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RESEARCH ARTICLE

Molecular and Immunogenic Properties of Recombinant Falgellin of Salmonella Typhimurium DT104

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Abstract

Molecular properties of recombinant flagellin from *Salmonella* Typhimurium DT-104 were characterized. To assess the immunogenicity of the recombinant flagellin, doses of 30 micrograms of the recombinant flagellin in 100 µl of the flagellin in PBS emulsified in equal volume of adjuvant, were inoculated into each of 10 BALB/c mice while a similar group of mice were injected with the same solution but without the flagellin protein, as a control. Three injections were administered into each mouse and blood was collected before each injection to determine the anti-flagellin serum titers. It was concluded that the recombinant flagellin has a good immunogenic properties. The relative protective effect of the anti-flagellin antibodies was assessed by subjecting the immunized and the control mice to lethal doses of the parent S. Typhimurium. A larger number of the immunized mice survived the challenge dose of S. Typhimurium than the control mice suggesting the protective value of the recombinant flagellin as a putative vaccine.

INTRODUCTION

Salmonella Typhimurium DT104 is recognized among the most serious emerging bacterial pathogens during the last two decades causing serious infections and mortalities among humans and animals (1). Human infection with *Salmonella* is commonly caused by ingestion of contaminated water and foods of animal origins such as cattle, poultry, swine, and eggs (2, 3). Livestock can be asymptomatic carriers of infections, making diagnosis difficult in live animals and adding to potential cross contamination during food harvest (4). Current treatments for S. enterica infections are insufficiently effective, therefore there is a need to develop effective vaccines (5). Vaccines against salmonellosis can be divided into three groups: whole-cell killed, live attenuated, and subunit vaccines. Subunit vaccines containing outer membrane proteins, porins, fimbriae, flagellin, and cell extracts have been tested in experimental animal models including poultry, mice, rabbits and calves (6). Flagellin has a number of advantages that make it an attractive candidate for use in *Salmonella* vaccines. It is effective at very low doses (7), does not induce IgE responses, prior immunity to flagellin does not impair its adjuvant activity (8). Bergman et al., (9) demonstrated that both innate (TLR 5) and adaptive (T-cell receptor) immune responses target the most conserved flagellin domains that are required for protein function. Host immune system uses pathogen-associated molecule patterns (PAMPs) to detect infection. These molecules can be found commonly throughout the sequence of the flagellin protein that composes the eleven filaments of the flagellum (10). There are over 494 amino acid sequences and domains known for Salmonella flagella. Researchers have found much homology in particular regions of the flagella that are highly conserved among *Salmonella* serovars, both the amino and carboxy termini of the flagellar sequence are conserved, while the central portion is often more varied (11). An additional series of studies investigated the use of a synthetic fliC polypeptide as a subunit vaccine when administered with an adjuvant (12,

13). The aim of this study was to evaluate the immunogenicity of recombinant flagellin (r-fliC) as a putative vaccine in BALB/c mice.

Materials and Methods

Bacterial strains and growth conditions:

Luria-bertani (LB) media for bacterial culture were purchased from ISC Bio Express (USA). TaqDNA polymerase enzyme, dNTP(HotstarTaq® plus PCR Master mix) (Qiagen/USA) restriction enzyme(Bio Labs/England), T4 DNA ligase enzyme (Quick ligation kit) (New England Bio Labs /England), DNA size marker was obtained from Invitrogen /USA and protein size marker was acquired from BioRad (USA).The oligonucleotide primers were synthesized by Integrated and technologies (IDT/USA). Ni2+-nitrilotriacetate (Ni2+-NTA) resin was obtained from Qiagen Inc. (Valencia, CA, USA). Reagents for polyacrylamide electrophoresis such as acrylamide, bis-acrylamide, ammonium persulfate and TEMED were obtained from Sigma/USA. *Salmonella* typhimurium DT104 is a clinical isolate from a human case of gastroenteritis from a survey conducted by Dr. Saeed. E. coli DH5α and E. coli SG13009 (Qiagen/USA).Plasmid PQE30 as expression vector was purchased from Qiagen/USA. Recombinant protein was produced by inoculation 20 ml of overnight culture (LB with 100 µg/ml ampicillin) with the recombinant E. coli carrying the flagellin genes of S. Typhimurium DT-104. Inoculated medium was incubated at 37C in a shaker incubator set at 160 rpm. Expression of the recombinant gene was induced by 0.5 mM for 3 hours using **(**The QIAexpressioist™ kit/ Qiagen/USA**).**

