[SII-1]

Immune Responses to Recombinant Antigen Delivered by Live Attenuated Recombinant *Salmonella* Vaccines

Ho Young Kang*, Tae Ho Lee, In Soo Lee[1], and Roy Curtiss III[2]

Division of Biological Sciences, Pusan National University, Busan, Korea 609-735

[1]Department of Microbiology, Hannam University, Taejon, Korea 306-791

[2]Department of Biology, Washington University, St. Louis, Missouri 63130

The immunogenicity and appropriate subcellular location of the recombinant antigen in the *Salmonella* vaccine strain may contribute to augmenting immune responses by facilitating adequate exposure of recombinant antigen to antigen presenting cells (APCs) for processing. To allow for secretion from gram-negative bacteria and overexpression of antigen, a DNA fragment encoding a highly antigenic α-helical region of PspA (pneumococcal surface protein A) was subcloned downstream to the β-lactamase signal sequence in the multicopy Asd^+ pYA3493 vector to create pYA3494. pYA3493 was derived from a class of Asd^+ vectors with reduced expression of Asd to minimize selective disadvantage and enhance immunization of expressed recombinant antigens. The *S. typhimurium* vaccine strain was constructed by the introduction of deletion mutations Δ*crp-28* and Δ*asdA16*. Approximately fifty percent of the recombinant PspA (rPspA) expressed in *Salmonella* strain harboring pYA3494 was detected in the combined supernatant and periplasmic fractions of broth-grown recombinant *Salmonella*. After a single oral immunization in BALB/c mice with 10^9 CFU of the recombinant *Salmonella* vaccine strain carrying pYA3494, IgG antibody responses were stimulated to both the heterologous antigen rPspA and *Salmonella* LPS and outer membrane proteins (OMPs). About half, and even more at later times after immunization, of the antibodies induced to rPspA were IgG1 (indicating a Th2-type response) whereas 60 to 70 percent of the antibodies to LPS and 80 to 90 percent to OMPs were IgG2a (indicating a Th1-type response). A sublethal infection with *Streptococcus pneumoniae* WU2 boosted PspA antibody levels and maintained similar IgG2a/IgG1 ratios as seen before the challenge. Oral immunization of *Salmonella*-PspA vaccine protected 60% of immunized mice from death against intraperitoneal challenge with a 50 X LD_{50} dose of virulent *S. pneumoniae* WU2.

Introduction

The immune responsiveness to orally administered *Salmonella* has been applied to develop live attenuated oral *Salmonella* vaccines (1). Attenuated *Salmonella* vaccine strains have been genetically modified to express another pathogen's antigen(s) specified by multicopy plasmids. These recombinant vaccines induce immunity to the pathogen whose antigen gene is expressed as well as to *Salmonella*. It is essential that the antigen specifying plasmids in *Salmonella* vaccines are stably maintained during the in vivo colonization

process. A "balanced-lethal host-vector system" based on the essential bacterial gene aspartate β-semialdehyde dehydrogenase (*asd*) has been used to specify recombinant antigens from Asd^+ plasmids that are retained in vivo in *asd* gene deleted *Salmonella* vaccine strains (2).

Streptococcus *pneumoniae* is a human pathogen that causes life-threatening diseases, including community-acquired pneumonia, otitis media, meningitis, and bacteremia in persons of all ages (3). Vaccination with the pneumococcal polysaccharide vaccine does not reduce the frequency of hospitalization, costs, and mortality caused by pneumococcal pneumonia (4), which reinforces the need for effective new vaccines.

In this work, we constructed a stable multi-copy Asd^+ antigen expression vector encoding the β-lactamase signal sequence fused in-frame to the immunogenic α-helical region of PspA. We report the immunogenicity, type of immune responses, and protection to both *Salmonella* and *S. pneumoniae* of mice immunized with a *Salmonella* vaccine expressing rPspA by an improved antigen expression system.

Results and discussion

Construction of Asd^+ vectors to use for antigen expression.

