Flagellin expressed by live Salmonella vaccine strains induces distinct antibody responses following delivery via systemic or mucosal immunization routes

M.E. Sbrogio-Almeida a,*, L.C.S. Ferreira b

a Divisão de Desenvolvimento Científico Tecnológico, Instituto Butantan, Avenida Vital Brasil 1500, 05503-900 São Paulo, Brazil
b Departamento de Microbiologia, Instituto de Ciências Biológicas II, Universidade de São Paulo, Av. Prof. Lineu Prestes 1374, 05508-900 São Paulo, Brazil

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Abstract

Salmonella flagellin, expressed as flagella in live attenuated vaccine strains, elicits distinct systemic (IgG) and secreted (IgA) antibody responses in mice following delivery via mucosal (nasal/oral) or parenteral (intraperitoneal (i.p.)) immunization routes. Reduced flagellin-specific antibodies were detected either systemically or locally following delivery of flagellated derivatives of aroA Salmonella enterica serovar Dublin SL1438 via the nasal route, the most effective mucosal site for activation of immune responses in mice. In contrast, flagellin represents the most potent Salmonella antigen for the generation of specific serum antibody (IgG) responses following i.p. inoculations. The distinct immunogenic properties of Salmonella flagellin could not be ascribed to deficient colonization, reduced invasive ability or loss of the flagellin expression by the flagellated vaccine strains. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Salmonella flagellin represents one of the most relevant antigens for the generation of protective immunity in mice [1,2]. Moreover, Salmonella flagellin has been shown to induce synthesis and secretion of proinflammatory cytokines either in humans or mice [2–5]. Nonetheless, the flagellin-specific systemic and secreted antibody responses induced following local delivery of attenuated Salmonella strains have been overlooked. Our previous observations indicated that Salmonella flagellin does not induce efficient systemic and secreted antibody responses following oral delivery of attenuated flagellated strains to mice [6].

The antibody inducing properties of Salmonella flagellin, following local administration of attenuated strains to mucosal sites, is particularly relevant since bivalent Salmonella vaccine strains expressing hybrid flagella fused to heterologous epitopes derived from different organisms have been intensively studied as a potential vaccine approach able to elicit a broad array of immune responses, including the local production of secreted IgA (sIgA) against both bacterial and passenger epitopes [7,8]. Particularly relevant is the fact that attempts to induce systemic and secreted antibody responses against heterologous epitopes fused to flagellin following oral administration of attenuated Salmonella strains have frequently failed [9–11].

In this work we investigated the ability of flagellated Salmonella vaccine strain to elicit systemic and secreted flagellin-specific antibody responses following inoculation of live Salmonella cells in the nasal cavities of mice, probably the most effective mucosal administration route, either in mice or humans, for the generation of local (sIgA) and systemic (IgG) antibody responses both for soluble and particulate antigens.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The attenuated Salmonella vaccine strains SL5928, SL5930 and ICB5 were derived from the Salmonella enter-
2.2. Immunization experiments

Female C57BL/6 mice, 8–12 weeks old, were from the Animal Breeding Center of the Biomedical Sciences Institute at the São Paulo University. Immunizations were carried out with exponential-phase cells (optical density (OD) of 0.8 at 600 nm). Bacteria were washed once with phosphate-buffered saline (PBS) and suspended in sterile water, or in 3% sodium bicarbonate for the peroral (p.o.) inoculations, to obtain different concentrations ranging from $5 \times 10^8$ colony forming units (CFU) ml$^{-1}$ to $5 \times 10^{10}$ CFU ml$^{-1}$. Groups of 10 mice under light anesthesia (etherization) were intranasally (i.n.) immunized with 20$\mu$l of bacterial suspension containing approximately $10^7$ CFU on days 0, 21 and 35 using a micropipette. Additional i.n. immunization experiments were carried out with $10^6$ and $10^9$ CFU per dose applied on days 0 and 21. Intraperitoneal (i.p.) immunizations were based on a three dose protocol containing $10^7$ CFU each, while the p.o. immunizations used three doses of $10^{10}$ CFU. The same immunization schedule (days 0, 21 and 35) was followed for both i.p. or p.o. experiments. Blood was drawn from the retro-orbital plexus 1 week after the last dose. Mice were killed by means of cervical dislocation and lungs were collected in 0.5 ml sterile PBS with the aid of a thin plastic cannula inserted in the trachea. Samples were transferred to microcentrifuge tubes and the supernatants harvested by centrifugation at 5000 $g$ for 5 min at 4°C. Blood-containing lung wash samples were discarded. Sera and lung wash samples were both stored at $-20°C$ until testing.

