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Protective immunity against enteral stages of *Trichinella spiralis* elicited in mice by live attenuated *Salmonella* vaccine that secretes a 30-mer parasite epitope fused to the molecular adjuvant C3d-P28

Ericka N. Pompa-Mera [a,b,c], Pablo Arroyo-Matus [a], Alicia Ocaña-Mondragón [b], César R. González-Bonilla [d], Lilián Yépez-Mulia [a,*]

[a] Unidad de Investigación en Enfermedades Infecciosas y Parasitarias, Hospital de Pediatría, Centro Médico Nacional Siglo XXI, IMSS, Mexico City, Mexico
[b] Unidad de Investigación Médica en Inmunología e Infectología, Hospital de Infectología, Centro Médico Nacional La Raza, IMSS, Mexico City, Mexico
[c] Facultad de Medicina, Universidad Nacional Autónoma de México, Mexico City, Mexico
[d] Coordinación de Vigilancia Epidemiológica, IMSS, Mexico City, Mexico

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ABSTRACT

The development of a veterinary vaccine against *T. spiralis* infection is an alternative strategy to control trichinellosis. In an effort to develop an efficient vaccine, BALB/c mice were immunized with attenuated *Salmonella enterica* serovar Typhimurium SL3261 that expresses a 30-mer peptide (Ag30) derived from the gp43 of *T. spiralis* muscle larvae fused to three copies of the molecular adjuvant P28 (Ag30-P28$_3$) and it was either displayed on the surface or secreted by recombinant *Salmonella* strains. *Salmonella* strain secreting Ag30-P28$_3$, reduced the adult worm burden 92.8% following challenge with *T. spiralis* muscle larvae compared to 42% achieved by recombinant *Salmonella* displaying Ag30-P28$_3$ on the surface. The protection induced by secreted Ag30-P28$_3$ was associated with a mixed Th1/Th2 with predominance of Th2 phenotype, which was characterized by the production of IgG1, intestinal IgA antibodies and IL-5 secretion. This finding could provide an efficient platform technology for the design of novel vaccination strategies.

1. Introduction

Trichinellosis is a worldwide zoonosis caused by nematodes of the genus *Trichinella*, which infect domestic and wild animal species and man (Cui et al., 2011; Ribicich et al., 2010). The wide geographical distribution of human trichinellosis affects both industrialized and non-industrialized countries (Pozio, 2007), and it is estimated that up to 11 million people may be infected with the parasite (Dupouy-Camet, 2000). Humans acquire *T. spiralis infection* by the ingestion of raw or undercooked meat contaminated with muscle larvae. Larvae moult to the adult stage, mate, and reproduce in the host's small intestine. The parasite's life cycle is completed when newborn larvae invade the host's striated muscle cells and differentiate to muscle larvae, transforming muscle cells into a structure called the nurse cell (Capó and Despommier, 1996).

The clinical features of trichinellosis vary from mild to severe disease, depending on several factors, such as the number and localization of the parasites and the intensity of the inflammatory reaction. Gastroenteritis, diarrhea, abdominal pain, cutaneous rash, facial and periorbital edema, fever, myalgia and persistent fatigue are the most common symptoms (Bruschi and Dupouy-Camet, 2014; Gottstein et al., 2009).The disease is associated with infiltrating inflammatory cells such as mast cells, eosinophils, monocytes, and lymphocytes. Besides, IgE production is responsible for allergic manifestations typical of trichinellosis and the presence of lactate deshidrogenase and creatinine phosphokinase in serum suggests muscle damage (Bruschi and Dupouy-Camet, 2014).

Although control of trichinellosis can be achieved with sanitary measures, such as epidemiological surveillance, adequate marketing systems, and good farming practices, the economic conditions in some developing countries prevent the implementation of these practices. In addition, the emergence of parasite resistance to traditional anthelminthic drugs has motivated the search for alternative strategies to control this zoonosis (Ortega-Pierres et al., 2000). Among these strategies, the development of a vaccine against trichinellosis has become an attractive and promising approach, especially in the veterinary field.