Since export of PspA into the periplasmic space *Salmonella* was inefficient and caused toxicity when the export depended on the signal sequence for PspA (5), we constructed a recombinant plasmid by cloning a DNA fragment specifying the signal sequence of β-lactamase which is efficiently transported into the periplasmic space in *Salmonella*. A 105 bp DNA fragment (nucleotides 4049 to 4153 of accession number J01749) of the β-lactamase gene was PCR-amplified from the pBR322 DNA template using a pair of primers, ([N-terminal], 5'GCATTCATGAGTATTCAACATTTCC3'-*Bsp*HI [underlined] and ([C-terminal], 5'CCGGAATTCTTCAGCATCTTTTACT3'-*Eco*RI [underlined]). The PCR-amplified fragment included the N-terminus of β-lactamase from the ATG start codon through the signal sequence (23 amino acids) plus 12 amino acids of the N-terminus of the mature β-lactamase These additional 12 amino acid residues were included to increase the efficiency of secretion of the recombinant protein (6). The 105 bp PCR product was digested with *Bsp*HI and *Eco*RI enzymes and cloned into the *Nco*I (compatible with the *Bsp*HI site) and *Eco*RI sites of the Asd^+ vector pYA3342, resulting in plasmid pYA3493 (**Fig. 1**).

Construction of the rPspA-expressing plasmid.

A highly immunogenic α-helical region of PspA from amino acid residues 3 to 257 (765 bp; 255 amino acids) of the mature $PspA_{Rx1}$ protein (588 amino acids) was selected to use as a test antigen in antigen delivery by a *Salmonella* carrier. The 765 bp DNA fragment of the *pspA* gene of *S. pneumonia* Rx1 was PCR-amplified from the pYA3193 DNA template with a pair of primers ([N-terminal], 5'CCGGAATTCTCTCCCGTAGCCAGTCAGTCT3', and the same C-terminal primer used in the construction of histidine [6X]-tagged PspA which introduces the TAA TAG stop codons after the *pspA* coding sequences). The PCR product, digested with *Eco*RI and *Hin*dIII enzymes, was cloned into *Eco*RI and *Hin*dIII sites of pYA3493, resulting in pYA3494.

Expression and subcellular localization of rPspA in *Salmonella*.

With the expectation of the periplasmic secretion of the rPspA, various subcellular fractions including cytoplasm, periplasm, outer membrane, and culture supernatant of χ8599 (pYA3494) were prepared to examine the location of rPspA. Although the calculated size of rPspA was approximately 30 kDa, PspA-specific monoclonal antibody Xi126 reacted with an approximately 35 kDa protein (**Fig. 2**). Densitometry analyses of immuno reactive bands showed that approximately 50% of the rPspA was located in the combined periplasm (25%) and culture supernatants (25%).

Recombinant *S. typhimurium* Δ*crp-28* vaccine expressing rPspA antigen.

pYA3493 (vector control) and pYA3494 encoding rPspA were electroporated into the Δ*crp-28* Δ*asdA16* strain χ8501. The *S. typhimurium* χ8501 (Δ*crp-28* Δ*asdA16*) vaccine strain containing pYA3494 expressed the rPspA protein at an approximate molecular mass of 35 kDa. In the analyses of Coomassie blue-stained SDS-PAGE gels, the amount of rPspA protein was as much as approximately 1-2% of the total χ8501 (pYA3494) proteins. With results consistent with those seen in the rPspA localization analysis [75% of rPspA cell-associated (50% cytoplasm and 25% periplasm) and 25% of rPspA secreted], the rPspA expressed in χ8501 vaccine strain was secreted into the culture supernatant along with other secreted proteins.

Immune responses in mice after oral immunization with the recombinant *S. typhimurium* vaccines.

