequence as M1-C1 but was catalytically inactive. These results suggest that the significant reduction of CSP expression in cells treated with the M1-C1-containing *Salmonella* is due to *Salmonella*-mediated gene delivery of the ribozyme.

4. Inhibition of HCMV growth by *Salmonella*-mediated gene delivery of ribozyme

To determine whether *Salmonella*-mediated gene delivery of ribozymes inhibits the growth of HCMV, differentiated THP-1 cells were first treated with *Salmonella* carrying the ribozyme sequences. The *Salmonella*-containing cells were then isolated by FACS analysis based on GFP expression and infected by HCMV at an MOI of 1–5. We harvested the infected cultures (cells and culture medium together) at one-day intervals through seven days postinfection and determined the viral titers of these samples. At six days postinfection, a reduction of at least 5,000-fold in viral yield was observed in cells that were treated with *Salmonella* carrying the vector containing the M1-C1 sequence (Figure-3.5). In contrast, no significant reduction was found in cells that were treated with *Salmonella* that carried the empty vector plasmid, or plasmids containing the M1-C2 and M1-TK sequence (Figure-3.5).

5. Blocking viral capsid maturation by *Salmonella*-mediated gene delivery of M1GS

HCMV CSP is essential for viral capsid maturation and assembly (1). Meanwhile, it is possible that the observed reduction of viral growth in cells that were treated with M1-C1 sequence-containing *Salmonella* is not necessarily due to specific M1GS RNA-mediated cleavage of CSP mRNA but is due to other effects of the ribozyme or *Salmonella* on viral lytic replication that are unrelated to the consequence of the ribozyme cleavage or the inhibition of viral CSP expression. To further determine the antiviral mechanism of the *Salmonella*-mediated gene delivery of M1GS against the CSP mRNA, two sets of experiments were performed to
investigate which step of the viral lytic cycle was blocked in cells treated with M1-C1 sequence-containing Salmonella. First, we examined the expression of other viral genes. Inhibition of CSP/assemblin expression is not expected to affect the expression of other viral genes, including immediate-early (α), early (β), and late (γ) genes (1). The levels of the IE2 (an α-transcript) and US2 mRNA (a β-transcript) were examined using Northern analyses, whereas the levels of viral protein UL44, a viral early late (βγ) protein and gB, a viral late (γ) protein were assayed with Western analyses. We observed no significant difference in the levels of these genes among Salmonella-treated cells (Table-3.1), suggesting that the Salmonella-mediated delivery of M1-C1 specifically inhibits the expression of its target and does not affect overall viral gene expression.

The second set of experiments was performed to determine whether viral genomic replication as well as capsid maturation is affected in cells treated with Salmonella that carried the ribozyme constructs. Cells were first treated with Salmonella, and the Salmonella-containing cells were then isolated by FACS analysis based on GFP expression and infected with HCMV. Total DNA was isolated from HCMV-infected cell lysates, and the level of intracellular viral DNA was determined by PCR detection of the HCMV IE1 sequence, using levels of β-actin DNA as the internal control (Figure-3.6). The amount of intracellular viral DNA detected by the PCR assay should represent the replication level of the viral genome because HCMV replicates only in an episomal form and does not integrate its DNA into the host genome (1). To determine the level of mature capsids assembled in infected cells and examine viral capsid formation, we assayed the level of encapsidated viral DNA. DNA samples were isolated from HCMV-infected cell lysates that were treated with DNase I. The encapsidated viral DNAs are resistant to DNase I digestion, whereas those that are not packaged in the capsid will be susceptible to degradation. When the DNA samples from cell lysates that were not treated with DNase I were assayed, no significant difference in the level of total intracellular (both encapsidated and unencapsidated) viral DNA were observed (Figure-3.6, lanes 1–3). However, when the samples were first treated with DNase I and then assayed, the “encapsidated” DNA was hardly detected in cells that were treated with Salmonella carrying the M1-C1 expression plasmid (Figure-3.6, lane 5). These observations suggest that Salmonella-mediated gene delivery of ribozymes against the CSP mRNA does not affect the replication of viral DNA but blocks DNA encapsidation and capsid formation.
DISCUSSION

Nucleic acid-based gene interference strategies, such as antisense oligonucleotides, ribozymes or DNAzymes, and RNA interference, represent powerful research tools and promising therapeutic agents against human diseases (2). The RNase P ribozyme-based technology represents an attractive approach for gene inactivation by using M1 RNA, a highly active RNA enzyme found in nature (3). The properties and activities of RNase P ribozyme, as well as the simple design of the guide sequence, make M1GS an attractive and unique gene-targeting agent that can be generally used for antiviral as well as other in vivo applications. For nucleic acid-based gene interfering approaches including M1GS ribozyme to be successful as a therapeutic tool for practical applications, one of the most important issues is targeted gene delivery of these agents to specific types of cells and tissues. This study demonstrates targeted gene delivery of RNase P ribozymes into specific human cells by Salmonella.

In the current study, we constructed a M1GS RNA targeting the overlapping region of HCMV CSP and assemblin mRNAs. The ribozyme cleaved the target mRNAs efficiently in vitro and, furthermore, reduced their expression levels by 87–90% and inhibited viral growth by about 5,000-fold in human macrophages that were treated with Salmonella carrying the plasmid of the M1-C1 sequence. In contrast, a reduction of less than 10% in the levels of CSP expression and viral growth was observed in cells that were treated with Salmonella carrying the plasmids of the M1-C2 and M1-TK sequences. M1-TK targets an unrelated mRNA, and M1-C2 is catalytically inactive and contains an identical guide sequence to M1-C1. Thus, the observed reduction in viral gene expression and inhibition of viral growth in cells treated with the M1-C1-containing Salmonella is primarily attributed to Salmonella-mediated gene delivery of the functional ribozyme sequence for targeted cleavage of HCMV CSP mRNA.

Several lines of evidence presented in our study indicate that the Salmonella-mediated gene transfer is efficient, and ribozymes expressed following the Salmonella-mediated gene transfer are active and specific. First, targeted gene transferring of the ribozyme constructs by Salmonella yields substantial expression of the ribozymes (Figure-3.3). Furthermore, more than 80% of the cells were GFP positive, suggesting efficient transfer of the ribozyme construct, which also contained the GFP expression cassette. Second, the presence of ribozyme sequences in Salmonella did not significantly affect the viability and gene transfer ability of the bacteria (Figure-3.3). Third, the ribozymes expressed following transfer effectively and specifically inhibited the expression of CSP/assemblin. No reduction in the expression levels of other viral genes examined (e.g., IE2, US2, UL44, and gB) was observed in cells treated with M1GS-containing Salmonella (Table-3.1). Furthermore, the ribozyme expression does not affect the replication of viral genomic DNA (Figure-3.6). Fourth, the inhibition of viral growth and capsid maturation appears to result
from the reduction of CSP and assemblin. We observed that the level of capsid formation (DNA encapsidation) and the expression of CSP greatly decreased in cells treated with Salmonella carrying the sequence of M1-C1 but not M1-C2 or M1-TK (Figure-3.4 and 3.6). Together, these results suggest that Salmonella can function as a gene transfer vector for efficient delivery of RNase P ribozyme in human macrophages and that the ribozymes expressed following gene transfer are active and specific in inhibiting only the expression of their target mRNA and do not affect the expression of other viral genes and genome replication.

The fundamental challenge in gene therapy is to develop approaches for delivering genetic material to the appropriate cells of the patient in a way that is tissue/cell specific, efficient, and safe (8, 9). Tissue-specific vectors have been only partially obtained by using carriers that, in nature, infect certain cell types, such as herpes simplex virus does for cells of the nervous system (8, 9). Salmonella-based vectors exhibit several unique and attractive features as a gene delivery tool. First, the Salmonella-based vector is low cost and easy to prepare, store, and transport. Second, one of the most interesting aspects associated with the use of attenuated Salmonella as a vector is the possibility of administering these bacteria via the oral route, a strategy that has proved to be successful in terms of efficacy and acceptability (18, 19). In fact, the antityphoid fever vaccine based on the attenuated strain Ty21a is one of the few live vaccines licensed for human use and has been extensively used to immunize both adults and children (19, 20). Third, it is conceivable to generate attenuated mutants with more than one independent deletion leading to an attenuated phenotype (21). This will eliminate the risk of reversion following horizontal gene transfer. Furthermore, different mutants have been identified, which are not harmful even for immunocompromised hosts (22). Fourth, while some safety considerations for the use of Gram-negative bacteria refer to the toxic effect of LPS, this concern has mostly been ruled out by oral delivery and by the fact that Salmonella strains have been widely used as vaccines both in human and veterinary medicine (9, 18). Finally, integration of bacteria-delivered DNA in host cell genome is not common (23), and oncogenesis promotion of the bacterial infection has not been known. Thus, the low-cost, safe, noninvasive administration and lifelong suitability of the therapy, combined with easy preparation of the bacterial carrier, support the notion that Salmonella represents an attractive and promising gene delivery tool for gene therapy for human diseases, including viral infections.

It is well established that bacterial infection elicits various innate immune responses, including activation of TLR4 and TLR9, which can be induced by bacterial LPS and the unmethylated CpG motifs of bacterial DNA, respectively (24, 25). Some of these responses are beneficial to the host (e.g., inhibiting bacterial infection and replication), however, some other responses may have unwanted side effects (e.g., induction of apoptosis), leading to potential cytotoxicity. To avoid these responses and reduce potential cytotoxicity, mutations have been introduced to bacterial vectors in order to inactivate the expression of specific bacterial components (e.g., LPS) (18).
Alternatively, bacteria carrying transgenes that modulate these responses (e.g., inhibition of TLR9 expression) can be used (25). Further experiments to generate mutant *Salmonella* strains and examine the innate immune responses and cytotoxicity associated with these mutants should facilitate the development of better *Salmonella*-based vectors for gene therapy applications.