Recombinant Flagellin Production and Purification

Primers were designed for the PCR amplification of the fliC gene from DNA extract of the *Salmonella* Typhimurium DT104. The amplified fliC gene was enzymatically cut at specific sites. Same enzymes were used to cut the vector pQE30 and the two components were ligated using T4 DNA ligase. Recombinant fliC was cloned into a competent E. coli (SG13009 [pRP4] component of "Qiagen Expressionist Kit".

Recombinant flagellin gene was cloned and expressed as Histidine-tagged protein in the bacterial expression system. Briefly, recombinant protein was produced by inoculation 20 ml of overnight culture (LB with 100 µg/ml ampicillin) with the recombinant E. coli carrying flagellin genes of S. Typhimurium DT-104. Inoculated medium was incubated at 37C in a shaker incubator set at 160 rpm. Expression of the recombinant gene was induced induced by 0.5 mM of IPTG for 3 hours during incubation at 37C and shaking at 160 rpm. Recombinant flagellin (r-fliC) was purified by (Ni-NTA) affinity chromatography, which was obtained from Qiagen Company as "QIA expressionist" kit. Cell pellet fraction was re-suspended in 15 ml denaturing buffer containing 100 mM NaH_2PO_4, 10mM Tris base and 8 M urea (pH 8.0) and was lysed by gentle vortexing at room temperature until the solution became translucent. The cell lysate was centrifuged at 12000 rpm for 20 minutes at 4^oC to pellet the cellular debris. The recombinant protein in the supernatant was purified by Ni-NTA chromatography under denaturing conditions according to the "QIA expressionist" kit manufacturer's directions (Qiagen). After unbound proteins were washed from the column, the His-tagged, recombinant protein was recovered by elution buffer containing 100 mM NaH_2PO_4, 10 mM Tris base and 8 M urea (pH4.5). QIA expressionist" kit was used to check if the recombinant protein expressed in the soluble or insoluble form. While the purity of the expressed proteins was verified by analysis of the product on (10%) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) along with protein molecular mass marker. Proteins were visualized by staining with Coomassie blue G-250. The eluted protein was dialyzed against distilled water overnight at 4^oC to remove the urea before freeze drying. The protein concentration was measured by Bradford method (14).

Animals

Michigan State University institutional animal care and use committee (IACUC) oversee the laboratory animal housing and management which follow the guidelines of the National Institute of Health (NIH) and the United State Department of Agriculture (USDA) and are subject to periodical inspection by these federal agencies. Approval from the IACUC was obtained before twenty, 6-8 weeks old BALB/c mice, were purchased from Charles River Laboratories (Boston, MA, USA). Mice were females between 6-8 weeks of age for easier handling. The mice were marked on the margins of their ears with numbers from 1-10 and kept at the University Laboratory Animal Resources (ULAR) facility during the 10 weeks of vaccination schedule under a standard laboratory conditions. Mice were kept in cages and provided with balanced pelleted mouse diet food (Ralston Purina) which meets or exceed the NRC (National Research Council) requirements. Food and water were provided ad libitum. Mice were moved to the University infectious disease containment facility during the challenge experiment.

