Drugs of the Future 2007, 32(11): 985-990

CCC: 0377-8282/2007
DOI: 10.1358/dof.2007.032.11.1132406

Review Article

The *Salmonella typhimurium* type III protein secretion system: an effective antigen delivery platform for cancer therapeutics

Jorge E. Galán
Section of Microbial Pathogenesis, Yale University School of Medicine, New Haven, CT 06536, USA. Correspondence: jorge.galan@yale.edu

CONTENTS

Abstract

Virulence-attenuated bacterial pathogens have been used in diverse cancer therapeutic strategies. Some strategies require bacterial growth within the tumor tissue to effect tumor regression, while others use recombinant bacteria expressing tumor antigens to induce antitumor responses that result in tumor rejection. The stimulation of cytotoxic $CD8^+$ T-cells against tumor antigens is central for effective tumor immunity. The *Salmonella typhimurium* type III secretion system offers a powerful means to deliver antigens to the class I antigen-presenting pathway to generate this type of response. In addition, this system is capable of sensitizing tumor cells to pre-existing circulating $CD8^+$ T-cells, thereby offering a novel cancer immunotherapeutic strategy that does not require prior knowledge of the antigenic composition of the tumor.

Introduction

The utility of bacteria in anticancer therapeutics has been long recognized. Since the pioneering yet anecdotal observations of William Coley (1), several studies in animals have clearly demonstrated that the administration of some bacterial species can lead to tumor eradication (reviewed in Refs. 2-4). The use of bacteria in cancer therapeutics has taken at least three different forms. In one type of strategy, bacteria are administered systemically or directly into the tumor and subsequent bacterial replication within the tumor leads to tumor regression. Several bacteria have been used for this purpose, notably several anaerobic (*e.g., Clostridium* spp., *Bifidobacterium* spp.) and facultative anaerobic (*e.g., Salmonella typhimurium*) bacteria (4-6). It has been argued that some bacteria (*e.g., S. typhimurium*) can specifically target tumor tissues (7, 8). However, this is most likely not the case and bacterial replication at tumor sites most likely occurs not because bacteria can specifically reach this site, but because the tumor site is permissive for replication, therefore giving the false impression of tumor target specificity. The reasons why bacteria more readily replicate within tumors compared to other tissues are not clear. It has been proposed that the hypoxia characteristic of tumor tissues may encourage bacterial growth. Although hypoxia may well help the growth of anaerobic bacteria, the most likely factor that leads to bacterial growth in tumors is the overall immunosuppressive environment that is known to exist in tumor tissues (9-11). Human clinical trials of systems whose effectiveness depends on bacterial growth within the tumor, however, have been disappointing since, in contrast to the mouse, in some cases bacteria appear to grow poorly in human tumors (12, 13). In other instances, toxicity due to oncolysis or tumor regrowth after treatment forced the interruption of the trials (14). Efforts have been made to enhance the effectiveness of bacteria-mediated anticancer therapies by combining them with the delivery of prodrugs into the tumor (3, 15). In any case, it appears that this approach would not be effective for the treatment of small metastatic tumors, where bacterial replication cannot occur.

Another strategy utilized in microbial-based cancer therapy has been the use of bacteria as antigen delivery vehicles to develop cancer immunotherapies. This approach has been used not only against cancers with a clear infectious cause or predisposing factor (*e.g.,* cervi-

cal or stomach cancer), but also against other types of cancers not thought to be of infectious origin. Thus, virulence-attenuated versions of different bacterial pathogens endowed with the ability to express tumor antigens have been successfully used to eradicate or prevent tumor development in mice (16-18). In addition, bacterial vectors have been used to deliver DNA expressing tumor antigens (19-22). In this article, the use of the *Salmonella* type III secretion system as an antigen delivery platform for cancer immunotherapeutics will be discussed.