HCMV is a member of the herpesvirus family, which includes different viruses such as herpes simplex virus and Epstein-Barr virus (1). Macrophages represent the major reservoirs for HCMV as this virus can establish both primary and latent infections in these cells, leading to pathogenesis such as life-threatening complications in immunocompromised individuals. Eliminating infection in macrophages is central to the treatment and prevention of HCMV-associated diseases. Our study provides direct evidence that *Salmonella*-mediated gene transfer of a RNase P ribozyme can specifically block HCMV infection and replication in human differentiated macrophages. Future studies, including the construction of *Salmonella* strains through mutagenesis strategies, should lead to the generation of *Salmonella* vectors with better gene transfer efficiency and less toxicity. Moreover, investigation of the expression and activity of the ribozymes following gene transfer should provide insight into the mechanism of *Salmonella*-mediated gene transfer of RNase P ribozymes. These studies will facilitate the development of RNase P ribozymes as a promising gene-targeting agent for in vivo applications.
MATERIALS AND METHODS

Antibodies

The anti-rabbit polyclonal antibodies against human cytomegalovirus (HCMV) assemblin and capsid scaffolding protein (CSP) were kindly provided by Annette Meyer of Warner Lambert Co (Ann Arbor, MI). The monoclonal antibodies that react with HCMV UL44 and gB were purchased from Goodwin Institute for Cancer Research, and the monoclonal antibody against human actin was purchased from Sigma Inc.

Growth of Viruses and Cells

The propagation of HCMV (AD169) in cells was carried out as described previously (7). Human fibroblasts and THP-1 cells (American Type Culture Collection) were maintained in DMEM and RPMI medium 1640 supplemented with 10% (vol/vol) fetal bovine serum, respectively. THP-1 cells (American Type Culture Collection) were differentiated with 100 ng/mL tetradecanoyl phorbol acetate (Sigma) for 24 h before infection (1, 26, 27).

Ribozyme and Substrate Constructs

The DNA sequence that encodes the CSP mRNA substrate was constructed by PCR using pGEM3zf(+) as a template and oligonucleotides AF25 (5′-GGAATTCTAATACGACTCACTATAG-3′) and sSCP3 (5′-CGGGATCCGTAACGCTCCATCCCGACGTTGTTCAATCTATAGTGGAGTCTGTATTT-3′) as 5′ and 3′ primers, respectively. Plasmid pV57 contained the DNA sequence coding for V57 RNA (16). The DNA sequence that encodes ribozyme M1-C1 was constructed by PCR using pV57 as the template and oligonucleotides AF25 as the 5′ primer and M1CSP3 (5′-CCCGCTCGAGAAAAAATGGTGTCCGGATGGGAGCGTTATGTGGAATTGTG-3′) as the 3′ primer. M1GS RNAs and the CSP mRNA substrate were synthesized in vitro by T7 RNA polymerase and cleavage and binding assays were carried out (7). M1-TK was generated from pV57 as described previously (16).

In vitro analysis of ribozyme activity

M1GS RNAs and the CSP mRNA substrate were synthesized in vitro by T7 RNA polymerase (Promega Inc.) and further purified on 8% polyacrylamide gels containing 8-M urea. The procedures to measure the equilibrium dissociation constants (K_d) of the M1GS-CSP mRNA substrate complexes were modified from Pyle et al (28) and have been described previously (7, 16). The values of K_d were the average of three experiments. The cleavage reactions were carried out by incubating the ribozyme and [32P]-labeled mRNA substrate at 37°C in a volume of 10 μL in buffer A (50 mM Tris, pH 7.5, 100 mM NH4Cl, and 100 mM MgCl2) (7). Cleavage
products were separated in denaturing gels and quantitated with a STORM840 PhosphorImager (Molecular Dynamics). The overall rate of cleavage \( \left( \frac{k_{\text{cat}}}{K_m} \right) \) was assayed following the procedure described previously (16). In the cleavage reaction shown in Fig. 1D, the substrate (20 nM) was incubated either alone or with 5 nM of M1-C1, M1-C2, or M1-TK. Reactions were carried out for 40 minutes in buffer A (50 mM Tris pH 7.5, 100 mM NH₄Cl, and 100 mM MgCl₂) at 37 °C.

**Growth analysis of Salmonella**

Growth analysis of *Salmonella* in LB broth was carried out by first inoculating a single colony in 2 mL of LB broth and culturing at 37 °C with shaking at 250 rpm overnight (about 16 hours) (29). Thirty microliters of the overnight culture were then inoculated into 3 mL of fresh LB broth and cultured at 37 °C and 250 rpm. At time points of 0, 2, 4, 6, 8, 10, 12, 14, 16, and 24 hours after inoculation, 100 μL of bacterial culture were collected and used for analysis by limiting dilution in sterile 96-well plates and then plated on LB agar plates to determine their cfu/mL. Each sample was analyzed in triplicates, and the analysis was repeated at least three times. The average value of cfu/mL was used to generate the growth curve (29).

**Expression of ribozymes by *Salmonella*-mediated delivery**

The auxotrophic *Salmonella typhimurium* aroA strain SL7207 was kindly provided by professor Bruce A. D. Stocker (Stanford University, Stanford, CA). *Salmonella* carrying different constructs were obtained by transforming SL7207 with plasmids pU6, pU6-M1-C1, pU6-M1-C2, and pU6-M1-TK. Construct pU6 contained the GFP expression cassette and, in addition, the small U6 RNA promoter, which was used for the expression of ribozymes in human cells. To study gene transfer of ribozyme by *Salmonella* vectors, THP-1 cells were allowed to differentiate into adherent macrophage-like cells in the presence of 100 ng/mL tetradeacanoyl phorbol acetate (Sigma) for 24 hours (27) and then seeded into cultured wells at a concentration of 1 × 10⁶ cells/mL. The differentiated cells were then infected with *Salmonella* at a MOI of 10–20 bacteria/cell. Cultures were centrifuged at 200 × g for 5 minutes and incubated at 37 °C for 30 minutes to allow phagocytosis to occur. Under these conditions, essentially most of cells were infected with bacteria. The medium was then replaced with fresh medium containing gentamicin (20 μg/mL) and incubated for the indicated times. Cells were harvested and the expression of ribozymes was assayed using Northern analyses (7).

For Northern analyses of the expression of the ribozymes, both nuclear and cytoplasmic RNA fractions from *Salmonella*-treated cells were isolated (30), separated in gels that contained formaldehyde, transferred to nitrocellulose membranes, hybridized with the [³²P]-radiolabeled DNA probes that contained the DNA sequence coding for M1 RNA and H1 RNA, and finally analyzed with a
STORM840 PhosphorImager. The radiolabeled DNA probe used to detect M1GS RNAs was synthesized from plasmid pFL117 (5, 7).

Studies of viral gene expression, growth, and genome replication

Differentiated THP-1 cells (approximately 1–5 × 10^6 cells) were first incubated with Salmonella carrying different constructs at a MOI of 10–20 bacteria/cell. The medium was then replaced with fresh medium containing gentamicin (20 μg/mL) and incubated for 16 h to allow the expression of the ribozymes. The Salmonella-containing cells were then subjected to FACS using a FACS Vantage SE sorter (Becton Dickinson), and a population of GFP-positive cells (usually 1–5 × 10^5 cells with a positive fluorescence of >99%) was isolated. The isolated cells were incubated for 8 hours and then either mock-infected or infected with HCMV as described previously (7). The multiplicity of HCMV infection (MOI) is specified in Results. The infected cells were incubated for another 8–72 hours. The RNA and protein samples were isolated from infected cells, and the expression of specific mRNAs and proteins was assayed by Northern and Western analyses, respectively (7). Total and encapsidated (DNase I-treated) DNAs were isolated and used as the PCR templates for a semiquantitative PCR, and inhibition of HCMV growth by ribozymes was studied as described previously (7).

Analysis of viral mRNA and protein expression

Viral mRNAs and proteins were isolated from HCMV infected cells as described previously (7). The multiplicity of infection (MOI) is specified in Results. To measure the levels of viral immediate-early (IE) transcripts, some of the cells were also treated with 100 μg/mL cycloheximide prior to and during infection. The RNA fractions were separated in agarose gels that contained formaldehyde, transferred to nitrocellulose membranes, hybridized with the [32P]-radiolabeled DNA probes that contained the HCMV or human β-actin DNA sequences, and analyzed with a STORM840 PhosphorImager. The DNA probes used to detect M1GS RNAs, human β-actin mRNA, HCMV immediate-early 5 kb RNA transcript, IE2 mRNA, US2 mRNA, and CSP mRNA were synthesized from plasmids pFL117, pβ-actin RNA, pCig27, pIE2, pCig38, and pCSP, respectively.

