Vaccine 28 (2010) 2980–2985

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Eimeria maxima recombinant Gam82 gametocyte antigen vaccine protects against coccidiosis and augments humoral and cell-mediated immunity

Seung I. Jang[a,1], Hyun S. Lillehoj[a,*], Sung Hyen Lee[a], Kyung Woo Lee[a], Myeong Seon Park[a], Sung-Rok Cha[a], Erik P. Lillehoj[b], B. Mohana Subramanian[c], R. Sriraman[c], V.A. Srinivasan[c]

[a] *Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705, USA*
[b] *Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD 21201, USA*
[c] *Indian Immunologicals Limited, Research and Development Center, Gachibowli, Hyderabad 500032, Andhra Pradesh, India*

ARTICLE INFO

Article history:
Received 18 November 2009
Received in revised form 5 February 2010
Accepted 8 February 2010
Available online 21 February 2010

Keywords:
Coccidiosis
Gam82
E. maxima

ABSTRACT

Intestinal infection with *Eimeria*, the etiologic agent of avian coccidiosis, stimulates protective immunity to subsequent colonization by the homologous parasite, while cross-protection against heterologous species is poor. As a first step toward the development of a broad specificity *Eimeria* vaccine, this study was designed to assess a purified recombinant protein from *Eimeria maxima* gametocytes (Gam82) in stimulating immunity against experimental infection with live parasites. Following Gam82 intramuscular immunization and oral parasite challenge, body weight gain, fecal oocyst output, lesion scores, serum antibody response, and cytokine production were assessed to evaluate vaccination efficacy. Animals vaccinated with Gam82 and challenged with *E. maxima* showed lower oocyst shedding and reduced intestinal pathology compared with non-vaccinated and parasite-challenged animals. Gam82 vaccination also stimulated the production of antigen-specific serum antibodies and induced greater levels of IL-2 and IL-15 mRNAs compared with non-vaccinated controls. These results demonstrate that the Gam82 recombinant protein protects against *E. maxima* and augments humoral and cell-mediated immunity.
Published by Elsevier Ltd.

1. Introduction

Avian coccidiosis is caused by multiple species of the apicomplexan protozoa *Eimeria* which invade discrete regions of the intestinal epithelium causing reduced feed conversion efficiency leading to decreased body weight gain [1]. Coccidiosis is one of the most economically costly diseases of the poultry industry, with estimated annual losses greater than three billion US dollars [2]. Prophylactic feeding of coccidiostat drugs is the major disease control method used in commercial settings. However, with increasing demands for high-protein meats and heightened consumer concerns over the use of antibiotics in poultry production, the search for alternative strategies against avian coccidiosis have intensified [1,3].

While immunization against avian coccidiosis has engendered some measure of success, the existence of numerous, antigenically distinct *Eimeria* species and their complex life cycle of asexual and sexual, as well as intracellular and extracellular stages, hinder further vaccine development [4,5]. An immunogenic *Eimeria* protein designated as 3-1E (profilin) has been shown to engender protective immunity using various immunization strategies. This protein was identified from the merozoite stage of *Eimeria acervulina* and encodes *Eimeria* profilin which is highly conserved across different stages of *Eimeria* life cycle and *Eimeria* species [6,7]. Several proteins associated with the sexual stage of *Eimeria maxima* including 14, 30, 56, 82 and 230 kDa antigens have been identified as potential vaccine targets for inducing transmission-blocking immunity [8]. Immunization with the 56 and 230 kDa antigens promoted cell-mediated immunity against experimental coccidiosis and reduced fecal oocyst shedding, an important component of the *Eimeria* life cycle [8]. Additionally, the 56 and 230 kDa antigens induced the production of antibodies that were transferred to the embryo via egg yolk [9–12]. In particular, Gam82, the 82 kDa tyrosine-rich sexual stage glycoprotein of *E. maxima*, has been implicated in oocyst wall formation [11]. These promising results led us to the hypothesis that other gametocyte proteins also may promote cell-mediated and transmission-blocking immunity against subsequent parasite infection.

Therefore, current study was undertaken to evaluate the effects of Gam82 vaccination on resistance to experimental *E. maxima* infection and to correlate disease resistance with humoral and cell-mediated immunity. In addition, we compared various aspects of host immune response to Gam82 to those induced by *Eimeria* profilin.

* Corresponding author. Tel.: +1 301 504 8771; fax: +1 301 504 5103.
E-mail address: Hyun.Lillehoj@ars.usda.gov (H.S. Lillehoj).
[1] This work was carried out during sabbatical leave from the Institute of Health and Environment, Daejeon Metropolitan City, Daejeon 305-338, Korea.

0264-410X/$ – see front matter. Published by Elsevier Ltd.
doi:10.1016/j.vaccine.2010.02.011

2. Materials and methods

2.1. Experimental animals

One-day-old Ross broiler chickens were obtained from the Longenecker's Hatchery (Elizabethtown, PA) and housed in Petersime starter brooder units in an *Eimeria*-free facility. The animals were provided with feed and water *ad libitum* and moved to larger hanging cages (2 animals/cage) until use. All protocols were approved by the Beltsville Area Institutional Animal Care and Use Committee.

2.2. Parasites

The wild type strain of *E. maxima* originally developed and maintained at the Animal and Natural Resources Institute (Beltsville, MD) was used. Oocysts were cleaned by floatation on 2.5% sodium hypochlorite, washed three times with PBS, and enumerated using a hemocytometer prior to infection.