A single dose of *S. typhimurium* χ8501 (pYA3494) (1.9 X 10^9 CFU) or χ8501 (pYA3493) (control, 2 X 10^9 CFU) was orally administered to 7 week-old female BALB/c mice. All immunized mice survived and we did not observe any signs of disease in the immunized mice during the entire experimental period. The serum IgG responses to LPS, SOMPs and rPspA are presented in **Figure 3**. At two weeks after administration, little IgG responses to the antigens were observed. Maximal anti-LPS, -SOMPs and –rPspA IgG levels without boost immunization were detected at 6 weeks post immunization.

At 17 weeks post immunization, we infected mice intravenously with a sublethal dose (3.8 X 10^5 CFU) of the virulent *S. pneumoniae* WU2 strain to monitor the changes of anti-rPspA antibody titers. Sublethal i.v. infection with *S. pneumoniae* did not kill mice immunized with the χ8501 (pYA3493) or χ8501 (pYA3494) vaccines. Because native PspA is a highly immunogenic pneumococcal surface protein, the pneumococcal challenge boosted rPspA specific immune responses in χ8501 (pYA3494) immunized mice (Fig. 3).

IgG isotype analyses.

The types of immune responses to *Salmonella* LPS and SOMPs and the rPspA were further examined by measuring the levels of IgG isotype subclasses IgG2a and IgG1. The Th1-helper cells direct cell-mediated immunity and promote class switching to IgG2a, and Th 2 cells provide potent 'help' for B cell antibody production and promote class switching to IgG1 (7). IgG2a isotype dominant responses were observed for the *Salmonella* LPS and SOMPs antigens (**Fig. 4**). The ratios of IgG2a/IgG1 for anti-LPS and –SOMPs in sera obtained from mice immunized with χ8501 (pYA3493) were not significantly different to those observed in mice immunized with χ8501 (pYA3494) (P=0.07, LPS; P=0.054, SOMPs). The ratios of IgG2a/IgG1 for

anti-SOMPs (ranging from 6.4 to 11.5) are higher than that for LPS (ranging from 1.1 to 2.5) with statistical significance (P=0.0004, χ8501 (pYA3493); P=0.0003, χ8501 (pYA3494)).

Evaluation of protective immunity.

To examine the ability of Salmonella-rPspA vaccines to protect against pneumococcal infection, BALB/c mice were immunized with either S. typhimurium χ8501 (pYA3493) (1.3 X 10^9 CFU dose) or χ8501 (pYA3494) (1.7 X 10^9 CFU dose). Ten weeks after initial immunization, a second 10^9 CFU dose of each vaccine was administered. At 5 weeks after the second immunization, mice were challenged intraperitoneally with 4.8 X 10^3 CFU (50 X LD_{50}) of S. pneumoniae WU2. Sixty percent of the mice immunized with χ8501 (pYA3494) were protected from pneumococcal challenge with statistical significance ($P<0.05$). This challenge dose killed 100% of unimmunized and χ8501 (pYA3493) immunized mice (**Table 1**) (8).

References

1. Curtiss, R. III, T. Doggett, A. Nayak, and J. Srinivasan. 1996. Strategies for the use of live recombinant avirulent bacterial vaccines for mucosal immunization, p. 499-511. *In* H. Kiyono and M. F. Kagnoff (ed.), Essentials of mucosal immunology. Academic Press, San Diego, CA.
2. Nakayama, K., S. M. Kelly, and R. Curtiss III. 1988. Construction of an Asd^+ expression-cloning vector: Stable maintenance and high level expression of cloned genes in a *Salmonella* vaccine strain. Bio/Technology **6**:693-697.
3. Mufson, M. A. 1990. *Streptococcus pneumoniae*, p. 1539-1550. *In* G. L. Mandell, R. G. Douglas, Jr., and J. E. Bennett (ed.), Principles and practice of infectious disease. Churchill Livingstone, Inc., New York, N. Y.
4. Hirschmann, J. V. 2000. Use of the pneumococcal polysaccharide vaccine is unwarranted in the U. S. ASM News **66**:326-327.
5. Nayak, A. R., S. A. Tinge, R. C. Tart, L. S. McDaniel, D. E. Briles, and R. Curtiss III. 1998. A live recombinant avirulent oral *Salmonella* vaccine expressing pneumococcal surface protein A induces protective responses against *Streptococcus pneumoniae*. Infect. Immun. **66**:3744-3751.
6. Summers, R.G., and J.R. Knowles. 1989. Illicit secretion of a cytoplasmic protein into the periplasm of *Escherichia coli* requires a signal peptide plus a portion of the cognate secreted protein. J. Biol. Chem. **264**:20074-20081.
7. O'Garra, A., and N. Arai. 2000. The molecular basis of T helper 1 and T helper 2 cell differentiation. Trends Cell. Biol. **10**:542-550.
8. Kang, H. Y., J. Srinivasan, and R. Curtiss III. 2002. Immune responses to recombinant pneumococcal PspA antigen delivered by live attenuated *Salmonella enterica* serovar Typhimurium vaccine. Infect. Immun. **70**:1739-1749.

