Parasite Immunology, 1999: **21:** 219–224

Brief communication

Immunogenicity of the extracellular copper/zinc superoxide dismutase of the filarial parasite *Acanthocheilonema viteae* delivered by a two-phase vaccine strain of *Salmonella typhimurium*

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SUMMARY

The recombinant extracellular copper/zinc superoxide dismutase of the filarial parasite Acanthocheilonema viteae *(AVSOD2) was cloned in an expression vector under control of the bacteriophage T7 promoter and the resulting plasmid pLAT7 was introduced in tha* aroA *attenuated* Salmonella typhimurium *vaccine strain SL3261::pYZ84. This vaccine strain carries a chromosomally integrated two phase expression system containing inducible T7 RNA polymerase. The recombinant AVSOD2 was efficiently expressed, constituting up to 5% of the total bacterial protein. Furthermore, the plasmid vector containing the AVSOD2 cDNA was shown to be stable over a long period of time in the vaccine strain without antibiotic selection* in vitro *and* in vivo. *Jirds which were immunised orally with the recombinant vaccine strain expressing the* A. viteae *EC-SOD produced a strong humoral immune response.*

Keywords *filariasis, superoxide dismutase, vaccination,* Salmonella, Acanthocheilonema viteae, Onchocerca volvulus

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Received: 18 May 1998
Accepted for publication: 11 September 1998
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INTRODUCTION

In the course of infection, many pathogens are confronted by activated oxygen metabolites generated by activated leukocytes which undergo an 'oxidative burst'. Consequently the expression of high levels of antioxidant enzymes is implicated to play an important role in immune evasion of pathogenic bacteria and parasites (Bannister *et al.* 1987, Callahan *et al.* 1988, Maizels et al. 1993). Superoxide Dismutases (SODs) protect cells by inactivating the superoxide radical ($\cdot O_2^-$) which is able to oxidize biological membranes and proteins (Brunori & Rotilio 1984). According to their metal cofactors, SODs are grouped into three classes, Fe SODs, Mn SODs, and CuZn SODs, the latter of which are primarily found in eukaryotes. Two SOD subtypes have been characterised, cytoplasmic (CY-SOD) and extracellular (EC-SOD) SOD. In various organisms SOD expression is correlated with extrinsic or intrinsic oxidative stress, and expression of SOD is up-regulated in response to the oxygen tension in prokaryotes and eukaryotes (Bannister *et al*. 1987). The immunoprotective potential of a vaccination with SOD has been evaluated for several bacterial species, leading to a protective immune response in experimental infection with *Mycobacterium leprae* (Gelber *et al*. 1994) and *Listeria monocytogenes* (Hess *et al*. 1997). SODs recently have also been characterized for several filarial species (Henkle-Dührsen *et al*. 1991, James *et al.* 1994, Tang *et al*. 1994) and there is evidence that filarial parasites release SOD activity into the surrounding environment (Batra *et al.* 1990, Henkle-Dührsen *et al*. 1991, Tang *et al*. 1994). Thus, SODs may participate in immune evasion of filarial infections and therefore are considered as a potential target molecule for an anti-filarial vaccine.

Attenuated vaccine strains of *Salmonella typhimurium*

and *S. typhi* have been shown to be efficient carriers for the delivery of recombinant antigens. *Salmonella* vaccine strains expressing various recombinant antigens have been shown to elicit humoral and T cell-dependent immune responses against these antigens in animals and humans (Brown *et al*. 1987, Turner *et al.* 1993, Khan *et al*. 1994, Maskell *et al*. 1987, Hopkins *et al*. 1995). The immune response induced by such recombinant *Salmonella* vaccines was shown to be protective against viral, bacterial, and protozoan infections in several animal models (Poirier *et al.* 1988, Tite *et al*. 1990, Yang *et al*. 1990, Oyston *et al*. 1995, Gómez-Duarte *et al*. 1997, Hess *et al*. 1997). To evoke an immune response, the recombinant antigen has to be presented in large amounts within a certain period of time. Nevertheless, the constitutive high level expression of foreign antigen often interferes with the viability of the recombinant *Salmonella* strain, thus resulting in the reduced stimulation of the immune system. Previously, we have reported the design of an attenuated *S. typhimurium aro*A vaccine strain by a rational approach combining the bacteriophage T7 polymerase/promoter system (Tabor & Richardson 1985) and a temperature-dependent phase variation system (Yan & Meyer 1994, 1996). This system guarantees the stable expression of foreign antigens *in vivo* since the vaccine strain population exists as two subpopulations. The non-expressing population is actively dividing and propagating like the parental vaccine strain, while the phase variable switching of a small proportion of this population constantly renews the subpopulation which expresses the recombinant antigen.