Avirulent *Salmonella* as antigen delivery vector

Avirulent strains of *Salmonella* have long been considered an effective platform for the development of vaccines to heterologous pathogens (23, 24). This vector offers several advantages: 1) it can be easily manipulated genetically; 2) there are several virulence-attenuated strains available; 3) it can be administered orally; 4) it can be grown easily and cheaply; 5) its pathogenesis is reasonably well understood; 6) it can be engineered to express many antigens; and 7) it can stimulate complex immune responses, both cellular and humoral. An additional advantage of *Salmonella* as a potential vaccine carrier is its unique ability to strongly stimulate the innate immune system. Indeed, stimulation of inflammation is a canonical feature of the *Salmonella* life cycle, as the induction of inflammatory diarrhea is central for its pathogenic program (25, 26). Consequently, *S. typhimurium* has developed redundant mechanisms to stimulate both canonical (*e.g.*, Toll-like receptor [TLR]-mediated), as well as noncanonical innate immune responses (26, 27). Since stimulation of innate immune responses is very important for mounting an efficient acquired immune response (28), this feature of *Salmonella* is likely to be at the center of its demonstrated ability to induce protective immune responses.

This antigen delivery platform, however, has some limitations that stem from certain aspects of its intracellular life style. *Salmonella* has the ability to enter cells that are normally nonphagocytic, such as cells of the intestinal epithelium (29). In addition, it has evolved the capacity to survive within macrophages and dendritic cells (30, 31). However, unlike other intracellular bacterial pathogens such as *Listeria monocytogenes* or *Shigella* spp., during its intracellular stage *Salmonella* remains localized within a membrane-bound compartment (26, 31). Consequently, heterologous antigens expressed by *Salmonella* are only inefficiently delivered to the class I antigen-presenting pathway (32-34). This shortcoming has hampered the use of *Salmonella* as a vaccine platform to protect against infections in which the generation of cytotoxic $CD8^+$ T-cells is crucial for protection. This includes not only infections with most viral and some bacterial pathogens, but also cancer therapeutics (35, 36). However, this shortcoming has been largely eliminated through the use of the type III secretion system (37).

The *Salmonella* type III secretion system

Many bacteria that have sustained close associations with eukaryotic hosts have evolved a specialized protein delivery device known as the type III secretion system (TTSS) (38). This includes bacteria that are pathogenic or symbiotic for animals, plants, or even insects. The central element of this protein delivery system is a bacterial envelope-associated multiprotein organelle known as the needle complex (39, 40). This organelle is composed of a multiring "base", firmly anchored within the bacterial envelope, and a slender "needle"-like structure that protrudes from the bacterial surface (Fig. 1). The entire organelle is traversed by a channel ~3 nm in diameter, which serves as a conduit for the proteins destined to travel this pathway. Other elements that are important for the function of this machine are: 1) a subset of membrane-associated proteins that presumably aid the transit of type III secreted proteins through the bacterial inner membrane; 2) a group of proteins which themselves are secreted via this pathway and whose function is to mediate the passage of type III secreted proteins through the membrane of the target eukaryotic cell; and 3) a family of customized chaperones which aid the targeting of proteins destined to travel this pathway to the secretion machine. *S. typhimurium* encodes two of these machines in different "pathogenicity islands" (*i.e.,* discrete genomic regions that encode clusters of virulence genes that are believed to have been horizontally acquired during evolution) (26). The system encoded in the pathogenicity island 1 (SPI-1) mediates the entry of *Salmonella* into host cells, while the other, encoded in the pathogenicity island 2 (SPI-2), is required for intracellular survival. The pattern of expression of these systems is consistent with these functions: the SPI-1 TTSS is expressed extracellularly, while the SPI-2 TTSS is induced when *Salmonella* reaches an intracellular location. Combined, these systems deliver more than 60 bacterial proteins (known as "effector" proteins) into eukaryotic cells, which have the capacity to modulate a number of cellular functions, including cytoskeleton dynamics, vesicular trafficking, programmed cell death and innate immune responses.

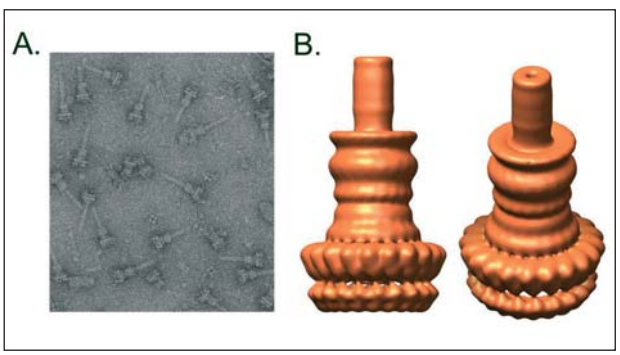

Fig. 1. The *Salmonella* type III secretion organelle. **A.** Electron micrographs of negatively stained isolated needle complexes from *Salmonella typhimurium*. **B.** Surface rendering of the high-resolution structure of the needle complex. Adapted from Ref. 38.