Immunization of mice

Total of 20 mice were divided into two groups (10 mice/group). Each mouse in the first group was injected subcutaneously at the neck region with 0.1 ml of the Complete Freund's adjuvant-flagellin emulsion in volume that contained 30 µg of recombinant flagellin. Three weeks later, every mouse was inoculated with similar dose of recombinant flagellin but emulsified in incomplete Freund's adjuvant. Three boosters were given before the LD50 challenge was performed. Mice in the second group were injected at the same time as the mice in the first group with the similar emulsion but without the flagellin protein as a control. Base line blood samples were obtained before immunization and at 3 week intervals for serological tests.

Determination of antibody titer by ELISA

The mice were bled prior to the immunization for a baseline measurement of the recombinant flagellin antibody and 7days after the primary immunization and each booster from the saphenous vein. Blood was allowed to coagulate for several hours at 37°C before placing the tubes at 4°C at a walking cold room to allow effective shrinkage of the clot before centrifugation at 3000 rpm for 3 minutes to collect the serum. Sera were kept at -20°C till used for ELISA measurement of the recombinant flagellin - specific antibody.

ELISA protocol:

Carbonate-bicarbonate buffer (0.05 M), pH 9.6 at 25°C was used to dissolve the freeze-dried recombinant flagellin to produce 2 µg protein per 0.1ml of the solution that was added to each well of the ELISA plates. The plate was incubated at 4°C overnight. To minimize non-specific binding of the antibody and other reagents to the walls of the plate, solution of 0.5% BSA in PBS-that contains 0.05 Tween 20 was used to block uncovered areas of the plate wells. Plates were incubated for 45minutes at 37C before several washings with PBS-that contains 0.05 Tween 20. One tenth milliliter of 1:1000 dilution of each tested serum samples in .5% BSA in PBS-that contains 0.05 Tween 20 was distributed into the ELISA plates in triplicates. Plates were then incubated for 45 minutes at 37C before washing three times with 0.5% BSA in PBS-that contains 0.05 Tween 20 to remove nonspecifically bound reagents. The detection of bound immunoglobulin was achieved using alkaline phosphate-tagged goat anti-mouse IgG antibody (Sigma/ USA) used as per the manufacturer's instruction. Plates were again incubated at 37C for 45minutes before similar washing cycle was performed. Finally, the Alkaline phosphate enzyme substrate p-Nitrophenyl Phosphate (p-NPP), Sigma product # N-9389, in 5mg tablets, was dissolved in 10% diethanolamine buffer to form a 1 mg p-NPP/1ml buffer, were added to each well, left at room temperature for 20 minutes. Reaction was stopped by adding 50 µl of 3 M NaOH solution to each well and plates were then read at a 405 nm in a Molecular devices ThermoMax® plate reader.

Preparation of recombinant flagellin immunogene

Protein concentration of the recombinant flagellin was measured by Bradford method (14). The preparation was filter-sterilized by passing through a sterile 0.22 µm filter before mixing with Complete Freund's adjuvant. Equal volumes of sterile flagellin in normal saline solution e and Complete Freund's adjuvant were emulsified using a Polytron 3000 mixer set at 14,000 rpm in a small vial dipped into crushed ice to minimize the heat generated by the fast prob. Mixing continued until true oil in water emulsion was produced by testing a drop of the emulsified product and demonstrating that it stays undispersed when dropped into water. A volume of the adjuvant flagellin that contains 30µg of protein was adjusted to be in 100 µl of the injected material per each mouse. Three doses of the adjuvant flagellin were given to each mouse :the Complete Freund's adjuvant in the first dose but in the 2nd and 3rd doses were emulsified into Incomplete Freund's adjuvant at 3 weeks interval between the primary immunizing dose and the first and second booster doses.

Challenge studies

Different dilutions of live parent strain of S. Typhimurium were produced after the strain was grown overnight in nutrient broth at 37°C in an incubator shaker set at 200 rpm. The culture was held for 24 hr at 4°C while viable counts were determined by plating. Broth cultures were then diluted in sterile saline and used for challenge via Intraperitonial inoculation. It was calculated that the overnight culture had 100^9 million cfu/ml of the broth. Dilutions were made to produce several levels of the LD50 doses before Intraperitonial inoculation of the immunized and control mice (15, 16). After 10 days of the last immunization dose, five immunized mice and five non-immunized controls were challenged IP with 0.1 ml of 100,000 cfu/ml of the live parent strain of S.