In this communication, we investigate the delivery of the recombinant EC-SOD of the small mammal filaria *Acanthocheilonema viteae* (AVSOD2, Genbank accession number AJ010164) expressed in the *aro*A attenuated *S. typhimurium* vaccine strain SL326l::pYZ84 (Yan & Meyer 1996). The natural parasite host system *A. viteaeMeriones unguiculatus*, is an important animal model to study the interaction of filarial parasites with the final host (Lok & Abraham 1992). We have analysed the immune response in jirds induced by oral inoculation of SL326l::pYZ84 expressing the recombinant AVSOD2, the stability of antigen expression *in vitro* and *in vivo* was determined, and the humoral immune response of the jirds was analysed.

Prior to vaccination, the expression level of recombinant AVSOD2 was examined in the vaccine strain SL326l::pYZ84 transformed with the expression vector pLAT7 which carries the recombinant AVSOD2. SDS-PAGE analysis demonstrates the efficient expression of

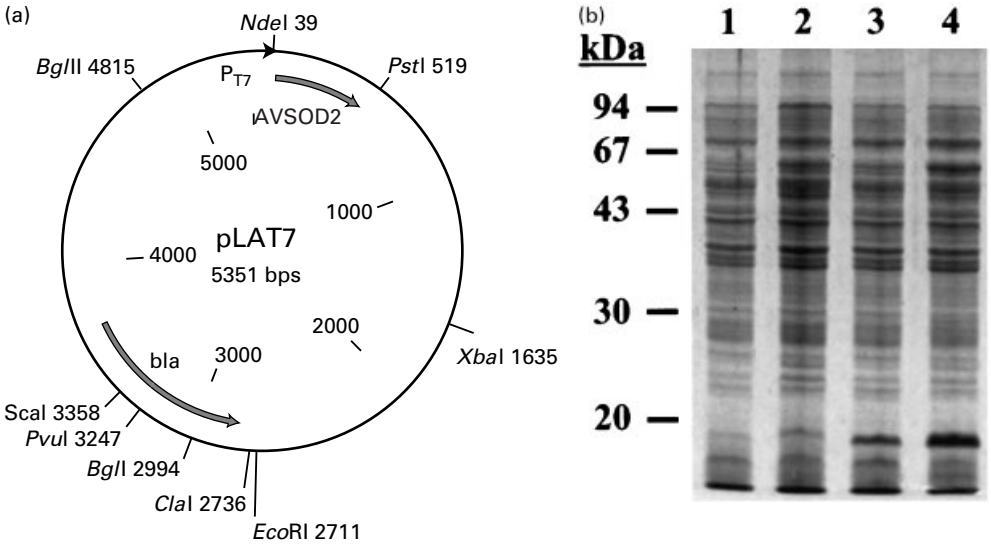

Figure 1 (a) *Construction of the expression vector for the AVSOD2*. The cDNA of the *A. viteae* AVSOD2 (Lattemann, Matzen & Apfel, in preparation) was subcloned into the expression vector pYZ97 (Yan & Meyer 1994) under control of the bacteriophage T7 promoter (P_{T7}). A fragment of the cDNA was amplified by PCR with the primers MAT2 (5′-GCGCCCCCGGGCATATGGCACGTGAAAGCAATTCTAAA-3′) and HA17 (5′-GGGCTGCAGACCAGCATTACCGGTTTTCA-3′). The sense primer MAT2 was designed to omit the putative signal peptide and carries a *NdeI* restriction site so that the cDNA could be directly fused into the start codon of pYZ97. Due to the PCR strategy which was initially chosen to amplify the AVSOD2 cDNA from total *A. viteae* cDNA, the cDNA was truncated by 42 nucleotides at the 3′-end of the coding region. (b): *Expression of recombinant AVSOD2 in* S. typhimurium *SL3261 Salmonella* strains SL3261::pYZ84 and SL3261::pYZ84/pLAT7 expressing recombinant AVSOD2 were cultivated overnight at 28°C and 37°C. Expression of recombinant AVSOD2 was determined by Coomassie stained SDS-PAGE loading equal amounts of bacteria in each lane. Lane 1: SL3261::pYZ84 (28°C); Lane 2: SL3261::pYZ84 (37°C); Lane 3: SL3261::pYZ84/pLAT7 (28°C); Lane 4: SL3261::pYZ84/pLAT7 (37°C).

the recombinant AVSOD2 migrating to 17 kDa (Figure 1). Cultivation of the bacteria at 37°C resulted in clearly enhanced expression of recombinant AVSOD2 leading to the accumulation of at least 5% of the cellular protein. Background expression of SOD is observed upon cultivation at 28°C. Next, we assessed *in vitro* the stability of the expression vector pLAT7 by passaging the vaccine strain four times for 12 h in medium without antibiotic selection, and found that 85% of the bacteria retained the construct (data not shown). To determine the *in vivo* stability of pLAT7, liver and spleen of jirds orally immunized with SL326l::pYZ84/pLAT7 were homogenized and the number of *Salmonella* recovered was determined (Figure 2). The total number of bacteria varied substantially among the individual animals, with a mean of 590 cfu in liver at day 7 which had declined to 20 cfu day 14. The spleen was colonized with an average of 14 cfu at day 7. At day 21 no bacteria could be recovered from liver or spleen, confirming the attenuation of this strain which allows safe antigen delivery. The proportion of bacteria resistant to