2.3. Measurement of antibody responses by enzyme-linked immunosorbent assay (ELISA)

Lipopolysaccharide (LPS)- and flagellin-specific serum IgG and lung wash IgA were measured following standard procedures [6]. Maxisorp plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 1 $\mu$g per well of group D Salmonella LPS (Sigma) or 0.1 $\mu$g per well of purified S. enterica serovar Dublin flagellin, prepared as previously described [6,11]. Plates were washed three times in PBS–0.05% Tween 20 (PBS-T) and blocked with 1% gelatin in PBS-T for 2 h at 37°C. Serial 2-fold dilutions of either sera or lung washes (100 $\mu$l per well) in PBS-T were incubated for 2 h at 37°C, followed by three washes with PBS-T and an additional incubation hour with PBS-T-diluted (1:1000), peroxidase-conjugated goat antibodies against mouse heavy chain-specific IgA or IgG isotypes (Sigma). Plates were finally washed with PBS-T and developed for bound peroxidase with O-phenylene diamine dihydrochloride and H$_2$O$_2$ as substrate. Endpoint titers were expressed as the reciprocal values of the last dilution with an OD of 0.1 at 492 nm above maximal absorbance values of samples collected from non-immunized animals (for anti-LPS responses) or from animals immunized with SL5928 (for anti-flagellin responses).

2.4. Immunoblot analyses

Detection of serum anti-flagellin antibodies in immunoblots was carried out with total protein extracts of SL5930 strains. The exponentially growing Salmonella cells were harvested by centrifugation, suspended in electrophoresis sample buffer and boiled for 5 min. Proteins were sorted in 10% polyacrylamide gels and transferred to nitrocellulose filters by standard procedures [12]. After blocking with 5% skim milk in PBS-T for 1 h, the nitrocellulose filters were incubated for 2 h at room temperature with sera from mice immunized with the Salmonella vaccine strains via different routes. Serum samples were diluted with PBS-T to concentrations ranging from 1:50 to 1:1000. Rabbit anti-flagellar antigen d serum (diluted 1:1000) (Difco, Detroit, MI, USA) was used as a positive control for flagellin detection. Development of reactive antibodies was carried out with peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Sigma), diluted in PBS-T containing 5% skin milk, and the ECL quimioluminescence kit (Amersham Corp., Arlington Heights, USA).

2.5. Detection of viable Salmonella cells in mouse tissues

Three groups of 15 female C57BL/6 mice were i.n. inoculated with a single $10^7$ CFU dose of the Salmonella vaccine strains (SL5928, SL5930 or ICB5). Mice were killed on various times after inoculation ranging from 1 to 20 days. Lungs and spleens were aseptically removed and homogenized individually in 1 ml of sterile PBS. Tissue homogenates were serially diluted in PBS and plated on LB agar plates for viable cell counting.

2.6. Evaluation of in vivo plasmid stability

The stability of pLS408 in strain SL5930 was determined by the number of ampicillin resistant colonies recovered from lungs, spleens, and Peyer’s patches of mice inoculated with a single dose of $10^7$ CFU (i.n.) or $10^{10}$ CFU (p.o.) of strains SL5930 or ICB5. Tissue homoge-
nates obtained from mice immunized with strain SL5930 were plated in LB (spleens or lungs) or MacConkey plates (Peyer’s patches) and incubated overnight at 37°C. Fifty colonies were replica-plated on L-agar plates containing ampicillin (100 μg ml⁻¹) and incubated for an additional night at 37°C. The number of ampicillin resistant colonies was counted and expressed as percentage of the total number of colonies analyzed. Flagellin expression in strain ICB5 was determined in Western blots developed with flagellin-specific rabbit serum using at least five colonies per organ per time point.

3. Results

3.1. Flagellin-specific antibody responses elicited in mice immunized with live attenuated Salmonella strains

CL57BL/6 mice immunized with three doses of live flagellated SL5930 or ICB5 strains via i.n. or p.o. inoculation routes developed reduced or undetectable serum flagellin-specific IgG responses (Fig. 1). On the other hand, mice i.p. immunized with strains SL5930 or ICB5 developed strong systemic (IgG) antibody responses against flagellin. Under the immunization conditions used in this work, flagellin represented by far the most potent Salmonella antigen as regards the generation of serum antibody responses detected in immunoblots (Fig. 1). Analysis in ELISA of the systemic and secreted antibody responses elicited by the Salmonella vaccine strains showed that the i.n. immunization resulted in serum LPS-specific IgG responses similar to those attained following parenteral administration, which confirms the performance of the i.n. route as an efficient mucosal immunization approach (Fig. 2A). On the other hand, the serum flagellin-specific IgG responses developed in mice following p.o. or i.n. inoculation of Salmonella SL5930 or ICB5 strains were marginal (Fig. 2B). Stabilization of flagellin expression in ICB5 did not change significantly the serum antibody responses elicited in mice immunized with the Salmonella vaccine strains (Fig. 2). Immunization trials carried out with larger doses (10⁸ or 10⁹ CFU) delivered via the i.n. route resulted in death of more than 50% of the inoculated animals. More-
ula (10^8 or 10^9 CFU) did not improve the local flagellin-specific IgA responses, as evaluated in survivors submitted to these immunization regimens (data not shown).