Typhimurium which approximately equals to 100 LD50 for this serotype. Another 5 immunized mice and 5 nonimmunized mice were challenged with 0.1 ml of 1000,000 cfu/ml of the strain which is approximately equal to 1,000 LD50 dose. Mice were placed in cages that minimize environmental contamination at the Infectious Disease Containment facility, Michigan State University. The caged mice were observed 3 times/day for the duration of the experiment. Dead mice were removed from the cages as soon as they were detected.

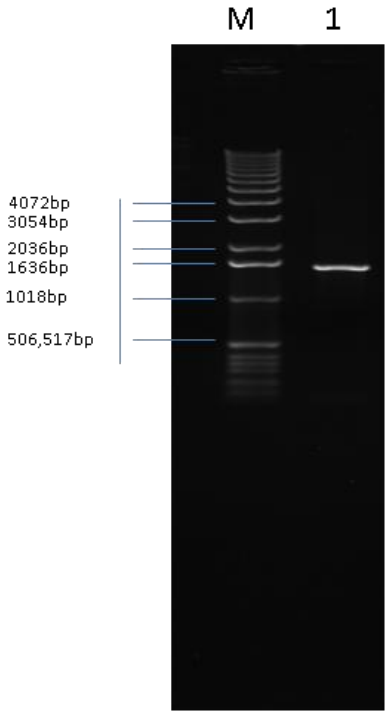
Statistical analysis

Significance of the differences in survival between the immunized and the control mice regarding their response to the challenge experiment was determined using Fisher's exact test. Differences associated with a p-value of < 0.00039 were regarded as significant (17).

Results and Discussion

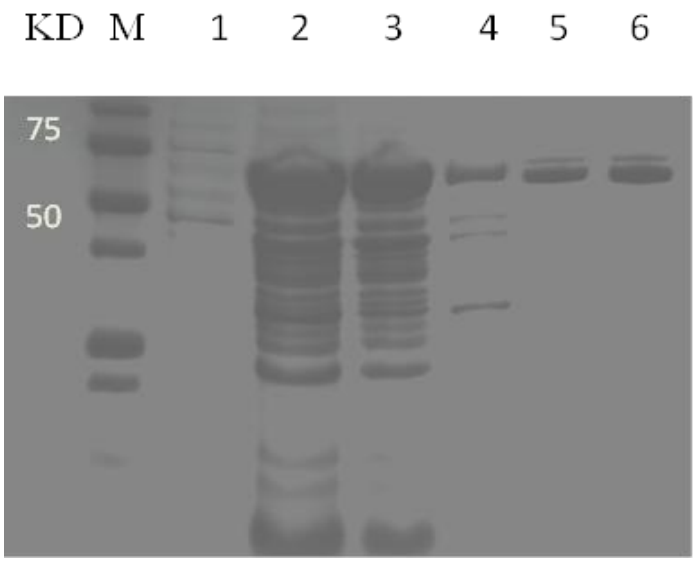
Expression, and purification of recombinant flagellin

The full coding sequence of fliC of S. Typhimurium DT104 was amplified from genomic DNA and the specific primers were used to amplify the entire gene, which yielded PCR product of approximately 1485 bp (Figure. 1).



(Fig. 1): Electrophoresis of PCR product on agarose gel (1% w/v).Lane 1: expected band a fliC approximately 1485bp. Lane M: 10 Kb DNA size markers.