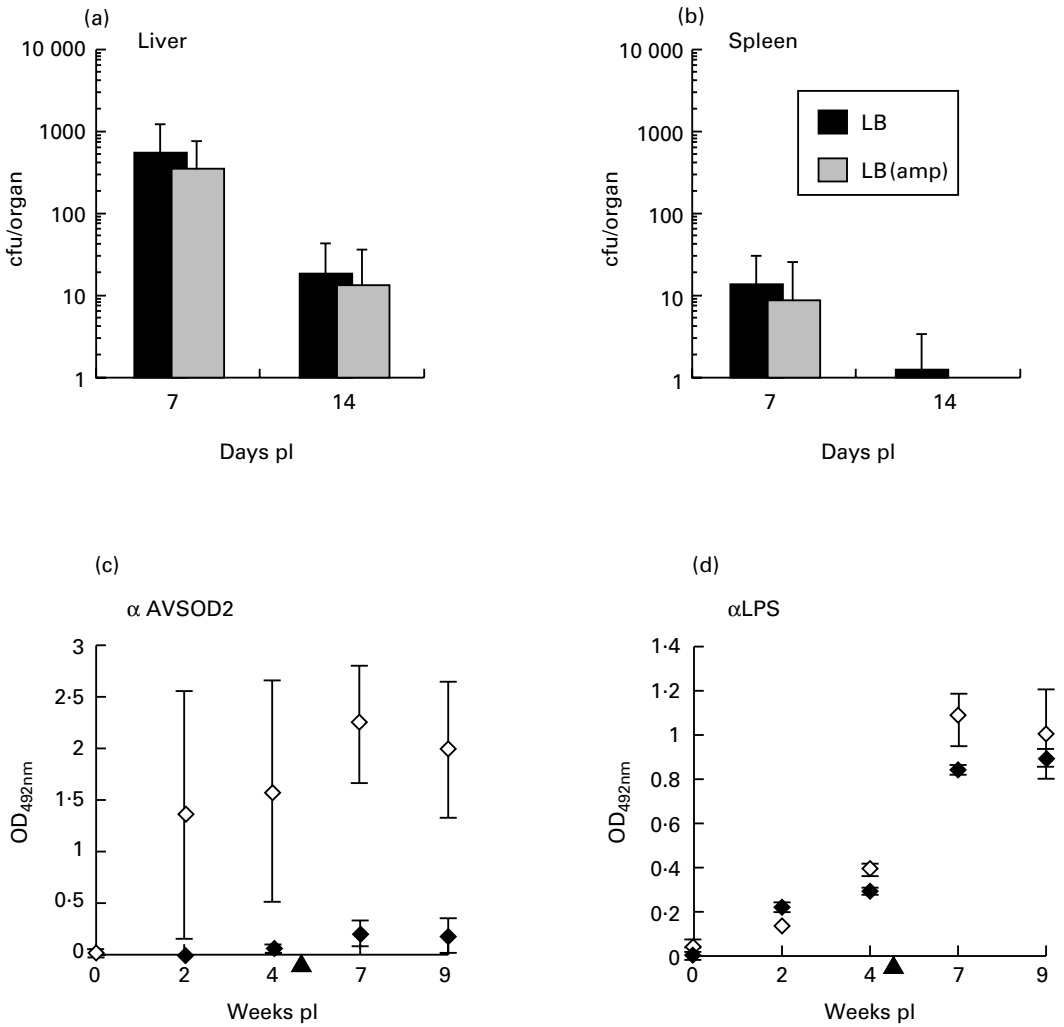

Figure 2 (a,b): *Recovery of SL3261: pYZ84/pLAT7 from liver and spleen of immunized jirds.* The organs of jirds oralIy immunized with 5×10^8 cfu SL3261::pYZS4/pLAT7 were removed at day 7 ($n=6$, two independent experiments) and at day 14 ($n=3$), homogenized and plated on LB plates with and without ampicillin. Viable cell counts were performed after cultivation overnight at 28°C. (c,d): *Vaccination of jirds with* S. Typhimurium *expressing recombinant AVSOD2.* The humoral immune response of jirds orally vaccinated with 5×10^8 cfu SL3261::pYZ84/pLAT7 (open diamonds, $n=6$), SL326l::pYZ84 (filled diamonds, $n=5$) was determined by ELISA against purified recombinant AVSOD2 (c) and *Salmonella* LPS (d) A booster immunization was performed on all jirds at week 5. Blood samples of the immunized jirds were collected at week 2, 4, 7 and 9 after the first immumization from the retroorbital venous plexus. These are representative results. One non-responding animal was excluded from the study.

ampicillin was about 70% of the total recovered at each time point of isolation. Additionally, 96% of the bacteria isolated from LB plates were resistant to ampicillin. These results demonstrate that pLAT7 is stable under *in vitro* and *in vivo* conditions and therefore is suitable for vaccination studies. The humoral immune response of jirds immunised orally with SL326l::pYZ84/pLAT7 was assessed by ELISA using a rabbit antiserum raised against jird total immunoglobulin and a goat anti rabbit peroxidase conjugate (Sigma) to detect the bound antibodies (Figure 2). After the first immunization, the immunized jirds developed a strong humoral immune response against recombinant AVSOD2, and one week after the booster immunisation this antibody response reached a maximum level before decreasing slightly at week nine. Control animals which were treated with SL3261::pYZ84 or saline produced no antibodies against AVSOD2 (Figure 2c). The antibody response against *Salmonella* LPS was similar in the groups of animals which were immunized either with SL326l::pYZ84/pLAT7 or SL3261::pYZ84, while no reaction was observed in animals which received saline (Figure 2d). Additionally, the sera of immunized jirds were analysed by Western blot against total worm homogenate of *A. viteae* and *Onchocerca volvulus*. These sera specifically recognised both the CY-SOD and the EC-SOD in both worm homogenates (data not shown).