For Western analyses, the polypeptides from cell lysates were separated on either SDS/7.5% polyacrylamide gels or SDS/9% polyacrylamide gels cross-linked with N,N"-methylenebisacrylamide and transferred electrically to nitrocellulose membranes. We stained the membranes using the antibodies against HCMV proteins and human actin in the presence of a chemiluminescent substrate and analyzed the stained membranes with a STORM840 phosphorimager. Quantitation was performed in the linear range of RNA and protein detection.
Analysis of the level of viral genome replication

Total and encapsidated (DNase I-treated) DNAs were isolated from HCMV infected cells essentially as described (31) and used as the PCR templates. Viral DNA was detected by PCR amplification of the viral IE1 sequence, using human β-actin sequence as the internal control. The 5′ and 3′ primers were CMV3 (5′-CCAAGCGGCCTCTGATAACCAAGCC-3′) and CMV4 (5′-CAGCACCATCCTCCTCTTCTCTGG-3′), respectively, while those used to amplify the actin sequence were Actin5 (5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′) and Actin3 (5′-CTAGAAGCATTGCGGTGGCAGATGAGGG-3′), respectively (32). PCR cycles and other conditions were optimized to assure that the amplification was within the linear range. The PCR reactions were performed in the presence of α-[32P]-dCTP, and the radiolabeled DNA samples were separated on polyacrylamide gels and then scanned with a STORM840 PhosphorImager. A standard (dilution) curve was generated by amplifying different dilutions of the template DNA. The plot of counts for both HCMV and β-actin vs. dilutions of DNA did not reach a plateau for the saturation curve under the conditions described above, indicating that quantitation of viral DNA could be accomplished. Moreover, we observed that the ratio of viral DNA to β-actin remained constant with respect to each DNA dilution in the standard curve, suggesting that the assay is adequately accurate and reproducible. The PCR results were derived from three independent experiments.

Analysis of the expression of Toll-like receptor 9 (TLR9) mRNA

Total RNA was isolated as described previously (7). The levels of TLR9 mRNA were determined using a qRT-PCR assay, in which the mRNA was amplified using a One-Step SYBR RT-PCR kit (TaKaRa) in an IQ5 real-time PCR instrument (Bio-Rad). The level of β-actin mRNA was used as the internal control. The 5′ and 3′ primers for amplification of TLR9 mRNA sequence were trlr9-5 (5′-GTGACAGATCCAGGTAAGT-3′) and trlr9-3 (5′-CTTCCTCTACAAATGCATCACT-3′), respectively, whereas those used to amplify the actin sequence were actin-5 (5′-CGTGGGCCGCCCTAGGCACCA-3′) and actin-3 (5′-TTGGCCTTAGGTTCCAGGGGG-3′), respectively (33, 34). Each reaction (25 μL) included 2 μL total RNAs, 12.5-μL One Step SYBR RT-PCR kit, 0.5-μL TaKaRa Ex Taq HS polymerase, 0.25-μL M-MLV RTase, 0.5-μL RNase inhibitor, 0.5-μL of each of forward and reverse primers (5 pmol/each) and 8.25 μL H2O.

Analysis of the inhibition of HCMV growth by M1GS ribozymes

To determine the level of inhibition of viral growth, 5 × 10^5 differentiated THP-1 cells were first treated with Salmonella carrying different constructs at a MOI of 20 bacteria per cell. At 16 hours post-treatment, the Salmonella-containing cells were isolated by FACS analysis based on GFP expression. The isolated cells were incubated for 8 hours and then either mock-infected or infected with HCMV at an MOI
of 2. The cells and medium were harvested at 1, 2, 3, 4, 5, 6, and 7 days post infection. Viral stocks were prepared, and their titers were determined by performing plaque assays on human foreskin fibroblasts (7). The values obtained were the average from triplicate experiments.
Figure 3.1 The schematic drawing showed once bacteria undergo lysis intracellularly, the plasmid content is released, leading to expression of the encoded transgenes in the construct by cellular machinery. (A) *Salmonella* (rod) transformed with pU6-M1GS (oval inside *Salmonella*) infects a macrophage and resides in SCV (*Salmonella*-containing vacuole). (B) Transformed *Salmonella* replicates inside SCV in macrophage. (C) Lysis of SCV and *Salmonella* and release of plasmid pU6-M1GS. (D) Transport of pU6-M1GS plasmid into nucleus and expression of transgenes (GFP in green and M1GS RNA in red).
Figure 3.2 (A and B) Schematic representation of a natural substrate (ptRNA) (A) and a complex formed between a M1GS RNA and its mRNA substrate (B). (C) Overall cleavage rate \([k_{cat}/K_m]s\) and binding affinity \(K_d\) in reactions with ribozymes (average values of triplicate experiments). (D) Cleavage of the CSP mRNA substrate by M1GS RNA. The substrate (20 nM) was incubated alone (lane 1), with 5 nM of M1-C1 (lane 2), M1-C2 (lane 3), or M1-TK (lane 4). Reactions were carried out for 40 min in buffer A (50 mM Tris.HCl, pH 7.5, 100 mM NH4Cl, and 100 mM MgCl2) at 37 °C.
Figure 3.3 (A) Analysis of growth in LB broth of Salmonella strain SL7207 and its derivatives that carried constructs of the sequence of M1-C1, M1-C2, and M1-TK. (B and C) Northern analyses of the expression of M1GS ribozymes in differentiated THP-1 macrophages that were treated with Salmonella carrying the empty vector (−, lane 1, 4) or with Salmonella carrying constructs that contained the sequence of M1-C1 (lanes 2 and 5) and M1-C2 (lanes 3 and 6). RNA samples (25 μg) were separated on 2% agarose gels that contained formaldehyde, transferred to nitrocellulose membranes, and hybridized to a [32P]-radiolabeled probe that contained the DNA sequence coding for M1 RNA (lanes 1–3) (B) or H1 RNA (lanes 4–6) (C), the RNA subunit of human RNase P (3).
Figure 3.4 (A–D) Expression levels of HCMV mRNAs (A and B) and proteins (C and D).
Differentiated THP-1 cells were first treated with Salmonella carrying the empty vector (-, lanes 1–2, 5–6, 9–10, and 13–14) or with Salmonella carrying constructs that contained the sequence of M1-C2 (lanes 3, 7, 11, and 15) and M1-C1 (lanes 4, 8, 12, and 16). The cells were then either mock-infected (lanes 1, 5, 9, and 13) or infected with HCMV (lanes 2–4, 6–8, 10–12, and 14–16) and were harvested at 12–72 h postinfection. In Northern analysis (A and B), RNA samples (25 μg) were separated on agarose gels, transferred to nitrocellulose membranes, and hybridized to [32P]-radiolabeled probes that contained the sequence of the HCMV 5-kb transcript (lanes 1–4) and CSP mRNA (lanes 5–8). For Western analyses (C and D), protein samples (35 μg) were separated in SDS-polyacrylamide gels. The membranes were stained with the antibodies against human actin (C) and HCMV CSP (D).
Figure 3.5 showed the growth of HCMV in differentiated THP-1 cells that were treated with Salmonella carrying the empty vector construct or constructs containing the sequence of M1-C1, M1-C2, and M1-TK.
Figure 3.6 Level of total intracellular and encapsidated viral DNA as determined by semiquantitative PCR. Total DNA (lanes 1–3) or DNase I-treated DNA samples (lanes 4–6) were isolated from differentiated THP-1 cells that were treated with Salmonella carrying the empty vector construct (−, lanes 1 and 4) or carrying constructs that contained the sequence of M1-C1 (lanes 2 and 5) or M1-C2 (lanes 3 and 6) and were infected with HCMV at MOI of 1. The levels of viral IE1 sequence were determined by PCR using human β-actin DNA as the internal controls. The radiolabeled PCR products were separated in 4% nondenaturing polyacrylamide gels.
Table 3.1 Levels of inhibition of HCMV gene expression in differentiated THP-1 cells that were treated with Salmonella carrying constructs that contained the sequence of M1-C1 (M1-C1), M1-C2 (M1-C2), and M1-TK (M1-TK), as compared to that in cells treated with Salmonella carrying an empty pU6 vector construct with no ribozyme sequence (THP-1); the values shown are the means of triplicate experiments and the values of standard deviation that were less than 5% are not shown.

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CHAPTER IV

_Salmonella_-mediated gene therapy (2):

Oral delivery of M1GS ribozymes by _Salmonella_ effectively inhibits MCMV infection in mice
ABSTRACT

Safe, effective, and tissue-specific delivery is a central issue for the therapeutic application of nucleic acid-based gene interfering agents, such as ribozymes and small interfering RNAs (siRNAs). In this study, we constructed a functional RNase P-based ribozyme (M1GS RNA) that targets the overlapping mRNA region of M80.5 and protease, two murine cytomegalovirus (MCMV) proteins essential for viral replication. In addition, a novel attenuated strain of *Salmonella*, which exhibited efficient gene transfer activity and little cytotoxicity/pathogenicity in mice, was constructed and used for delivery of anti-MCMV ribozyme. In MCMV-infected macrophages treated with the constructed attenuated *Salmonella* strain carrying the functional M1GS RNA construct, we observed an 80-85% reduction in the expression of M80.5/protease and a 2,500-fold reduction in viral growth. Oral inoculation of the attenuated *Salmonella* strain in mice efficiently delivered antiviral M1GS RNA into targeted organs, leading to substantial expression of ribozyme without causing significant adverse effects in the animals. Furthermore, the MCMV infected mice that were treated orally with *Salmonella* carrying the functional M1GS sequence displayed reduced viral gene expression, decreased viral titers and improved survival compared to the untreated mice or mice treated with *Salmonella* containing control ribozyme sequences. Our results provide the first direct evidence that oral delivery of M1GS RNA by *Salmonella*-based vectors effectively inhibits viral gene expression and replication in mice. Moreover, this study demonstrates the utility of *Salmonella*-mediated oral delivery of RNase P ribozyme for gene targeting applications *in vivo.*
INTRODUCTION

Cytomegalovirus (CMV), a member of the herpesvirus family that includes herpes simplex virus 1 (HSV-1) and Epstein-Barr virus, is the leading viral cause of mental retardation in newborns and causes life-threatening complications in immunocompromised individuals including AIDS patients (1). The emergence of drug resistant strains of CMV has posed a need to develop new antiviral agents and treatment procedures. Macrophages and their progenitor cells, such as monocytic cells, represent the major reservoir for CMV as this virus can establish both primary and latent infections in these cells (1). Thus, blocking CMV infection and replication in macrophages is central for treatment and prevention of CMV-associated diseases. There is currently lack of a suitable animal model for human CMV infection due to the inability of this virus to propagate in non-human cells (1). Murine cytomegalovirus (MCMV) infection of mice resembles its human counterpart with respect to pathogenesis, and thus represents an excellent animal model for studying CMV infection in vivo and for screening new drugs and developing novel treatment strategies (1).