The purified PCR products of S. Typhimurium DT104 that were submitted to the Research Technology Support Facility (RTSF) at Michigan State University for sequencing were found to be identical to the fliC sequence of (NCBI Accession No: M 11332). The gene was inserted into pQE30 expression vector was then cloned into E. coli SG 13009 (pRP4). Expression of the recombinant protein was induced by 0.5 mM of IPTG for 3 hours during incubation at 37C. The expressed of protein was purified by (Ni-NTA) affinity chromatography under denaturing conditions. SDS-PAGE was carried out on 10% acrylamide. Analysis of the purified protein shows a single band of approximately 56 kDa (Figure. 2).

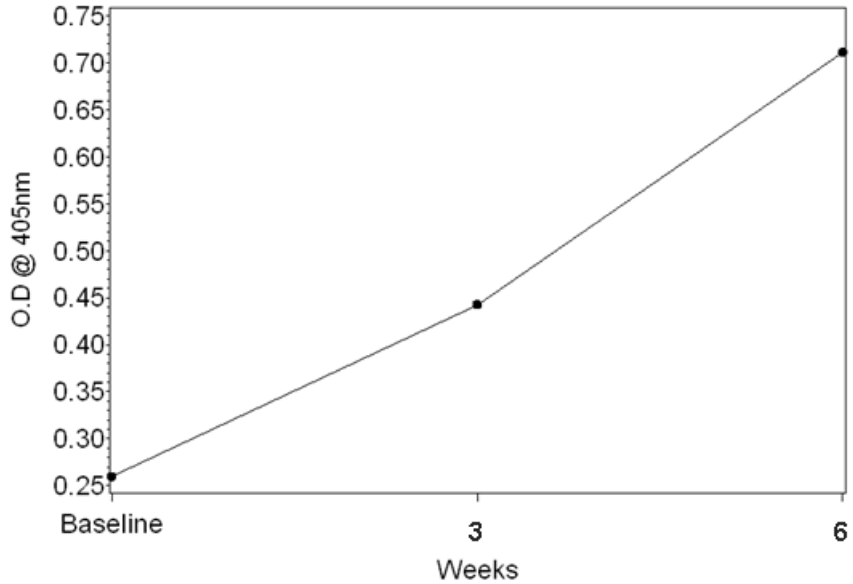


(Fig. 2): SDS- PAGE (10% w/v): profile for expression and purification under denaturing condition of flagellin gene in E.coli SG13009 strain at 200 volts for 40 min. M: BIO RAD protein ladder 250 KD. Lane (1): Culture prior to induction with IPTG. Lane (2): Cell lysate induced with IPTG. Lane (3): Flow- through. Lane (4): Wash with buffer C. Lane (5): Eluated 1 (purified recombinant flagellin). Lane (6): Eluated 2 (purified recombinant flagellin).

The eluted protein was dialyzed against distilled water overnight at 4^{o}C to remove the urea, and then the protein was filter-sterilized, freeze-dried, and stored at -80C. The reagent was reconstituted before using for immunization of BALB/c mice.

ELISA Measurement of the flagellin-specific Antibody in Sera of the Vaccinated Mice

The results of the ELISA testing of the sera from the immunized mice during the 9 weeks of immunization are depicted in fig (3).

