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SCIENCE DIRECT®
Microbes and Infection 7 (2005) 860–866
Microbes and Infection
www.elsevier.com/locate/micinf

Original article

Rapid clearance of a recombinant *Salmonella* vaccine carrier prevents enhanced antigen-specific CD8 T-cell responses after oral boost immunizations

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Received 13 January 2005; accepted 7 February 2005

Available online 13 April 2005

Abstract

The type III secretion system of *Salmonella enterica* serovar Typhimurium can be used to target heterologous antigens directly into the cytosol of antigen-presenting cells. Our laboratory has previously reported that the single oral immunization of mice with a recombinant *Salmonella* strain expressing the translocated *Yersinia* outer protein E fused to the immunodominant antigen p60 from *Listeria monocytogenes* results in the efficient induction of p60-specific CD8 T cells and confers protection against a lethal wild-type *Listeria* challenge infection. In the present study, we investigated whether these antigen-specific cytotoxic T lymphocytes induced by the prime immunization contribute to a more rapid clearance of the vaccine carrier after subsequent boost immunizations and whether oral boost immunizations lead to an augmented p60-specific CD8 T-cell response. We found that the ability of recombinant *Salmonella* strains to colonize the intestine, mesenteric lymph nodes, and spleen was markedly impaired after boost immunizations but that this effect was independent of existing CD8 T cells reactive with $p60_{217–225}$. A significant elevation of antigen-specific CD8 T cells could not be detected by enzyme-linked immunospot assay after the second or the third oral immunization, possibly due to the rapid clearance of the bacterial vaccine carrier from lymphatic organs.

Keywords: *Salmonella enterica* serovar Typhimurium; Bacterial vaccine carrier; Type III secretion system; Heterologous antigen; CD8 T cell; Boost immunization; Antivector immunity

1. Introduction

Live attenuated *Salmonella enterica* serovar Typhimurium strains have been widely used as vaccine carriers for antigens derived from other organisms to induce both humoral and cell-mediated immunity [1–4]. After gaining access to eukaryotic target cells, *Salmonella* replicates within a membrane-bound compartment termed *Salmonella*-containing vacuole (SCV) [5], thus facilitating delivery of antigens to the exogenous antigen presentation pathway and priming of peptide-specific CD4 T cells [6]. However, the confinement of serovar Typhimurium to the SCV complicates endogenous major histocompatibility complex (MHC) class I-restricted antigen presentation of heterologous antigens and subsequent CD8 T-cell induction. In an attempt to circumvent this problem, our laboratory has focused its research on the development of a new vaccination strategy by using *Salmonella*'s type III secretion system (TTSS) to translocate foreign antigens directly into the cytosol of antigen-presenting cells (APC) [7–9]. The immunodominant proteins p60 and listeriolysin O from *Listeria monocytogenes* were fused to the defined N-terminal translocation domain of the *Yersinia* outer protein E (YopE). In vitro experiments revealed that *Salmonella* allows secretion and translocation of these chimeric proteins in a TTSS-dependent fashion [8].

Abbreviations: APC, antigen-presenting cell; CFU, colony-forming units; Elispot, enzyme-linked immunospot; IFN-γ, interferon-gamma; MHC, major histocompatibility complex; SCV, *Salmonella*-containing vacuole; TTSS, type III secretion system; YopE, *Yersinia* outer protein E.

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doi:10.1016/j.micinf.2005.02.007

Translocation and cytosolic delivery of hybrid proteins into host cells, but not secretion into the endosomal SCV, led to efficient MHC class I-restricted antigen presentation of listerial nonamer peptides. As determined by enzyme-linked immunospot (Elispot) assay, mice orally vaccinated with a single dose of attenuated *Salmonella* expressing either translocated YopE/LLO or YopE/p60 proteins revealed high numbers of interferon-gamma (IFN-γ)-producing antilisterial CD8 T cells reactive with $LLO_{91–99}$ or $p60_{217–225}$, respectively. These T lymphocytes conferred protection against a lethal challenge with a *Listeria* wild-type strain [8,10].

There are conflicting reports concerning the impact of prior vector priming on the immunogenicity of recombinant *Salmonella*-based vaccines. Some data indicated that prior exposure to *Salmonella* enhanced antibody responses to a foreign antigen delivered orally by *Salmonella* [11,12]. These findings were contradicted by studies reporting that prior exposure to *Salmonella* can dramatically reduce serum antibody responses to a foreign antigen [13–17]. However, the goal of the present study was to determine whether (i) antilisterial immunity induced by the first immunization with recombinant *Salmonella* expressing YopE/p60 contributes to a more rapid clearance of the vaccine carrier after subsequent immunizations of mice and whether (ii) oral boost immunizations result in an enhanced p60-specific CD8 T-cell response.