Nucleic acid-based gene interference technologies, including ribozymes and small interfering RNAs (siRNAs), represent promising gene-targeting strategies for specific inhibition of mRNA sequences of choice (2, 3). For example, siRNAs effectively induce the RNA interference (RNAi) pathway to block gene expression in vitro and in vivo (2). Altman and colleagues have previously shown that RNase P of Escherichia coli contains a catalytic RNA subunit (M1 RNA) (4, 5), which can be engineered into a sequence-specific ribozyme (M1GS RNA) (Figure-4.1 A-B) (6, 7). M1GS RNAs efficiently cleave target cellular and viral mRNAs in vitro and block their expression in cultured cells (8, 9). M1GS-based strategy represents a unique nucleic acid-based interference approach because of the use of M1 RNA, one of the most efficient catalytic RNAs found in nature (4).

A fundamental challenge to use nucleic acid-based gene interfering approaches for gene therapy is to deliver the gene interfering agents to appropriate cells in a way that is tissue/cell specific, efficient and safe. Many of the currently-used vectors are based on attenuated or modified viruses, or synthetic vectors in which complexes of DNA, proteins, and/or lipids are formed in particles, and tissue-specific vectors have been only partially obtained by using carriers that specifically target certain cell types (10, 11). As such, efficient and targeted delivery of M1GS sequences to specific cell types and tissues in vivo is central to developing this technology for gene targeting applications.

Invasive bacteria, such as Salmonella, possess the ability to enter and transfer genetic material to human cells, leading to the efficient expression of transferred genes (12-15). Attenuated Salmonella strains have been shown to function as a carrier system for delivery of nucleic acid-based vaccines and anti-tumor transgenes.
In these studies, plasmid constructs, which contained the transgenes under the control of a eukaryotic expression promoter, were introduced to *Salmonella*. These attenuated strains can target specific cells such as dendritic cells, macrophages, and epithelial cells, leading to efficient transgene expression, although the mechanism of how plasmid DNA from a bacterial vector is transferred to the host is not completely understood (14). *Salmonella*-based vectors are low cost and easy to prepare. Furthermore, they can be administrated orally *in vivo*, a non-invasive delivery route with significant advantage. Thus, *Salmonella* may represent a promising gene delivery agent for gene therapy. Macrophages represent the major *in vivo* reservoir for *Salmonella* following their systemic dissemination and therefore, are considered an optimal target for a *Salmonella*-based gene therapy (14, 16). However, it has not been reported whether *Salmonella* can efficiently deliver ribozymes, such as RNase P ribozymes, for expression in animals. Equally unclear is whether *Salmonella*-mediated delivery of ribozymes would also function to inhibit gene expression *in vivo*.

In this study, a new attenuated strain of *Salmonella*, SL101, was constructed. This new strain exhibited high gene transfer activity and low cytotoxicity/pathogenicity. Using MCMV infection of mice as the model, this study showed that oral inoculation of SL101 in animals efficiently delivered RNase P-based ribozyme sequence into specific organs, leading to substantial expression of ribozyme and effective inhibition of viral infection and pathogenesis. M1GS ribozymes were constructed to target the mRNA coding for MCMV protein M80.5. The coding sequence of M80.5 is completely within the 3’ coding sequence of viral protease (PR). Thus, the ribozyme would be expected to target both the M80.5 and PR, which are essential for MCMV capsid assembly and replication (1). These results provide the first direct evidence that ribozymes expressed following targeted gene transfer with *Salmonella*-based vectors are highly active in blocking viral infection in animals. Moreover, these results demonstrate the utility of *Salmonella*-assisted oral delivery of RNase P ribozymes as a general approach for gene targeting applications *in vivo*.
RESULTS

1. **In vitro cleavage of MCMV mRNA substrate by M1GS ribozyme**

   To achieve efficient targeting, it is crucial to choose a target region that is accessible to binding of M1GS ribozyme since most mRNAs inside cells are usually either present in folded conformations or associated with proteins. An *in vivo* mapping approach with dimethyl sulphate (DMS) (7) was used to determine the accessibility of the region of the M80.5 mRNA in MCMV-infected cells and have chosen a highly accessible region as the cleavage site for M1GS RNA.

   Functional ribozyme M1-A was constructed by linking the 3' terminus of M1 RNA with a guide sequence of 18 nucleotides that is complementary to the targeted M80.5 mRNA sequence. Control “inactive” ribozyme M1-B was constructed to contain the same guide sequence and derived from C102 RNA, a M1 mutant that contained point mutations at the active P4 domain abolishing its catalytic activity (9). To determine if M1GS ribozyme with an incorrect guide sequence could affect the level of the target mRNA, ribozyme M1-TK1, which was derived from M1 RNA and targeted the HSV-1 thymidine kinase (TK) mRNA (9), was also used in the analysis.

   *In vitro* cleavage of a M80.5 mRNA substrate by M1-A was observed, but not M1-B or M1-TK1 (Figure-4.1C, lanes 2-4). The binding affinity of M1-B to the substrate ($K_d=0.32+0.05$ nM) is similar to that of M1-A ($K_d=0.36+0.05$ nM). Since M1-B contains the same antisense guide sequence and exhibits similar affinity to the M80.5 mRNA sequence as M1-A but is catalytically inactive, this ribozyme can be used as a control for the antisense effect in our experiments.

2. **Salmonella-assisted gene delivery of M1GS sequence for expression in cultured cells**

   DNA sequences encoding M1-A, M1-B, and M1-TK1 were cloned into vector pU6, which contains the small nuclear U6 RNA promoter for expressing ribozyme and a green fluorescence protein (GFP) expression cassette (18). The pU6-M1GS constructs were transformed into *Salmonella* strain SL101 for gene delivery studies. SL101 was derived from auxotrophic strain SL7207 (15) and in addition, contained a deletion of *ssrA/B* genes. SL7207 is attenuated in virulence and pathogenesis *in vivo* and has been shown to function as a gene delivery carrier for the expression of several transgenes in mammalian cells (16, 17, 19). *SsrA/B* regulates the expression of *Salmonella* Pathogenicity Island-2 (SPI-2) genes, which are important for *Salmonella* intracellular survival in macrophages and virulence *in vivo* (20). Deletion of *ssrA/B* is expected to further reduce the virulence of *Salmonella* and facilitate intracellular lysis of bacteria and release of the transgene construct, leading to efficient expression of the delivered gene in target cells.
The presence of the ribozyme sequence did not affect the viability of the bacterial carrier as we observed no difference in the growth kinetics of *Salmonella* carrying no constructs or various pU6-M1GS constructs in LB broth (Figure-4.2 A). When cultured *in vitro*, neither the GFP nor M1GS transcript was detected in *Salmonella* carrying ribozyme constructs, suggesting that M1GS, which was under the control of the U6 promoter, was not expressed in *Salmonella*. When mouse J774 macrophages were infected with *Salmonella* carrying pU6-M1GS constructs, more than 80% of cells were GFP positive at 24 hours postinfection, demonstrating efficient gene transfer mediated by *Salmonella*. Northern analysis confirmed M1GS expression in these cells (Figure-4.2B). The level of M1GS RNAs in cells treated with SL101 carrying pU6-M1-A was about 3-fold higher than those with SL7207 carrying the same construct (Figure-4.2B, lanes 2-3), suggesting that SL101 is a more effective delivery vector, possibly as a result of more efficient intracellular lysis of *Salmonella* and release of pU6-M1-A due to the deletion of ssrA/B, leading to a higher level of gene expression.

3. Inhibition of MCMV gene expression and growth in cultured cells by *Salmonella*-mediated gene delivery of M1GS sequence

To determine the effect of *Salmonella*-mediated delivery of M1GS on MCMV gene expression, we first treated J774 cells with SL101 carrying ribozyme constructs. The *Salmonella*-containing cells were then isolated by FACS analysis based on GFP expression and infected with MCMV. The expression levels of M80.5/PR mRNAs were determined by Northern analyses. The level of the 7.2 kb long viral transcript (7.2 kb RNA), whose expression is not regulated by M80.5 or PR under the assay conditions (1), was used as an internal control for the quantitation of expression of M80.5/PR mRNAs (Figure-4.3A). At 48 hours postinfection, a reduction of 81 + 6% and 81 + 8% in the level of the target M80.5 and PR mRNA was observed in cells treated with SL101 carrying pU6-M1-A while no significant reduction was observed in cells with SL101 containing pU6-M1-B or pU6-M1-TK1 (Figure-4.3A and Table-4.1). The protein expression of M80.5 was determined using Western analysis with the expression of actin as the internal control. A reduction of about 85% in the protein level of M80.5 was detected in cells treated with SL101 carrying pU6-M1-A (Figure-4.3B). A low level of inhibition (~7-8%) was found in cells treated with SL101 carrying pU6-M1-B (Table-4.1), presumably due to an antisense effect because M1-B exhibited similar binding affinity to the target sequence as M1-A but was catalytically inactive.