The *Salmonella* type III secretion system as an antigen delivery device

Proteins destined to travel the type III secretion pathway possess specific signals which target them for recognition by the secretion machine (38, 41). These signals have two components: 1) a small (~10-30) amino acid segment located at the amino terminal end of the secreted protein; and 2) a ~50-100-amino-acid domain (also located at the amino terminus) that serves as a binding site for specific chaperones. Proteins targeted to the TTSS machine are unfolded by an associated ATPase prior to their delivery to the secretion channel (42). This ATPase also serves to remove the associated chaperone, which remains in the bacterial cytoplasm, presumably to be recycled. The type III secretion signals fused to the amino terminus of a heterologous protein are able to direct its secretion and translocation into a target cell (43). This property was utilized to engineer the type III secretion machine to deliver proteins to the class I antigen presentation pathway (37). Thus, signal sequences from *S. typhimurium* effector proteins from the SPI-1 TTSS were shown to be able to direct viral proteins to the class I antigen-presenting pathway both *in vitro* and *in vivo*. Consequently, avirulent recombinant *S. typhimurium* engineered to deliver viral antigens from influenza, HIV or lymphocytic choriomeningitis viruses were able to prime robust $CD8^+$ T-cell responses that in some cases were shown to be protective in animal models of infection (37, 44-46). Therefore, the harnessing of the type III secretion protein delivery machine to deliver antigens has overcome a significant limitation in the use of *Salmonella* as a universal antigen delivery platform.

The *Salmonella* type III secretion system for the development of therapeutic cancer vaccines

S. typhimurium has been used in different anticancer strategies. One of these strategies involves the systemic administration of avirulent strains that retain the ability to grow within tumor tissues (7, 8). Bacterial growth within the tumor results in its regression. However, this system requires *Salmonella* to be administered systemically, which presents significant toxicity challenges. Furthermore, this strategy is ineffective for treating small metastatic tumors and initial human trials have been disappointing since *Salmonella* apparently did not replicate within human tumors (12).

Another anticancer therapeutic strategy based on *Salmonella* involves the elimination of the tumor not by bacterial growth within the tumor but by stimulating an immune response against the tumor (17). It is widely accepted that efficient tumor eradication requires the stimulation of cytotoxic $CD8^+$ T-cells directed to tumor surface antigens (35). Therefore, the stimulation of $CD8^+$ responses by the *Salmonella* vector is essential if this strategy is to be effective. This has been attempted through the delivery of DNA encoding tumor antigens by avirulent strains of *Salmonella* (19-22). However, the intracellular location of *Salmonella* within a membrane-bound compartment makes DNA delivery by these bacteria very inefficient.

Another strategy has used the *S. typhimurium* type III secretion system encoded in the SPI-1 to deliver tumor antigens to the class I antigen-presenting pathway, thereby stimulating $CD8^+$ cytotoxic T-cells against the tumor antigen (47, 48). This system has been shown to be effective in different murine tumor models. In one study, a single oral dose of *S. typhimuriu*m delivering the *L. monocytogenes* antigen p60 through the SPI-1 TTSS was able to protect 80% of mice challenged 30 days after immunization with an aggressive mouse fibrosarcoma engineered to express the bacterial antigen (48). Although the mechanism by which *S. typhimurium* afforded protection in this model was not specifically investigated, it was shown that immunization resulted in stimulation of antigen-specific effector and memory $CD8^+$ T-cells.

In another study, a virulence-attenuated strain of *S. typhimurium* was engineered to express the NY-ESO-1 human tumor antigen fused to type III secretion signals from the SPI-1 TTSS (47). The resulting strain was able to deliver the NY-ESO-1 antigen to the class I and class II antigen-presenting pathways of peripheral blood mononuclear cells (PBMCs) from cancer patients. Oral administration of the *S. typhimurium* NY-ESO-1 strain to mice that had been previously inoculated with a fibrosarcoma stably expressing NY-ESO-1 resulted in the development of antigen-specific $CD8^+$ T-cells and complete regression of the tumor (Fig. 2). Tumor regression was not due to bacterial replication within the tumor since: 1) no bacteria were detected within the tumor; and 2) tumors did not regress in animals inoculated with a control strain that did not express NY-ESO-1. Interestingly, $CD8^+$ T-cells specific for tumor antigens not contained in the vaccine were also detected in the vaccinated animals, a phenomenon known as “epitope spreading”. It is therefore possible that these responses also contributed to tumor eradication. Depletion of $CD8^+$ T-cells from immunized animals effectively abolished the therapeutic effect of *S. typhimurium*, whereas depletion of $CD4^+$ T-cells did not. Taken together, these results further demonstrate that the therapeutic effect of the administration of *S. typhimurium* NY-ESO-1 was due to the stimulation of antigen-specific $CD8^+$ cells.