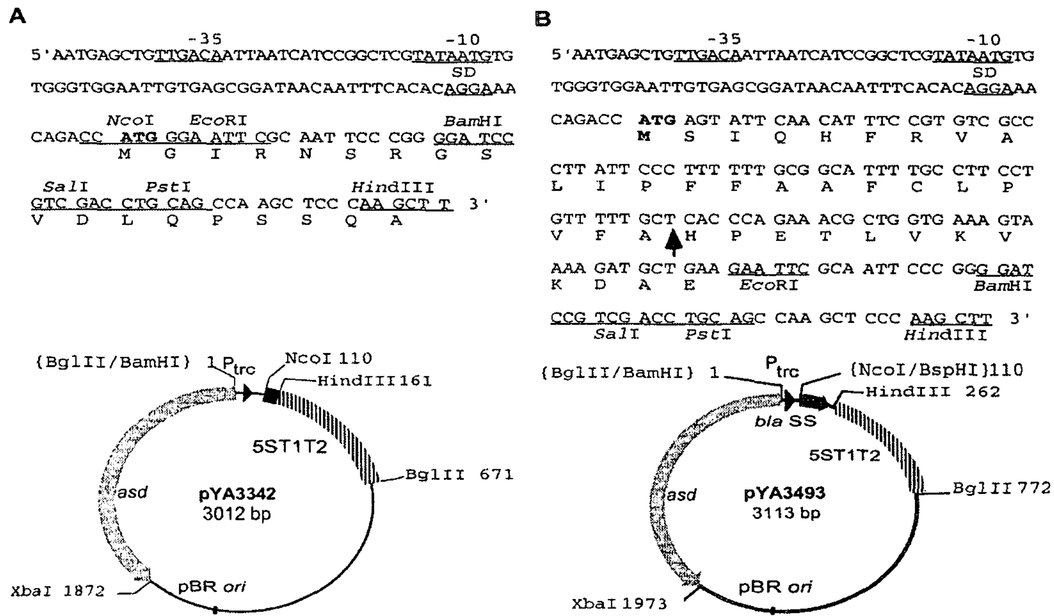

Figure 1. Asd$^+$ antigen expression vectors. A. Asd$^+$ vector pYA3342. The map of pYA3342 and the nucleotide sequences of the P_{trc} promoter region and multicloning sites are depicted. B. Periplasmic secretion Asd$^+$ vector pYA3493. A DNA fragment encoding the β-lactamase signal sequence and 12 amino acid residues of the N-terminus of mature β-lactamase of plasmid pBR322 was positioned under the control of the P_{trc} promoter of the Asd$^+$ vector pYA3342 (pBR*ori*).