In the past, various efforts to develop effective vaccines against trichinellosis have included antigenic preparations such as attenuated irradiated larvae (Agye-Frempong and Catty, 1983; Nakayama et al., 1998), crude homogenate from *T. spiralis* muscle larvae and parasite synthetic peptides (McGuire et al., 2002; Robinson et al.,

* Corresponding author. Tel.: +52(55) 56276940; fax: +52(55) 56276949.
E-mail address: lilianyepez@yahoo.com (L. Yépez-Mulia).

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1995a, 1995b), and excretory–secretory (ES) products from muscle larvae (Dea-Ayuela et al., 2006). DNA vaccines coding for different muscle larvae antigens have been also explored (Tang et al., 2012; Wang et al., 2006a; Yang et al., 2010) and more recently parasite specific antigens by phage display technology (Cui et al., 2013). However, only partial protection against *T. spiralis* infection has been obtained with these approaches.

Among the most promising technologies for inducing immune protection against enteric pathogens are live *Salmonella*-based vaccine systems (Cheminay and Hensel, 2008; Galen et al., 2010; Petavy et al., 2008). We recently developed an attenuated *Salmonella enterica* serovar Typhimurium SL3261 that expressed a 30-mer peptide (named Ag30) on its surface, which was derived from *T. spiralis* gp43 (Pompa-Mera et al., 2011). This recombinant *Salmonella* vaccine (*Salmonella* pAg30) developed significant protection in mice against intestinal stage of *T. spiralis* (61.8%), producing low titres of IgG1 and IL-5. The enhancement of humoral and cellular antigen-specific immune response could contribute to enhance protection against *T. spiralis* infection. To improve the efficacy of *Salmonella* pAg30, MisL autotransporter was used instead of ShdA, in combination with an intraperitoneal boost with the recombinant protein. This vaccine induced a higher level of protection (76%) against the enteral phase of *T. spiralis* infection (Castillo-Alvarez et al., 2013).

It is well accepted that in addition to surface display, an alternative strategy to enhance the immune response to heterologous antigens is their secretion out of recombinant *Salmonella*, facilitating the exposure of recombinant antigen to antigen-presenting cells for processing (Gómez-Duarte et al., 2001; Hess et al., 1996; Spreng et al., 2006). In fact, the high immunogenicity of heterologous antigens secreted by *Salmonella* vaccines has been demonstrated (Dietrich et al., 2003; Kang and Curtiss, 2003; Kang et al., 2002).

In addition, several studies showed that the fusion of multiple copies of C3d (or its minimum binding domain, P28) to an antigen improved antigen-specific immune responses by directly interacting with complement receptor 2 (CR2/CD21) on B cells (Bower and Ross, 2006; Dempsey et al., 1996). Proteins fused to multiple copies of C3d/P28 induced a Th2 immune response characterized by high levels of IL-4 (Liu et al., 2010; Mitchell et al., 2003; Ross et al., 2001; Wang et al., 2006b).

In this study, in order to enhance the protection elicited by *Salmonella* pAg30 against *T. spiralis* infection, Ag30 was fused to three copies of P28 (Ag30-P28$_3$) and it was either expressed on the surface or secreted to the external milieu by recombinant *Salmonella* strains. The protection induced by these recombinant strains against the enteral stages of *T. spiralis* infection was evaluated. In addition, the humoral and cellular immune responses elicited by these recombinant carriers were characterized.

2. Materials and methods

2.1. Bacterial strains, plasmids, and genetic engineering

Escherichia coli DH5α (*supE44 lac169 80lacZ M15 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was used as the host strain for the construction of plasmid vectors encoding the fusion proteins of interest. Attenuated *Salmonella enterica* serovar TyphimuriumSL3261 (*aroA::*Tn*10*) (Hoiseth and Stocker, 1981) was transformed with the plasmids and used for the delivery of the recombinant proteins.