The type III secretion system has also been used in the context of a different therapeutic strategy (47). This strategy makes use of the ability of the TTSS to inject proteins into tumor cells when *S. typhimurium* is administered intratumorally. In this strategy, *S. typhimurium* is engineered to deliver an antigen into the tumor cells that is not expressed by the tumor, but which renders the tumor cells susceptible to pre-existing $CD8^+$ T-cells specific for that antigen. Mice were immunized with NY-ESO-1 DNA and inoculated with a tumor that did not express NY-ESO-1. Administration of *S. typhimurium* NY-ESO-1 into the tumor resulted in rapid regression of the tumor (Fig. 2). Tumor regression was due to the delivery of NY-ESO-1 by *S. typhimurium* since intratumoral administration of a control

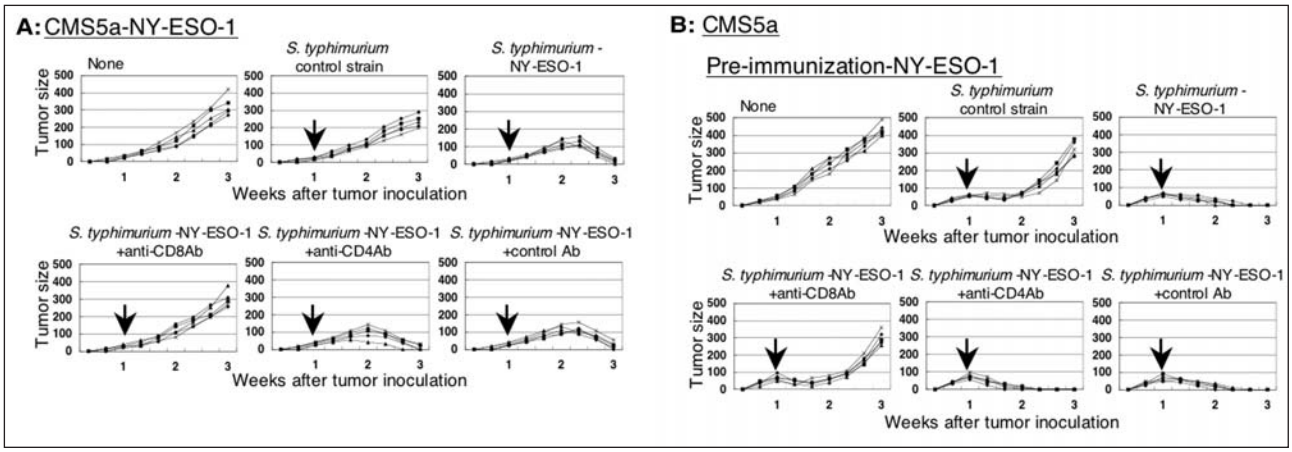

Fig. 2. **A.** Oral administration of *Salmonella typhimurium* endowed with the capacity to deliver NY-ESO-1 through the type III secretion system (TTSS) causes tumor regression in mice bearing NY-ESO-1-positive tumors. BALB/c mice were inoculated with CMS5a-NY-ESO-1 cells and 7 days later with *S. typhimurium* expressing NY-ESO-1 or a control strain. Some groups of mice were also injected i.v. with an anti-CD4 or anti-CD8 monoclonal antibody (MAb) or a control MAb. Arrows indicate the time of *S. typhimurium* oral administration. **B.** Local tumor antigen delivery by *S. typhimurium* TTSS causes regression of tumors in mice by epitope spreading. BALB/c mice were immunized with a plasmid encoding NY-ESO-1 by gene gun. Immunized mice were inoculated with CMS5a tumor cells (NY-ESO-1-negative), and 7 days later were given *S. typhimurium*-NY-ESO-1 or the control strain at the tumor site. Some groups of mice were also injected i.v. with an anti-CD4 or anti-CD8 MAb or a control MAb. Arrows indicate the time of *S. typhimurium* administration. Each line represents the tumor growth of an individual mouse. Adapted from Ref. 50.