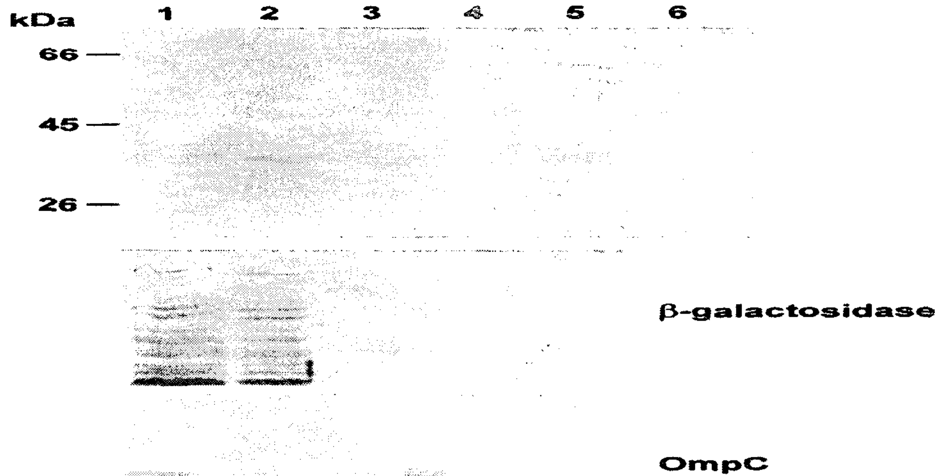

Figure 2. Subcellular location of expressed rPspA in *S. typhimurium*. Subcellular fractions were prepared from *S. typhimurium* χ8599 (pYA3494) cells grown in LB broth at 37°C by the procedures described in Materials and Methods. Fractions equivalent to 30 μl volumes of the 0.8 OD_{600} culture, except for supernatant fluids were analyzed by SDS-PAGE and the rPspA was detected by immunoblot with PspA specific monoclonal antibody Xi126. β-galactosidase and OmpC were used as fractionation controls for cytoplasmic and outer membrane fractions, respectively. Standards are indicated to the left. Lanes; 1, total cell lysate; 2, cytoplasm; 3, periplasm; 4, outer membrane; 5, concentrated supernatant (750 μl); 6, supernatant (10 μl).

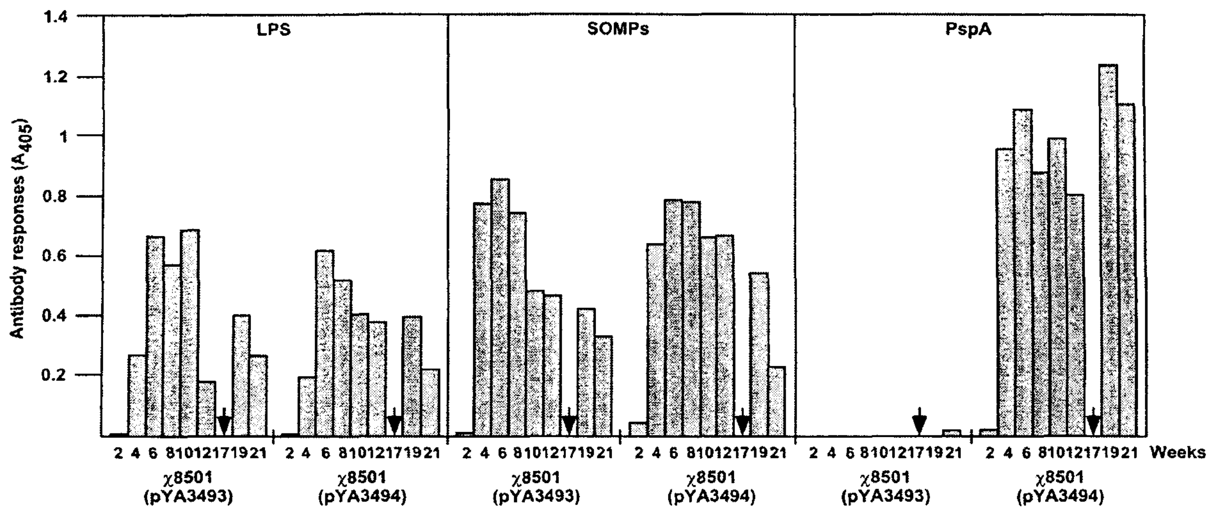

Figure 3. Serum IgG responses to *S. typhimurium* LPS and SOMPs, and recombinant PspA. The data represent IgG antibody levels induced in mice orally immunized with χ8501 (pYA3493) (vector control) and χ8501 (pYA3494) (expressing rPspA) at designated weeks after immunization. ELISA and data analysis are described in Materials and Methods. Black arrows indicate sublethal i.v. infection with *S. pneumoniae* WU2.