Inhibition of M80.5/PR expression is not expected to affect the expression of other viral genes, including immediate-early (α), early (β), and late (γ) genes (1). To determine if this is the case, the levels of the mie1 (an α transcript) and m155 mRNA (a γ transcript) were examined using Northern analyses while the levels of viral protein M112, a viral early-late (βγ) protein and M99, a viral late (γ) protein were assayed with Western analyses. We observed no significant difference in the levels
of these genes among *Salmonella*-treated cells (Table-4.1), suggesting that the *Salmonella*-mediated delivery of M1-A specifically inhibits the expression of its target, and does not affect overall viral gene expression.

*Salmonella*-mediated gene delivery of anti-M80.5 ribozyme also effectively inhibited MCMV growth. In these experiments, mouse macrophage J774 cells were first treated with SL101 carrying the ribozyme sequences. The *Salmonella*-containing cells were then isolated by FACS analysis based on GFP expression, and infected by MCMV at a multiplicity of infection (MOI) of 1. The infected cultures were harvested at 1-day intervals through 5 days postinfection and viral titers of these samples were determined. At 4 days postinfection, a reduction of at least 2,500-fold in viral yield was observed in cells treated with *Salmonella* carrying pU6-M1-A, while no significant reduction was found in cells treated with SL101 containing pU6-M1-B or pU6-M1-TK1 (Figure-4.3C).

4. Inhibition of MCMV infection and pathogenesis in mice by *Salmonella*-mediated oral delivery of M1GS sequence

Immunodeficient SCID mice are extremely susceptible to MCMV infection and represent an excellent animal model for evaluating therapeutic approaches in blocking CMV infection and preventing viral associated diseases *in vivo* (1). To study *Salmonella*-assisted delivery of M1GS *in vivo*, we intragastrically inoculated SCID mice with SL101 carrying pU6-M1GS constructs. Gene delivery mediated by SL101 was efficient *in vivo* as substantial amounts of M1GS and GFP-positive cells were detected in the liver and spleen of the *Salmonella*-treated mice (Figure-4.4A). M1GS expression was also detected in the lung of these animals (Figure-4.4A). Furthermore, SL101 exhibited much less virulence *in vivo* than the parental strain SL7207 and a wild type strain ST14028s. All mice infected with SL101 (1x10^9 CFU/mouse) remained alive even after 70 days post-inoculation (Figure-4.4D). In contrast, mice inoculated with a much lower dose of ST14028s (1x10^5 CFU/mouse) and SL7207 (5x10^5 CFU/mouse) died within 7 and 15 days, respectively (Figure-4.4D). Thus, SL101 appeared to be efficient in gene transfer and exhibited little virulence/pathogenicity *in vivo*.

To study the antiviral effect of *Salmonella*-assisted oral delivery of M1GS *in vivo*, SCID mice were intraperitoneally infected with MCMV, followed by oral inoculation of *Salmonella* carrying ribozyme constructs 36 hours later. To further allow sustained expression of M1GSs, we repeated oral inoculation of *Salmonella* every 5 days. Three sets of experiments were carried out to study the effect of *Salmonella*-mediated delivery of M1GSs on MCMV virulence and infection *in vivo*. First, the survival rate of the animals was determined. Treatment of SL101 carrying pU6-M1-B or pU6-M1-TK1 had no effect on animal survival compared with untreated animals as all mice died within 25 days postinfection with MCMV (Figure-4.5A). In contrast, in MCMV-infected mice treated with SL101 expressing M1-A, life span
improved significantly as no animals died within 50 days postinfection (Figure-4.5A). Second, viral replication in various organs of the animals was studied during a 21-day infection period before the onset of mortality of the infected animals. At 21 days postinfection, the viral titers in the spleen and liver of animals treated with pU6-M1-A-containing SL101 were lower than those from animals receiving SL101 carrying control constructs by 400 and 600 fold, respectively (Figure-4.5B-C). Third, viral gene expression in the tissues was also examined. At 14 days postinfection, substantial expression of viral M80.5/PR mRNAs as well as M80.5 protein was readily detectable in livers and spleens of mice receiving SL101 carrying pU6-M1-B and pU6-M1-TK1, while little expression of M80.5/PR was detected in mice treated with SL101 carrying pU6-M1-A (Figure-4.4B-C). Thus, *Salmonella*-assisted oral delivery of M1GS blocked MCMV infection in the treated mice.
DISCUSSION

For nucleic acid-based gene interfering agents such as M1GS ribozyme to be successful as a therapeutic tool for practical applications, a central issue is the targeted delivery of these agents to specific tissues and cells in vivo. To our best knowledge, this study represents the first to demonstrate targeted delivery of M1GS RNAs in animals by Salmonella. In this study, we have constructed a M1GS RNA targeting the overlapping region of MCMV M80.5 and PR mRNAs. Furthermore, we have generated a novel attenuated strain of Salmonella, SL101, which exhibited high gene transfer activity and low cytotoxicity/pathogenicity in vivo. The ribozyme cleaved the target mRNAs efficiently in vitro and furthermore, reduced their expression levels by 80-85% and inhibited viral growth by 2,500-fold in cells that were treated with SL101 carrying pU6-M1-A. When MCMV-infected SCID mice were orally inoculated with SL101 carrying different M1GS sequence, the expression of M1GS RNAs was detected in several tissues including spleen, liver, and lung. All MCMV-infected animals that received SL101 only or SL101 carrying pU6-M1-B or pU6-M1-TK1 died within 25 days postinfection while those receiving SL101 carrying pU6-M1-A remained alive until 50 days postinfection. Furthermore, viral titers found in the spleens and livers of the animals receiving SL101 carrying pU6-M1-A were significantly lower than those in animals that received SL101 only or SL101 with pU6-M1-B or pU6-M1-TK1. M1-TK1 targets an unrelated mRNA and M1-B is catalytically inactive and contains the identical guide sequence to M1-A. Thus, the observed reduction in viral gene expression and viral growth in the cells and animals that were treated with Salmonella carrying pU6-M1-A is primarily attributed to the specific targeted cleavage by the ribozyme as opposed to the antisense effect of the guide sequence.

Our results also suggest that the Salmonella-mediated gene transfer is efficient and that M1GS RNAs expressed following the Salmonella-mediated gene delivery are active and specific in mice. Firstly, targeted gene transfer of the ribozyme constructs by SL101 yields substantial expression of ribozyme in cultured cells and in different organs of animals, suggesting efficient gene transfer in vitro and in vivo. Secondly, the ribozymes expressed following transfer specifically inhibited the expression of M80.5/PR. Only the levels of the target M80.5/PR but not other viral genes examined (e.g. mie1, M99, M112, and m155) were reduced in cells treated with SL101 carrying pU6-M1-A (Table-4.1). Thirdly, the viability and gene transfer ability of the Salmonella vectors were not significantly affected by the presence of ribozyme sequences (Figure-4.2). Furthermore, animals that received SL101 carrying M1GS constructs via oral inoculation at over 1x10⁹ CFU exhibited no adverse signs for at least 70 days (Figure-4.4D), suggesting that oral inoculation of SL101 and the expression of ribozymes exhibited little pathogenicity or cytotoxicity in vivo. Fourthly, ribozyme M1-A expressed following the SL101-mediated gene delivery appeared to be active in cleaving its target mRNA in animals. Reduced M80.5/PR expression, decreased viral titers, and increased survival were observed in mice that were
inoculated with SL101 carrying pU6-M1-A but not control constructs pU6-M1-B or pU6-M1-TK1. These results suggest that *Salmonella*-mediated oral delivery of M1GS for cleavage of its target mRNA is effective and specific *in vivo* in inhibiting only the expression of the target mRNA, leading to blocking viral infection and increasing survival of infected animals.

A fundamental challenge in gene therapy is to develop approaches for delivering genetic material *in vivo* in a way that is tissue/cell specific, efficient, and safe. As a gene delivery tool, *Salmonella*-based vectors exhibit several unique and attractive features. First, *Salmonella*-based vectors are low cost and easy to prepare. Second, they can be administered orally *in vivo*. The oral route of administration is non-invasive and has proved to be successful in terms of efficacy and acceptability in vaccine trials with attenuated *Salmonella* strains (21, 22). Third, it is easy and feasible to generate new attenuated mutants with different deletions (e.g. SL101), which can be tolerated even by immunodeficient hosts. Fourth, safety is the first and foremost concern for any gene delivery vector. *Salmonella* is not known to be tumorigenic and integration of its delivered DNA in the host cell genome has not been reported. Furthermore, the anti-typhoid fever vaccine based on the attenuated *Salmonella* strain Ty21a is one of the few live vaccines licensed for human use, and has been extensively used to immunize both adults and children since the late 1980s (21, 22). Thus, attenuated *Salmonella* strains may represent promising gene delivery agents with a favorable safety profile.

It is known that different bacterial components such as lipopolysaccharides (LPS) and unmethylated CpG motifs elicit various immune responses, including activation of TLR4 and TLR9 (23, 24), some of which are beneficial to the host while others are detrimental. To reduce the potential cytotoxicity, mutations can be introduced to bacterial vectors to inactivate specific bacterial components (25). Alternatively, bacteria carrying transgenes that modulate specific responses can be used (24). Indeed, our newly constructed mutant SL101 was highly efficient for gene delivery while exhibiting little if any virulence. These results demonstrate the feasibility of developing novel vector strains exhibiting high gene delivery efficiency and low pathogenicity/toxicity *in vivo*.