3.2. Colonization/invasion abilities and flagellin expression of the Salmonella vaccine strains

The reduced immunogenicity of flagellin following mucosal delivery of attenuated *Salmonella* strains might reflect deficient colonization or reduced ability to invade deeper tissues. Colonization, measured by survival at the lung environment, and spleen invasion were determined for periods up to 20 days following inoculation of 10^7 CFU of the *Salmonella* SL5928, SL5930 and ICB5 strains. As observed in Fig. 4, the tested *Salmonella* strains showed similar behaviors as regards to colonization and invasion abilities, evaluated by the number of viable cells in lungs or in spleens of inoculated mice, respectively.

Reduced antigen load due to loss of flagellin expression by the SL5930 or ICB5 strains could have a negative effect on the activation of flagellin-specific secreted and systemic antibody responses. Therefore, stability of flagellin expression was monitored following p.o. or i.n. inoculation of mice either by the number of ampicillin resistant colonies, for the SL5930 strain, or by positive results in colony blots developed with flagellin-specific antibodies, for the ICB5 strain. As shown in Table 1, stability of flagellin-encoded plasmid measured in colonies recovered from Peyer’s patches or spleens of mice p.o. inoculated with a single dose of 10^7 (i.n.) or 10^10 (p.o.) CFU of strain SL5930 and processed as described in Section 2. Values represent the means of three determinations.

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4. Discussion

Recombinant attenuated Salmonella strains expressing hybrid flagellin have been designed as an elegant and simple approach to deliver heterologous epitopes derived from diverse pathogens to the mammalian immune system inductive sites [8,9]. One of the main appeals of such vaccine approach relied on the possibility to induce local and systemic antibody responses against host and heterologous antigens after mucosal delivery of the attenuated strains. However, our findings shows that Salmonella flagellin does not efficiently activate systemic or secreted antibody responses after p.o. or i.n. administration to mice. The low immunogenicity of flagellin following delivery at the nasal inoculation route was surprising since it has been shown to be the most sensitive mucosal site for generation of antibody responses both for humans and rodents [13-15]. These results suggest that flagellin does not represent an efficient protein carrier for activation of secreted and systemic antibody responses to B-cell epitopes fused to it when delivered at mucosal sites as a cellular component of live Salmonella strains.

The reduced immunogenicity of flagellin delivered at mucosal sites contrasted with the strong antibody responses attained after parenteral delivery of flagellated Salmonella cells. Some explanations for the distinct immune responses elicited by Salmonella flagellin delivered at different immunization sites would be a deficient mucosal colonization or reduced invasive ability of flagellated Salmonella cells. Nonetheless our present data and previous observations [6] have demonstrated that the flagellated SL5930 and ICB5 strains show no significant impairment, in relation to the non-flagellate strain, in their colonization or invasive properties. The lack of flagellin-specific antibody responses in mice mucosally inoculated with ICB5 strain demonstrated also that loss of flagellin-encoding gene does not contribute to the low antibody yields following delivery at mucosal sites. Moreover, low immunogenicity of flagellin following delivery at mucosal sites does not represent a case of tolerance since mice orally immunized with live flagellated Salmonella cells can induce strong antibody responses against parenterally administered flagellin (unpublished observations).

The low immunogenicity of Salmonella flagellin delivered at mucosal sites may reflect the action of gene regulation mechanism operating during in vivo replication. The flagellin-coding gene present in the Salmonella vaccine strains remains under the control of the native promoter, which, although strong, may be negatively regulated during replication inside mammalian cells, reducing the amount of antigen available for activation B-cell dependent responses. On the other hand, some authors suggested that local and systemic antibody responses induced against a heterologous antigen expressed by Salmonella strains do not require in vivo expression or even viability of the bacterial cells [16]. Expression of Salmonella flagellin under the control of constitutive or in vivo induced promoters is an attempt worth to pursue in order to improve the immunogenicity of mucosal delivered flagellated strains. Moreover, elucidation of the gene regulation mechanism affecting flagellin expression during in vivo growth should also help to design more rational vaccine approaches based on attenuated Salmonella strains.

The lack of antibody responses to flagellin in mice immunized at mucosal sites with live Salmonella cells could also mean that the elicited immune responses do not activate B-cells and, therefore, do not lead to antibody production. Previous evidences indicated that flagellin represents the most relevant antigen for the development of a protective cellular immune response in mice orally immunized with attenuated Salmonella strains [1,2]. Moreover, recent reports have shown that Salmonella flagellin is a strong inducer of inflammatory cytokines in vitro cultured human mononuclear cells [3,4]. Taken together, these results suggest that Salmonella flagellin can induce cellular immune responses in mice, but not antibody production, when attenuated strains are delivered at mucosal sites. Further experiments are required to demonstrate that delivery of flagellated Salmonella vaccine strains at murine mucosal sites can activate cell-mediated immune responses against heterologous epitopes fused to flagellin.

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References