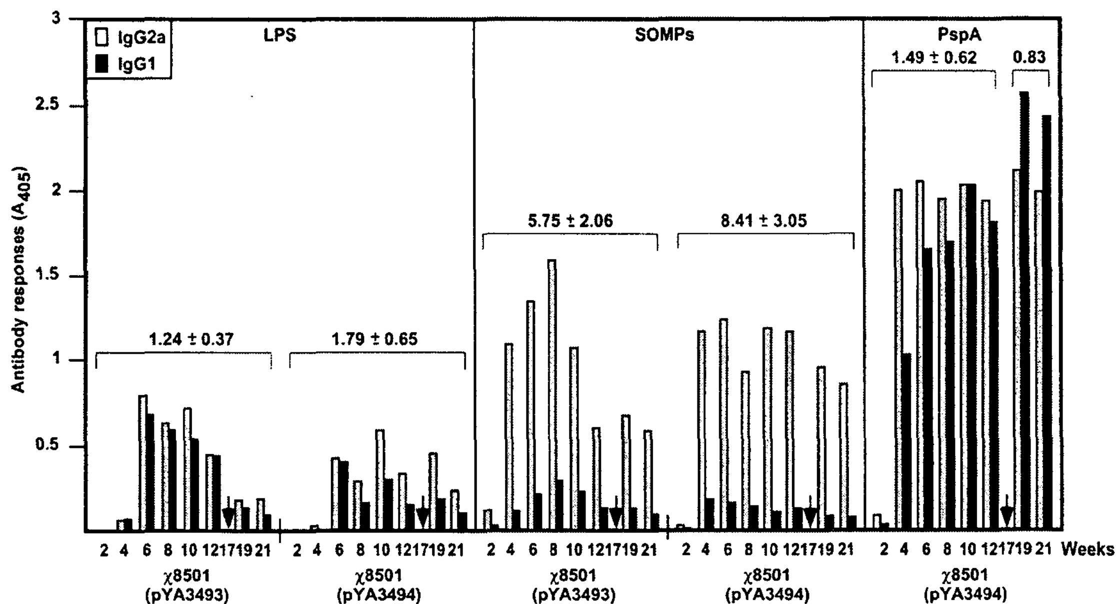

Figure 4. Serum IgG2a and IgG1 responses to *S. typhimurium* LPS and SOMPs, and recombinant PspA. The data represent IgG2a and IgG1 subclass antibody levels to *Salmonella* LPS and SOMPs and rPspA in sera of BALB/c mice orally immunized with χ8501 (pYA3493) (vector control) and χ8501 (pYA3494) (expressing rPspA) at designated weeks after immunization. Black arrows indicate sublethal i.v. infection with *S. pneumoniae* WU2. Anti-rPspA IgG2a and IgG1 responses of χ8501 (pYA3493) (negative control) were not shown. The overall IgG2a/IgG1 ratios (mean±SD) of each antigen are shown above the columns.

Table 1. Oral immunization of rPspA-expressing *S. typhimurium* χ8501 (pYA3494) vaccine protects BALB/c mice against challenge with virulent *S. pneumoniae* WU2 strain

Vaccines[a]	rPspA expression[b]	Protection (% alive)	Days to death
χ8501 (pYA3494)	+	60*	5, 5 >21, >21, >21
χ8501 (pYA3493)	-	0	1, 2, 2, 3, 3
Unimmunized	N/A	0	1, 2, 2, 2, 3

[a] Mice were orally immunized a total of two times at 10 week intervals with $\sim 10^9$ CFU dose of indicated vaccine strains

[b] +, rPspA expressed; -, rPspA not expressed; N/A, not applicable