Human CMV causes significant morbidity and mortality in immunoimmature or immunodeficient individuals (1). MCMV infection of SCID mice represents an excellent animal model to study CMV pathogenesis and to assess the efficacy of novel antivirals for blocking viral infection and virulence. Intraperitoneal infection of SCID mice leads to a biphasic infection, initially with viral infection and replication in the spleen and liver, followed by dissemination of the virus via leukocyte-associated viremia from the spleen and liver to peripheral organs (1, 26, 27). SCID mice are highly susceptible to MCMV, and can succumb to as little as 10 PFU virus, primarily due to liver damage and failure associated with viral lytic replication in the organ (1, 27). Our results indicate substantial expression of M1GS RNAs in the liver and
spleen of the Salmonella-treated animals. Furthermore, MCMV M80.5 expression and titer in the spleen and liver was found to be substantially reduced in mice treated with SL101 carrying pU6-M1-A. These results suggest that the delivery of pU6-M1GS constructs and the subsequent expression of M1GS RNAs in the spleen and liver resulted in the inhibition of viral infection in these two organs, leading to an overall diminished systemic infection and viral dissemination in other organs. The improved survival of animals receiving SL101 carrying pU6-M1-A is likely due to the reduced viral load found in the liver of these animals. This is consistent with the notion that a high level of viral lytic replication and production usually leads to severe damage of hepatic tissues and liver failure, and contributes significantly to MCMV virulence and killing of SCID mice (1, 26, 28). Thus, our results suggest that oral inoculation of Salmonella efficiently deliver M1GS sequence for expression in the spleen and liver, and that Salmonella-mediated oral delivery of M1GS can effectively block viral systemic infection and increase host survival by inhibiting viral infection in spleens and livers.

The properties and activities of RNase P ribozyme, as well as the simple design of the guide sequence, make M1GS an attractive and unique gene-targeting agent that can be generally used for antiviral as well as other in vivo applications (4). Our study represents the first to use Salmonella-mediated oral delivery of RNase P ribozymes for gene targeting applications in vivo. Future studies, including the generation of novel and more active M1GS through in vitro selection and the construction of new Salmonella strains through mutagenesis strategies, should facilitate the development of Salmonella-mediated gene delivery of RNase P ribozymes as a promising gene targeting approach for in vivo applications.
MATERIALS AND METHODS

Viruses, cells, and antibodies

The Smith strain of MCMV (ATCC, Rockville, MD) was grown in NIH3T3 cells (ATCC) or mouse J774 macrophages (ATCC) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Nu-Serum (BD Biosciences, Bedford, MA) as described previously (28). The antibodies against MCMV proteins were kindly provided by Annette Meyer (Pfizer, Inc., Ann Arbor, MI) and John Wu (Promab, Inc., Albany, CA). The anti-mouse actin antibody was purchased from Sigma Inc (St Louis, MO).

In vitro studies of ribozymes

The DNA sequence for the M80.5 mRNA substrate was constructed by annealing primers AF25 (5'-GGAATTCTAATACGACTCACTATAG-3') and sm80.5 (5'-CGGGATCCGCCCGACTGAGGTAGACCGTGGTTCATCCTATAGTGAGTCGTATTAT-3'), followed by PCR. Mutant ribozyme C102 contains several point mutations (e.g. A347C348 -> C347U348, C353C354C355G356 -> G353G354A355U356) at the catalytic domain (P4 helix) (9). The DNA sequences that encode ribozymes M1-A and M1-B were constructed by PCR using constructs pFL117 and pC102 (9), which contained the DNA sequences of the M1 and C102 ribozymes, as the templates and primers AF25 and M1m80.5 (5'-CCCGCTCGAGAAAAAATGGTGCGTCTACCTGGGGGTGTGGAATTGTG-3') as 5' and 3' primers, respectively. M1-TK1 was generated from pFL117 (9). Cleavage and binding assays were performed as described previously (9)(Supporting Information).

In vitro cleavage and binding studies of ribozymes

M1GS RNAs and the M80.5 mRNA substrate were synthesized in vitro by T7 RNA polymerase (Promega Inc. Madison, WI) following the manufacturer's recommendations and further purified on 8% polyacrylamide gels containing 8M urea. Subsequently, the M1GS RNAs were mixed with the [32P]-labeled mRNA substrate. The procedures to measure the equilibrium dissociation constants ($K_d$) of the M1GS-M80.5 complexes were modified from Pyle et al (29) and have been described previously (30). The values of $K_d$ obtained were the average of three experiments. The cleavage reactions were carried out at 37°C in a volume of 10 μl for 40 minutes in buffer A (50 mM Tris, pH 7.5, 100 mM NH₄Cl, and 100 mM MgCl₂) (30). Cleavage products were separated in denaturing gels and quantitated with a STORM840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA).
Construction of *Salmonella* strains

*Salmonella* strain SL101 was derived from the auxotrophic *Salmonella typhimurium* *aroA* strain SL7207 (a gift from Bruce A. D. Stocker (Stanford University, CA, USA)) (15) by deleting the coding sequence of *ssrA/B*. The plasmid construct pKan-clone7 was used as template to amplify the DNA fragment to delete *ssrA/B* in the genome of *Salmonella* SL7207 by homologous targeting. Primers P5 (5'-TGTACTGCGATAGTGAATATCCTCCTTAGTTCC-3') and P3 (5'-TGACCAATGCTTAATACCATCGGACGCCCTGGTGTAGGCTGGAGCTGCTT-3') were designed to amplify the kanamycin resistance gene sequence in construct pKan-clone7. The resulting PCR products were transformed into SL7207 carrying plasmid pKD46. The *ssrA/B* deletion mutant was constructed using the λ Red recombinase method (31), following the procedures described previously (32). The non-polar strain SL101 was selected for its sensitivity to kanamycin and further confirmed using PCR.

Analysis of *in vitro* growth kinetics of *Salmonella*

Growth kinetics of *Salmonella* in LB broth was analyzed by first inoculating a single colony in 2 ml LB broth and culturing at 37°C with shaking at 250 RPM overnight (33). An aliquot (~30 µl) of the overnight culture was then inoculated into 3 ml fresh LB broth and cultured at 37°C and 250 RPM. At time points of 0, 2, 4, 6, 8, 10, 12, 14, 16, and 24 hours after inoculation, an aliquot (~100 µl) of bacterial culture was collected and used for analysis by limiting dilution in 96-well plates, and then plated on LB agar plates to determine their CFU/ml. Each sample was analyzed in triplicates and the analysis was repeated at least three times. The average value of CFU/ml was used to generate the growth curve (33).

Expression of ribozymes by *Salmonella*-mediated delivery in cultured cells

*Salmonella* carrying different constructs were obtained by transforming SL101 with plasmid pU6, pU6-M1-A, pU6-M1-B, or pU6-M1-TK1. Construct pU6 contained the GFP expression cassette and the small U6 RNA promoter used for the expression of ribozymes in mammalian cells. To study gene transfer of ribozyme by *Salmonella* vectors, mouse J774 cells (1x10^6 cells/m) pre-treated with IFN-γ (150 U/ml) (R&D Systems Inc., Minneapolis, MN) for at least 12 hours were infected with *Salmonella* opsonized with normal mouse serum at a multiplicity of infection (MOI) of 10-20 bacteria/cell. Cultures were centrifuged at 200xg for 5 minutes and incubated at 37°C for 30 minutes to allow phagocytosis to occur. Culture medium was then replaced with fresh medium containing gentamicin (20 µg/ml) and incubated for the indicated time periods. Cells were harvested and the expression of ribozymes was assayed using Northern analyses (9) (Supporting Information).
Studies of viral gene expression and growth

Mouse J774 cells (approximately 1-5x10^6 cells) were first incubated with Salmonella carrying different constructs at a MOI of 10-20 bacteria/cell at 37°C for 30 minutes. The medium was then replaced with fresh medium containing gentamicin (20 μg/ml) and incubated for 8 hours to allow the expression of the ribozymes. The Salmonella-containing cells were then subjected to FACS using a FACSVantage SE sorter (BD Biosciences, San Jose, CA), and a population of GFP-positive cells (usually 1-5x10^5 cells with a positive fluorescence of >99%) was isolated. The isolated cells were cultured for 4 hours and then either mock-infected or infected with MCMV (a MOI of 0.5-1) for another 8-72 hours (9). The expression of specific mRNAs and proteins in infected cells was assayed by Northern and Western analyses, respectively.

Northern and western blot analyses

The RNA and protein samples were isolated from cells and tissues as described previously (30). The RNA fractions were separated in 1% agarose gels that contained formaldehyde, transferred to a nitrocellulose membrane, hybridized with the [32P]-radiolabeled DNA probes that contained the MCMV DNA sequence or the DNA sequence coding for mouse mP1 RNA, and analyzed with a STORM840 PhosphorImager (9). The DNA probes used to detect M1GS RNAs, mouse mP1 RNA, MCMV 7.2kb RNA transcript, and M80.5 and PR mRNA were synthesized from plasmids pFL117, pmP1 RNA, pM7.2KB, and pPR, respectively.

For western analyses, the polypeptides from cell lysates were separated on SDS/9% polyacrylamide gels cross-linked with N,N''methylenebisacrylamide, transferred electrically to nitrocellulose membranes, and stained using the antibodies against MCMV proteins and mouse actin in the presence of a chemiluminescent substrate (GE Healthcare, Sunnyvale, CA), and analyzed the stained membranes with a STORM840 PhosphorImager (9). Quantitation was performed in the linear range of RNA and protein detection. The levels of the mouse RNase P RNA (mP1), MCMV 7.2 kb transcript, and mouse actin protein were used as the internal controls in Northern and Western analyses, respectively.