2. Materials and methods

2.1. Plasmid, Salmonella strain and growth conditions

Previously, the construction of plasmid pHR241 was outlined in detail [8]. Briefly, this derivative of pWSK29 [18] is a low-copy-number expression vector and bears the genetic information for translocated chimeric $YopE_{1–138}/p60_{130–477}$/M45 under expression control of the *lac* promoter which is constitutively active in *S. enterica* serovar Typhimurium. The C-terminal M45-tag (MDRSRDRLPPFETETRIL) is derived from the E4-6/7 protein of adenovirus [19]. Proper expression of the hybrid protein was determined by using a monoclonal antibody directed against M45 for immunoblot analysis [8]. Plasmid pHR241 was transformed into *S. enterica* serovar Typhimurium strain SB824 by electroporation [7]. Strain SB824 was engineered by introducing the *sptP::kan* mutant allele from strain SB237 [20] into the *aroA* mutant strain SL3261 [21] by P22HT*int* transduction. Serovar Typhimurium was grown in Luria–Bertani medium supplemented with 0.3 M NaCl, pH 7.0, to allow expression of components and targets of the TTSS encoded by the *Salmonella* pathogenicity island 1 which mediates *Salmonella* invasion of the host cell [22,23].

2.2. Oral immunization of mice

Specific-pathogen-free female BALB/c mice, 6–8 weeks old, were purchased from Harlan-Winkelmann (Borchen, Germany). For the experiments, animals were housed in groups of up to five mice under standard barrier conditions in individually ventilated cages (Tecniplast, Buguggiate, Italy) equipped with steel grid floors and autoclaved filter paper. Water and food were withdrawn for 4 h before groups of five mice were orally immunized with 10^8 colony-forming units (CFU) of SB824 or SB824 (pHR241), respectively, by using round-bottomed gavages. Thereafter, drinking water ad libitum was offered immediately and food 2 h post immunization. Prime (day 0) and boost immunizations (day 30 and day 60) were separated by a period of 30 days. Each experiment was performed at least twice with similar results.

Animal experiments were approved by the German authorities and performed according to the legal requirements.

2.3. Analysis of Salmonella loads in intestines, mesenteric lymph nodes and spleens

At the indicated time points post immunization, mice were sacrificed by CO_2 asphyxiation, and samples from the intestinal tract, mesenteric lymph nodes, and spleens were removed for analysis.

Intestinal contents from the cecum were weighed before resuspending them in 500 µl of 4 °C phosphate-buffered saline, pH 7.4. The numbers of CFU per 1000 mg from intestinal content were determined by plating appropriate dilutions on MacConkey agar plates containing kanamycin at 50 µg/ml.

To analyze the colonization, three mesenteric lymph nodes per animal and the spleen were removed aseptically. Spleens were weighed before homogenizing them in 4 °C phosphate-buffered saline, pH 7.4 (0.5% Tergitol, 0.5% bovine serum albumin). The numbers of CFU per 1000 mg from splenic tissue or from three mesenteric lymph nodes, respectively, were determined by plating appropriate dilutions on MacConkey agar plates containing kanamycin at 50 µg/ml.

2.4. IFN-γ Elispot

Thirty days after the first, second and third immunization, splenocytes were analyzed directly ex vivo for epitope-specific CD8 T-cell responses using an IFN-γ Elispot assay as described previously [24,25]. Assays were performed in nitrocellulose-backed 96-well microtiter plates (Nunc, Wiesbaden, Germany) coated with a rat anti-mouse IFN-γ monoclonal antibody (clone RMMG-1; Biosource, Camarillo, USA). Unseparated splenocytes (6×10^5 per well) were stimulated for 6 h in round-bottomed microtiter plates in the presence of a 10^{-6} M concentration of the CD8 T-cell epitope $p60_{217–225}$. Subsequently, activated cells (4×10^5 per well) were transferred to Elispot plates and incubated overnight. Elispot plates were developed with a biotin-labeled rat anti-mouse IFN-γ monoclonal antibody (clone XMG1.2; Pharmingen, San Diego, USA), horseradish peroxidase-streptavidin conjugate (Dianova, Hamburg, Germany) and aminoethylcarbazole dye solution. The frequency of antigen-specific cells was calculated as the number of spots per splenocyte seeded.