strain that did not express NY-ESO-1 did not result in tumor regression. Interestingly, vaccinated mice developed $CD8^+$ responses to tumor antigens that were not contained in the vaccine, indicating that this strategy also led to epitope spreading. In fact, since a very small proportion of the tumor cells were directly sensitized by TTSS-mediated antigen delivery, most likely the generation of these secondary responses was largely responsible for tumor eradication. Removal of $CD8^+$ cells from immunized animals effectively abrogated the ability of *S. typhimurium* NY-ESO-1 to induce tumor regression, further demonstrating that cytotoxic T-cells are central to this protective immune response. Taken together, these results demonstrate that the delivery of a heterologous antigen into tumor cells by the *S. typhimurium* TTSS sensitizes these cells to pre-existing $CD8^+$ T-cells directed to the heterologous antigen, leading to epitope spreading and tumor eradication. Although this study utilized NY-ESO-1 as a model antigen, this strategy would not require the use of a tumor antigen. The only requirement would be for the patient to have pre-existing $CD8^+$ T-cells to the antigen delivered by the *S. typhimurium* TTSS. In fact, it is very likely that this strategy could be implemented using viral antigens against which patients are likely to have circulating $CD8^+$, such as influenza or Epstein-Barr virus (EBV). The main advantage of this strategy is that prior knowledge of the tumor antigen composition would not be required for its implementation.

Optimization of the *Salmonella* type III secretion system for antigen delivery

Due to the narrow diameter of the secretion channel, proteins destined to travel the type III secretion pathway must be unfolded prior to secretion. Protein unfolding is largely mediated by a TTSS-associated ATPase (42). Proteins that naturally transit the TTSS pathway have presumably evolved so that protein unfolding can occur rapidly and efficiently. However, some heterologous proteins that are particularly stable may be difficult to deliver through the TTSS (42). Strategies have therefore been devised to improve the efficiency of delivery of such proteins (49). One of the strategies involves the identification of the domain(s) that interfere with secretion. For example, in some cases, discrete domains such as Zn fingers have been shown to interfere with secretion. In this case, removal or destabilization of the interfering domains may be sufficient to render a given protein permissive for secretion via the TTSS. A second strategy is based on the observation that type III secreted proteins most often have flexible domains immediately adjacent to the secretion and translocation signals. The presence of flexible regions in this position is thought to help the unfolding of the entire molecule. Therefore, type III secretion of non-permissive proteins has been achieved by placing a flexible region between the secretion signal and the heterologous protein. Using either of these strategies virtually any protein can be rendered permissive for delivery through the TTSS.

Concluding remarks

Virulence-attenuated strains of *S. typhimurium* endowed with the capacity to deliver tumor antigens via the type III secretion system have been shown to be effective at both preventing tumor development and causing regression of already established tumors. Unlike other strategies that have used *Salmonella* as an anticancer

therapy, the TTSS-dependent strategy does not require bacterial replication within the tumor. In fact, tumor eradication is strictly dependent on the immune response to the antigens delivered by the *S. typhimurium* TTSS, and more specifically, on the induction of $CD8^+$ T-cells against the tumor antigens. A potentially very exciting application of the TTSS for tumor eradication does not even require prior knowledge of the antigenic composition of the tumor. In this strategy, *S. typhimurium* administered into the tumor sensitizes the tumor cells to an antigen against which there are pre-existing circulating $CD8^+$ T-cells. The rather effective antitumor immune response induced by even a single dose of *Salmonella* is remarkable. In fact, immune responses to antigens not present in the vaccine (*i.e.,* "epitope spreading") were readily detected in immunized animals. It is therefore likely that this vaccine platform has unique properties that allow it to break the immunological tolerance that usually prevents the mounting of a robust immune response to the tumor. It is likely that the intrinsic ability of *S. typhimurium* to induce potent innate immune responses may be central for its effectiveness as an anticancer platform.

Acknowledgements

Work in my laboratory is supported by Grants for the National Institutes of Health (NIH). I thank María Lara-Tejero for critical review of this manuscript.

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