Analysis of the inhibition of viral growth in macrophages

To determine the level of inhibition of viral growth, 5x10^5 mouse J774 macrophages were first treated with Salmonella carrying different constructs at a MOI of 20 bacteria per cell. At 8 hours posttreatment, the Salmonella-containing cells were isolated by FACS analysis based on GFP expression. The isolated cells were incubated for 4 hours and then either mockinfected or infected with MCMV at an MOI of 1. The cells and medium were harvested at 1, 2, 3, 4, and 5 days postinfection. Viral stocks were prepared and their titers were determined by performing plaque
assays on mouse NIH3T3 cells (9). The values obtained were the average from triplicate experiments.

Plaque assays to determine the viral titers

Plaque assays were performed in NIH3T3 cells plated overnight in 6-well cluster plates (Costar, Corning, NY). Ten-fold serial dilutions of virus samples were inoculated onto each well of NIH3T3 cells. After 90 minutes of incubation, the cells were washed with DMEM then overlaid with DMEM containing 1% low melt agarose (Sigma, St Louis, MO). Viral plaques were counted after 3-5 days under an inverted microscope. Each sample was titered in triplicate, and the titer of the sample was the average of the three values and recorded as PFU/ml of organ homogenate. The limit of virus detection in the organ homogenates was 10 PFU/ml of the sonicated mixture. Those samples that were negative at a 10-1 dilution were designated a titer value of 10 (10^1) PFU/ml.

Salmonella-mediated gene delivery and MCMV infection in animals

Four to six weeks old CB17 SCID mice (Jackson Laboratory, Bay Harbor, Maine) were infected intraperitoneally with 1x10^4 PFU of MCMV and at 36 hours postinfection, were intragastrically inoculated with *Salmonella* in oral delivery experiments. For intragastric inoculation of mice, animals were first anesthetized with isoflurane and then intragastrically inoculated with 0.1-0.2 ml phosphate-buffered saline (PBS) containing 1x10^8 CFU *Salmonella*, using a gavage needle (32). The oral inoculation procedure was repeated every 5 days. The gene delivery efficiency was evaluated by detecting the expression of M1GS RNAs in mouse tissues (e.g. livers) using Northern analyses and by examining the GFP signal of the transfected cells in the tissues using fluorescence microscopy. The mortality of infected animals (five animals per group) was monitored for at least 60 days postinfection, and the survival rates were determined. Groups of MCMV-infected animals (at least five animals per group) were also sacrificed at 1, 3, 7, 10, 14, and 21 days postinoculation. Spleens and livers were harvested and sonicated as a 10% (wt/vol) suspension in a 1:1 mixture of DMEM medium and 10% skim milk. Viral titers of the tissue samples were determined using plaque assays (28) (Supporting Information). In gene expression experiments, tissues were homogenized, and the expression of M1GS RNA and viral mRNAs was determined using Northern analyses while the expression of viral proteins was assayed using Western analyses (9, 28) (Supporting Information). To determine the virulence and toxicity of *Salmonella*, SCID mice (five animals per group) were intragastrically inoculated with *Salmonella* strain ST14028s (1x10^3 CFU), SL7207 (5x10^5 CFU), and SL101 (1x10^9 CFU) carrying pU6-M1-A. Their mortality was monitored for at least 70 days postinfection, and the survival rates were determined.
Figure 4.1 (A-B) Schematic representation of a natural substrate, precursor tRNA (ptRNA) (A) and a complex formed between a M1GS RNA and its target mRNA substrate (B). (C) Cleavage of the M80.5 mRNA substrate by M1GS RNA in vitro. The substrate (20 nM) was incubated alone (lane 1), with 5 nM of M1-A (lanes 2), M1-B (lane 3), or M1-TK1 (lane 4).
Figure 4.2 (A) Analysis of growth in LB broth of *Salmonella* strain SL101 and its derivatives that carried constructs pU6-M1-A, pU6-M1-B, and pU6-M1-TK1. (B-C) Northern analyses of the expression of M1GS ribozymes in mouse J774 macrophages that were treated with strain SL101 carrying the empty vector pU6 (-, lane 1, 4) and pU6-M1-A (lanes 3 and 6), or with strain SL7207 carrying pU6-M1-A (lanes 2 and 5). The levels of the mouse RNase P RNA subunit (mP1 RNA) were used as the internal control (C).
Figure 4.3 (A-B) Expression levels of MCMV mRNAs (A) and proteins (B). Mouse J774 cells were first treated with Salmonella carrying the empty vector pU6 (-, lanes 1-2, 5, 8) or constructs that contained the sequence of M1-B (lanes 3 and 7) and M1-A (lanes 4 and 6). The cells were then either mock-infected (lanes 1 and 5) or infected with MCMV (lanes 2-4 and 6-8) and harvested at 48 hours postinfection. The levels of the MCMV 7.2 kb transcript and mouse actin protein were used as the internal controls in Northern (A) and Western (B) analyses, respectively. (C) Growth of MCMV in mouse J774 cells that were treated with SL101 carrying pU6 (SL101), pU6-M1-A (M1-A), pU6-M1-B (M1-B), or pU6-M1-TK1 (M1-TK1).
Figure 4.4 Expression of M1GS RNA (A), viral mRNAs (B), and proteins in vivo (C). Spleens, livers, and lungs were isolated from SCID mice that were intragastrically inoculated with SL101 carrying different constructs and either mock-infected (lanes 1-7, 8, and 12) or infected with MCMV (lanes 9-11 and 13-15), and were harvested at 14 days postinfection. Northern and Western analyses were carried out using RNA (A-B) or protein samples (C) isolated from different organs of animals that received SL101 carrying pU6 (-, lanes 1, 8-9, 12, and 15), pU6-M1-B (lanes 3, 5, 7, 10, and 14), or pU6-M1-A (lanes 2, 4, 6, 11, and 13). The levels of the mouse RNase P RNA (mP1) and actin protein were used as the internal controls. (D) Virulence and toxicity of Salmonella in SCID mice. SCID mice (5 animals per group) were infected intragastrically with ST14028 (1x10^5 CFU), SL7207 (5x10^5 CFU), or SL101 (1x10^9 CFU) carrying pU6-M1-A, and their survival was recorded.
Figure 4.5 (A) Mortality of the SCID mice infected with MCMV, followed by oral inoculation of *Salmonella* SL101 (1x10⁸ CFU/animal) carrying pU6 (SL101), pU6-M1-A (M1-A), pU6-M1-B (M1-B), or pU6-M1-TK1 (M1-TK1). SCID mice (5 animals per group) were infected intraperitoneally with 1x10⁴ PFU MCMV, 36 h prior to *Salmonella* inoculation. Oral inoculation of *Salmonella* was repeated every 5 days. (B-C) Titers of MCMV in the spleen (B) and liver (C) of the infected SCID mice. At different time points postinfection, the animals were sacrificed. Spleens and livers were collected, and the viral titers in tissue homogenates were determined. The limit of detection was 10 PFU/ml of the tissue homogenate. The viral titers represent the average obtained from triplicate experiments. The error bars indicate the standard deviation. Error bars that are not evident indicate that the standard deviation was less than or equal to the height of the symbols.
REFERENCES


CHAPTER V

Conclusions
Goal of this research

A number of ideas for using catalytic nucleic acids to inactivate viral genes inactivation has been enthusiastically proposed and tested. Among the different nucleotide-based gene interference technologies, Ribonuclease P (RNase P), especially its derivative M1GS catalytic RNA, has proved to be very effective and specific in blocking viral gene expression and replication in cultured cells (1-10). Compared to other nucleic acid-based interference approaches, M1GS ribozyme possesses several unique features such as high catalytic efficiency, high target specificity, no detectable cytotoxicity and low target sequence requirement. Thus, M1GS catalytic RNA is a powerful gene targeting tool which exhibits promising antiviral activity for future clinical application. However, before the pre-phase clinical trial stage can be reached, several technical barriers have to be overcome. This dissertation focused on how to improve the application of M1GS antiviral methodology in tissue culture cells and live animals.

Intravenous administration

Since M1GS ribozyme can efficiently inhibit human cytomegalovirus (HCMV) gene expression and replication in cultured human cells, the first question needed to be addressed is how would this technique work *in vivo*? To answer this question, we first need to address two other issues. The first is the animal model. A good animal model to study CMV infection and antiviral effect of M1GS RNA needed to be identified. Murium cytomegalovirus (MCMV) shares many similar features with its human counterpart, HCMV. Furthermore, MCMV infection of SCID mice which lack functional T and B lymphocytes represents an excellent model system to study CMV pathogenesis in immunocompromised hosts. As such, mouse infected by MCMV provides a good animal model for studying CMV pathogenesis *in vivo* and was selected in this research for testing the engineered M1GS RNA as antiviral agent *in vivo* (11-14). The second issue was how to deliver and express M1GS RNA in the live animal. An efficient delivery system needed to be developed to either directly or indirectly mediate M1GS RNA expression inside the target organs of the CMV infected animals to achieve antiviral effect. Considering that RNA is less stable and there are numerous nucleases inside the blood system, we decided to deliver DNA plasmids carrying M1GS sequence into animals and synthesize M1GS RNA by exploiting host transcriptional machinery. Compared to conventional vector mediated gene delivery techniques, such as virus-based or non-viral systems, hydrodynamic transfection has advantages. This method simply uses intravenous injection to force the naked DNA plasmids from blood stream into tissues to achieve the delivery purpose. This is a relatively simple transfection technique without the time consuming process of preparation of infectious virus or non-viral vectors. Furthermore, no infectious virus particles were synthesized during delivery and the method significantly reduces the safety concern. Thus, this technique was chosen in my
research to demonstrate the feasibility of delivering plasmid which can express M1GS RNAs into animals and to test their antiviral activity in vivo.