All plasmids used in this study were derived from plasmid pAg30 (3511 bp), which encodes a fusion protein (a 30-mer epitope of *T. spiralis* muscle larvae) that is translocated and expressed on the surface of *Salmonella* by the autotransporter ShdA (Pompa-Mera et al., 2011).

Three copies of the gene sequence encoding the murine CR2 binding domain of C3d (p28) corresponding to amino acids 1187–1214 (KFLTTAKDKNRWEDPGKQLYNVEATSYA) were fused to the 3′ end of the Ag30 sequence in plasmid pAg30. The construction of plasmids encoding the fusion protein Ag30-P28$_3$ was achieved as described in Sambrook and Russell (2001). Briefly, a DNA fragment encoding a single copy of a synthetic p28 gene (p28 template) was generated by PCR using oligonucleotides p28-1 and p28-2. The resultant amplicon included a linker composed of two repeats of four glycines and two serines $(G_4S_2)_2$ and a p28 sequence, as described by Bower and Ross (2006). DNA fragments p28-A, p28-B and p28-C were amplified by PCR from the p28 template using the primers shown in Table 1.

The p28-A, p28-B and p28-C sequences were cut with XhoI and HindIII, HindIII and NdeI, NdeI and SpeI restriction enzymes, respectively, and cloned into the transit vector pRL-null (Promega). Oligonucleotides Null-SEQ-1 and Null-CDS-2 were employed in a second round of PCR to obtain the p28$_3$ cassette (three tandem repeats of p28 adjuvant). It contained an upstream XbaI, downstream NheI and the BamHI restriction sites used to link with the pAg30 plasmid. The shdA gene from pAg30 plasmid was excised, purified and then reinserted into the pAg30-p28$_3$ plasmid (3943 bp) (Fig. 1). A secreted version of Ag30 or Ag30-P28$_3$ proteins was

Table 1
List of oligonucleotides used to amplify three copies of p28 and SCOT sequences.

Fragment	Name	Sense	Sequence	Restriction endonuclease[a]	Size (bp)
p28 template	p28-1	Foward	5′CGAATCTAGAGGCGGTGGCGGTAGCAGCGGCGGTGGCGGCA GCAGCAAATTCCTGACCACCGCGAAAGATAAGAACCGCTGG3′	XbaI	144
	p28-2	Reverse	5′CGCGGCTAGCACTAGTCGCATAGCTGGTCGCAACCACGAAATA CAGCTGTTTCCCCGGATCTTCCCAGCGGTTCTTATCTTTCGCGG3′	NheI, SpeI	
p28-A	p28-a	Foward	5′TATCCTCGAGGGCGGTGGCGGTAGC3′	XhoI	144
	p28-b	Reverse	5′GGCCAAGCTTCGCATAGCTGGTCG3′	HindIII	
p28-B	p28-c	Foward	5′ATAGAAGCTTGGCGGTGGCGGTAGC3′	HindIII	144
	p28-d	Reverse	5′GGCCCATATGCGCATAGCTGGTCGC3′	NdeI	
p28-C	p28-e	Foward	5′AATTCATATGCTCGAGGGCGGTGGCGGT3′	NdeI	144
	p28-f	Reverse	5′GGCAACTAGTCGCATAGCTGGTCGC3′	SpeI	
p28$_3$	Null-SEQ-1	Foward	5′TGTCTAGAACAGATCTCGAGGGCG3′	XbaI	432
(tandem repeats)	Null-CDS2	Reverse	5′AGGATCCATGCATGCTAGCCGCATAGCTGGTCGCTTCCAC3′	BamHI, NsiI, NheI	
SCOT	Scot-E1	Foward	5′CTAGAAAACGCCCGGGCGGTGGCGGTGGCAAACGCGGC	XbaI	76
	Scot-E2	Reverse	GGTGGCGGTGGCCCGAAACGCGCTAGCATGCATG3′ 5′GAT CCATGCATGCTAGCGCGTTTCGGGCCACCGCCACCGCCGC GTTTGCCACCGCCACCGCCCGGGCGTTTT3′	BamHI, NsiI, NheI	

[a] Restriction enzyme sites underlined.