Our results demonstrate that oral immunization with attenuated *S. typhimurium* strains expressing recombinant filarial antigens is a promising strategy for the development of anti-filarial vaccines. We have efficiently expressed the AVSOD2 in the *aroA S. typhimurium* vaccine strain SL326l::pYZ84 to generate a strain which stimulates a strong humoral immune response against the enzyme in vaccinated jirds. Only three out of 16 animals produced negligible levels of antibodies against the AVSOD2 whereas the other animals responded well to the recombinant antigen in ELISA. The immune sera were seen to specifically recognise AVSOD2 by Western blot analysis of total worm homogenate, thus confirming the results obtained in ELISA. Our results contrast the finding from natural infections of jirds with *A. viteae*, where only a small proportion of animals produce antibodies against recombinant AVSOD2 (Lattemann, Matzen & Apfel in preparation). Immunization studies with purified recombinant EC-SOD from *O. volvulus* that was applied with different adjuvants also failed to induce SOD-specific immune response in jirds (Lucius 1995). The induction of antibody response by recombinant *Salmonella* has been shown to depend on the amount of antigen produced by recombinant vaccine strains for the efficient stimulation of an immune response (Fayolle *et al*. 1994). However, overexpression of foreign antigens is often toxic for the antigen producing cell (Dong, Nilsson & Kurland 1995), and bacteria constitutively producing high levels of foreign antigen are often rapidly cleared *in vivo* by the immune system or lose the expression plasmid (Khan *et al*. 1994, Oyston *et al*. 1995, author's unpublished observations). To avoid this, we successfully utilized the natural phenomenon of phase variation to generate a heterogeneous population within the vaccine strain which guarantees the continuous formation of an antigen-expressing pool of bacteria (Yan & Meyer 1994, 1996). Due to this strategy, the *Salmonella* expressed recombinant AVSOD2 at high rates and did not lose the plasmid. Most importantly, overexpression of the recombinant AVSOD2 did not impair the invasion and colonization properties of SL326l::pYZ84/pLAT7 compared to the wild type strain, as illustrated by the similar immune response against *Salmonella* LPS in animals immunized with both strains.

The effector mechanisms of protective anti-filarial immunity are not yet understood, however, some studies underline the importance of the mode of antigen presentation for the design of anti-filarial vaccines. In experimental infection with *Brugia malayi*, mice of the H-2d haplotype do not produce antibodies against gp29, the cuticular glutathione peroxidase (Cookson *et al.* 1992). Also immunizations with parasite extract administered in incomplete Freund's adjuvant failed to induce gp29 specific immune response in this mice, while delivery of recombinant gp29 by live attenuated *Salmonella* could at least partially overcome this restriction in H-2d mice (Chacón *et al*. 1996). Chacón *et al*. contribute this effect to the different mode of antigen presentation. Despite the fact that *Salmonella* remain inside phagolysosomal compartments, infected macrophages have been shown to present antigenic peptides by both MHC class I and class II molecules (Pfeifer *et al*. 1993, Turner *et al*. 1993, Wick *et al*. 1994). Consequently, both cytotoxic and humoral immune responses are potently triggered by recombinant *Salmonella* vaccines. Furthermore, *Salmonella* are capable of inducing a broad spectrum of antibody isotypes against helminth antigens, indicating that T helper cells expressing Th1 and Th2 type cytokines are induced (Chacón *et al*. 1996, Comoy *et al.* 1997). To investigate the potential of recombinant AVSOD2 delivered by SL3261::pYZ84 to protect jirds from a challenge infection with *A. viteae*, we have performed protection studies in jirds. Preliminary results show a reduction of the worm burden of about 30% in jirds vaccinated with SL326l::pYZ84/pLAT7 in comparison to the control groups which received the non recombinant vaccine strain or saline (unpublished results). This indicates that the EC-SOD indeed is a promising vaccine candidate for an anti-filarial vaccine. Protective immunity may be further enhanced by the application of pools of *Salmonella* expressing a repertoire of anti-oxidant enzymes of filariae, and our future studies will

thus be aimed at optimizing antigen delivery of *Salmonella* by pooling filarial antigens. We also plan to express the AVSOD2 in different bacterial compartments in order to assess whether periplasmic or surface expression has beneficial effects on antigen presentation and the immune response.

ACKNOWLEDGEMENTS

The authors wish to thank Dr S. Gray-Owen for reviewing this manuscript.

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