As described in Chapter Two, the results for this delivery system are positive. Plasmid constructs carrying M1GS that target MCMV mAP/M80 were successfully delivered into MCMV-infected SCID mice. M1GS RNA expression was detected in the CMV infected animal organs, including liver and spleen. Compared to the control groups, animals receiving the functional ribozyme constructs exhibited a significant reduction of viral gene expression and infection. Viral titers in different organs, such as spleen, liver, lung, and salivary gland of the functional M1GS-treated animals were much lower than those in the control groups. Moreover, the survival of the infected animals significantly improved when receiving the functional ribozyme construct. This study successfully demonstrated that using M1GS ribozyme for inhibition of viral gene expression in animals is feasible and technically attainable.

However, we do realize that, as a delivery system, hydrodynamic transfection has its limitations. One major concern is the toxicity issue. Although all animals appeared to recover well from the intravenous injection procedure, transient increase of serum concentration of alanine aminotransferase (ALT) was detected. This finding suggested that there is some minor liver damage which is most likely caused by the large volume of saline used for injection. After a short period of time, the ALT value returned to the normal range which indicated that the rodent (lab mouse) can recover from the liver damage quickly. However, whether human beings can tolerate such high-volume intravenous saline injections and recover from unpredictable organ damages would be an unavoidable safety concern for future clinical applications. It seems that this method is useful for testing the M1GS antiviral activity in an animal model (15-17), but not suitable for future clinical applications. To achieve safe and efficient delivery purpose, new system needs to be developed and tested.

**Oral administration**

The hunt for a new delivery system led us to *Salmonella*, an invasive bacterium. During the past twenty years, *Salmonella* has been investigated as an efficient delivery system for a variety of different molecules. *Salmonella* has several advantages as an M1GS delivery vector. First, attenuated *Salmonella* strains are low cost, easy to prepare and use oral route administration (18-20). Second, *Salmonella* has been widely used as a vaccine and as a delivery vector in cancer therapy and has proved to be safe for human use (18, 21-25). Third, methods for genetic manipulations are readily available and it’s feasible to generate new attenuated strains (26). Fourth, plasmids carrying M1GS sequence are easy to construct and transform into *Salmonella* strains. Fifth, oral infection of animal (SCID mice) by *Salmonella* can be consistently performed without ill effect on animals. Sixth, and most importantly, *Salmonella* will bring the constructs carrying M1GS to the tissues where CMV resides. During natural infection, cytomegalovirus can infect and
replicate in monocyte-derived macrophages. During latent infection, CMV can be detected in macrophage progenitor cells in the bone marrow. For *Salmonella*, macrophages represent the major *in vivo* reservoir following bacterial systemic dissemination. Thus, we decided to use differentiated human macrophage cells to evaluate the *Salmonella*’s ability to mediate the ribozyme delivery into cultured cells and test for an antiviral effect.

As described in Chapter Three, the experimental results showed that attenuated *Salmonella* strain SL7207 can efficiently deliver M1GS sequences into cultured human cells, leading to the expression of M1GS catalytic RNA and effective inhibition of HCMV infection. M1GS RNAs targeting CSP/assembly were detected in differentiated human macrophages treated with attenuated *Salmonella* strains carrying the ribozyme sequence constructs. Compared to control groups, significant reductions of target viral protein expression and viral replication were observed in cells treated with *Salmonella* carrying the sequence of the functional ribozyme. This study for the first time showed *Salmonella*-mediated delivery and expression of M1GS ribozyme are highly active and specific in blocking HCMV infection in cultured cells. Moreover, the positive results in cultured cells encourage us to develop *Salmonella* strains more efficient as gene delivery vector of M1GS ribozyme for future animal study.

For the purpose of delivering M1GS sequence into live animals, the *Salmonella* strain has to meet at least two requirements. First, the bacteria need to have low pathogenicity in animals. Less virulent *Salmonella* will not only minimize the morbidity and mortality from bacteria themselves, but also make it possible for using higher doses of bacteria to carry as much M1GS sequences into animals as possible. Second, the bacterial vehicle can efficiently deliver the constructs carrying M1GS sequence to specific tissues and cells where CMV resides. Auxotrophic SL7207 strain which was used in cultured-cell assay is attenuated and cannot replicate inside mammalian cells, however, pilot study showed that the animals orally inoculated with $10^5$ CFU of bacteria per mouse died within 15 days. This dosage of bacteria won’t bring enough M1GS into animals. Moreover, given the fact that SCID mice infected intraperitoneally with MCMV ($10^4$ pfu) can usually survive at least 20 days, the SL7207 strain cannot meet experimental requirement for our future animal study. New mutant strains have to be generated for ribozyme animal delivery.

The newly constructed attenuated *Salmonella* strain, SL101, was generated from its parental SL7207 strain and contained the deletion of ssrA/B genes which function as transcriptional activators and regulate the expression of *Salmonella* Pathogenicity Island-2 (SPI-2). This island is known to be crucial for *Salmonella*’s intracellular survival in macrophages as well as systemic infection in host. The deletion of ssrA/B is expected to further reduce the virulence of the attenuated SL7207 and facilitate intracellular lysis of *Salmonella*. The M1GS plasmids would be released from bacteria and transported into nucleus in a faster way, leading to
efficient expression of ribozyme in target cells. As described in Chapter Four, study showed that animals orally inoculated with SL101 at $10^9$ CFU per mouse remained alive even after 70 days post-inoculation. The level of M1GS RNAs in cultured mouse macrophage cells treated with SL101 carrying pU6-M1GS was about 3-fold higher than those with SL7207 carrying the same construct, suggesting that SL101 is a more efficient delivery vector due to the deletion of ssrA/B. In mouse macrophages, M1GS RNAs targeting M80.5/protease were successfully delivered by SL101. A significant reduction in viral gene expression and viral growth was observed. Furthermore, oral inoculation of the SL101 strain in live animals efficiently delivered antiviral M1GS into targeted organs, leading to a substantial expression of M1GS RNA without causing significant adverse effects in the animals. Compared to the control groups, mice received SL101 carrying functional M1GS sequence showed reduced viral gene expression, decreased viral titers and greatly improved survival. In summary, this study provides the first direct evidence demonstrating the feasibility of developing novel attenuated *Salmonella* strains that exhibit high gene delivery efficiency with low pathogenicity and toxicity in vivo.

**Future direction**

The final antiviral outcome using *Salmonella*-mediated ribzyme expression depends on the combination of the dose of *Salmonella*, the route of delivery, the virulence of vector bacteria, and the genetic materials carried by the bacteria, in our case, M1GS ribozyme sequence. Although newly constructed SL101 was highly efficient for gene delivery in mice and exhibiting low virulence, there is still room for improvement before applying this technique to primate study.

First and foremost, the virulence of the vector needs to be further reduced. At the same time, lower bacterial pathogenicity will make a higher inoculation dose feasible. As we know, different bacterial components and bacterial virulent factors can activate various immune responses, including innate immunity, adaptive innate immunity and antigen specific immunity. Some of these defenses are beneficial to the host while others are harmful. To reduce the potential cytotoxicity, further mutations can be introduced into bacterial vectors to inactivate specific bacterial components (18). For example, many cytokines in the host are activated by and respond to *Salmonella* lipopolysaccharides (LPS), such as platelet activating factor, chemokines, eicosanoids, TNFα, IL1β, IL6, IL12 and IFNγ (27). During *Salmonella* infection, these cytokines are involved in the endotoxic septic shock syndrome characterized from bacteraemia to multi-organ failure. Even though attenuated auxotrophic strain was used in the study, cytokine factors had to be seriously taken into consideration. To reduce the proinflammatory immune response caused by LPS, deletion mutants can be introduced into LPS biosynthesis pathway. For example, deletion of *waaN*, a gene involved in lipid A biosynthesis, reduced enteropathogenic responses induced by *S.typhimurium* (28). The *waaN* mutants lack a fatty acyl chain in LPS molecules and can grow unusually high numbers with most animals surviving the infection. Previous
study showed that compared to the wild type *S.typhimurium* strain, this mutant induces lower levels of TNFα and IL1, and reduced inducible nitric oxide synthase (iNOS) response (29). If we introduce the *waaN* deletion into SL101 background, it will be of interest to test whether the new mutant is lower in virulence and induces less host cytokine response. If so, animal studies can be performed to test the delivering function. Another possibility, *msbB*, lipid A biosynthesis (KDO) 2-(lauroyl)-lipid IVA acyltransferase, also functions in LPS biosynthesis pathway. MsbB mutant prevents the addition of a terminal myristyl group to the lipid-A domain and reduces the induction of proinflammatory cytokines and nitric oxide synthase (29). This mutation suppressed *Salmonella* virulence *in vivo* and mice inoculated orally with this mutant have a better survival rate compared to those infected by the wild type strain (30, 31). Moreover, *msbB* mutant has been used in Phase I study of the intravenous administration for cancer therapy which make this mutant more reliable and feasible as delivery vector for M1GS *in vivo* (32). *MsbB/ssrAB, waaN/ssrAB* and *msbB/ssrAB/waaN* mutants in the SL7207 background will provide more opportunities for us to screen better delivery vectors for antiviral gene therapy. One more concern here is that newly constructed attenuated strains of *Salmonella* may not be very efficient in disseminating via blood stream to get to the target organs. Related assays have to be considered and carried out during the screening process.
References