2.3. RNA extraction and amplification of the Gam82 gene

Total RNA was isolated from intestinal scrapings of *E. maxima*-infected chickens using Trizol (Invitrogen, Carlsbad, CA) and cDNA was prepared using Thermoscript reverse transcriptase (Invitrogen) with a Gam82-specific reverse primer. The Gam82 coding sequence was amplified by PCR using Proof Start DNA polymerase (Qiagen, Valencia, CA) with the following primer sequences containing Bam HI and Not I restriction enzyme sites (underlined): forward, 5′-AGCTGGATCCACCAGCTCTGGCCAGGATCAGGTG-3′; reverse: 5′-TCTAGAGCGGCCGCTGCCCACATCTCTGATTGTTC-3′. Amplicons were cloned into the pET28a (+) plasmid vector (Novagen/EMD Chemicals, Gibbstown, NJ) downstream from an NH_2-terminal His_6 epitope tag, plasmid clones were verified by sequence analysis, and used to transform competent *Escherichia coli* BL21 Star (Invitrogen).

2.4. Bacterial expression and purification of Gam82 recombinant protein

Recombinant *E. coli* were induced for 4 h with 0.75 mM IPTG (Bangalore Genei, Bangalore, India) at OD_{600} = 0.6, the cells harvested by centrifugation, and lysed with 10 μg/ml of lysozyme (Sigma–Aldrich, St. Louis, MO) and sonication (Vibra-Cell, Sonics & Materials, Inc., Newtown, CT). The lysate was applied to a Ni^{2+}-chelating affinity column (HiTrap, GE Healthcare, Piscataway, NJ), the column was washed with PBS, pH 7.0 to remove unbound proteins, and bound proteins were eluted step-wise with PBS, pH 7.0 containing 0.5 or 1.0 M imidazole (Sigma). The eluted protein fractions were visualized on 10% SDS-acrylamide gels stained with Coomassie brilliant blue and on Western blots probed with horseradish peroxidase-conjugated anti-His monoclonal antibody (1:3000; Qiagen), and stored at −20 °C.

2.5. Gam82 immunization and parasite-challenge infection

The experimental design is illustrated in Fig. 1. At 1 wk of age, chickens (30/group) were intramuscularly immunized with 100 μl of 300 or 600 μg/ml of recombinant Gam82 in Freund's complete adjuvant (FCA). Control animals received PBS in FCA. At 1 wk post-immunization, animals were given a booster injection with 100 μl of 300 or 600 μg/ml of recombinant Gam82, 3-1E, or PBS in Freund's incomplete adjuvant. At 7 d post-secondary immunization, animals were given PBS or 2.0×10^4 *E. maxima* sporulated oocysts by oral gavage using an 18-gauge needle. Body weight gains were determined between 0 and 10 d post-infection. Fecal oocysts numbers were determined between 5 and 10 d post-infection using a McMaster counting chamber as described [13]. Lesion scores were determined at 6 d post-infection on a scale of 0 (none) to 4 (high) in a blinded fashion by two independent observers as described by Johnson and Reid [14].

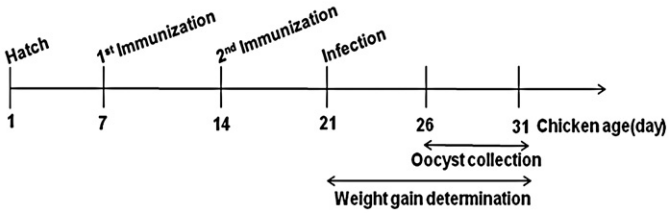

Fig. 1. Schematic outline of the experimental design.

2.6. Anti-Gam82 antibody response

Blood was collected by venipuncture from the wing vein at 7 d following the primary and secondary immunizations, clotted for 4 h at 4 °C, sera were collected by centrifugation, and anti-Gam82 antibodies were measured by ELISA [15]. Microtiter plates were coated overnight with 10 μg/well of purified Gam82 recombinant protein, washed with PBS containing 0.05% Tween 20, and blocked with PBS containing 1% BSA (Sigma). Diluted sera were added, incubated for 4 h with continuous shaking, the plates were washed, and bound antibody detected with horseradish peroxidase-conjugated rabbit anti-chicken IgG antibody (Sigma) and peroxidase-specific substrate. Optical density at 450 nm (OD_{450}) was determined with a microplate reader (Bio-Rad, Richmond, CA).

2.7. Cytokine quantitative RT-PCR

Intestinal tissues were collected between the jejunum and the ileum at 3, 6, and 10 d post-secondary immunization, and total RNA was isolated from the intestinal tissues using Trizol™ reagent (Invitrogen, USA). Five micrograms of total RNA from each sample were incubated for 15 min at room temperature with 1.0 U of DNase I and 1.0 μl of 10× reaction buffer (Sigma), 1.0 μl of stop solution was added, and the mixture was heated for 10 min at 70 °C. RNAs were reverse-transcribed using the Affinity Script Multiple Temperature cDNA Synthesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. PCR amplification and detection were preformed using the Mx3000P system and Brilliant SYBR Green QPCR master mix (Stratagene) using oligonucleotide primers for interleukin-2 (IL-2) and IL-15 (Table 1) as described [16,17]. Standard curves were generated using $\log_{10}$ diluted standard RNA and the levels of individual transcripts were normalized to those of GAPDH using the Q-gene program [18]. Each analysis was performed in triplicate. To normalize RNA levels between samples within an experiment, the mean threshold cycle (C_t) values for the amplification products were calculated by pooling values from all samples in that experiment.

2.8. Statistical analysis

All data were expressed as mean ± S.D. values and subjected to one-way analysis of variance using SPSS software (SPSS 15.0 for Windows, Chicago, IL). Duncan's multiple range test was used to analyze differences between the mean values. Differences were considered statistically significant at $P < 0.05$.