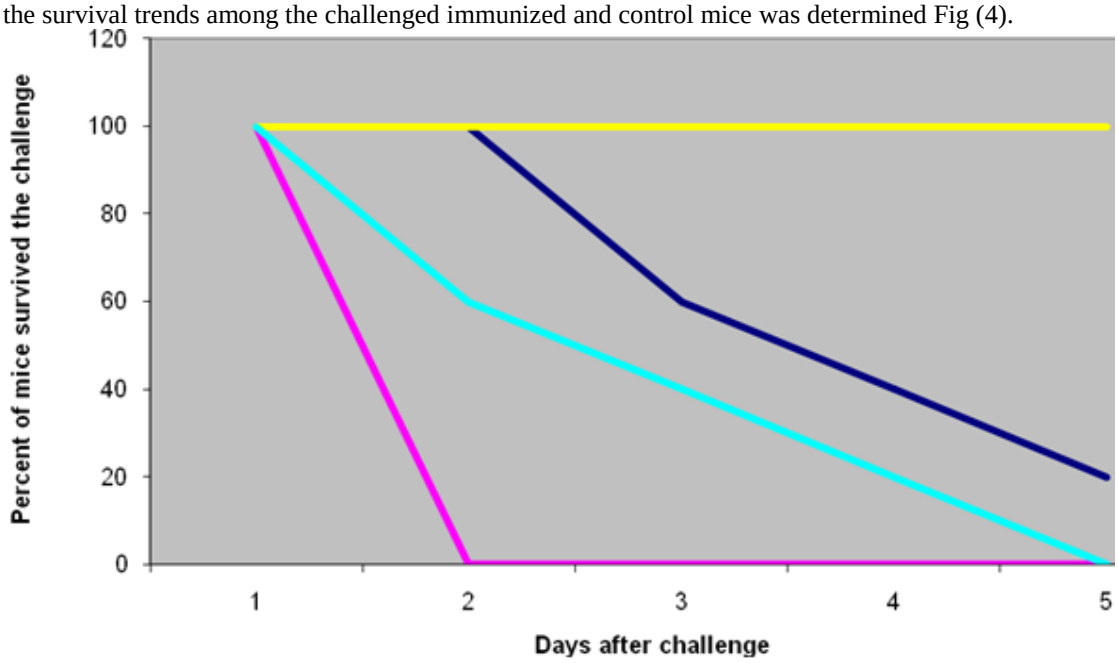


(Fig. 3): Serum Immune response to Immunoglobulin G (IgG) of BALB/c mice immunized with recombinant Salmonella flagellin. Optical density was measured at 405 nm using (ELISA). Sera were screened at a single dilution of 1:100**.**

The figure showed that 3 points which represent the mean of the triplicate values of the optical density measured at 405 nm for sera from the 10 immunized mice. The results suggested a rise in the recombinant flagellin-specific IgG serum antibodies by the 3rd week post immunization from an optical density of 0.264 to 0.439. After the 3rd week booster, a progressive rise in the ELISA titer to an optical density of 0.711 was measured by ELISA at 6 weeks post immunization. This suggests that the adjuvanted recombinant flagellin injected into the BALB/ c mice has good immunogenic properties. Sera obtained from the control mice that were injected with the same material without flagellin did not reveal any significant level of the anti-flagellin antibody.

Challenge Experiment

To demonstrate the significance of survival differences between the immunized and control mice, a comparison of the survival trends among the challenged immunized and control mice was determined Fig (4).



(Fig. 4): Shows the patterns of survival of flagellin-immunized and control mice challenged with 100LD50 and1000LD50 doses of S. Typhimurium DT104. The overall survival of the immunized mice compared to the control mice throughout the challenge experiment suggest the protective value of the flagellin among the immunized mice in comparison to the control mice. **Yellow line**: immunized mice challenged with 100 LD50 dose**, Blue line:** Control mice challenged with 100 LD50 dose, **Sky blue line:** Immunized mice challenged with 1000 LD50 dose, **Red line:** Control mice challenged with 1000 LD50 dose**.**

The patterns shows of survival of flagellin-immunized and control mice challenged with 100LD50 and1000LD50 doses of S. Typhimurium DT104. Immunized mice challenged with 100LD50 dose of S. Typhimurium survived for five days after the challenge, whereas two of the control mice challenged with the same dose died on the 2nd day. The other 3 mice died on the 3rd, 4th, and 5th days after the challenge with the same dose (100 LD 50). After challenging other groups of immunized and control mice (five of each) with 1000LD50 dose of S. Typhimurium DT104, all the control mice (100%) and 2 (40%) of the immunized mice died by the second day after challenge. All of the immunized mice died by the 5th day after the challenge. Immunized mice survived significantly for longer times than the control mice after challenge with the low and high LD50 doses of the S. Typhimurium (Fisher exact test P-value < 0.00039). Previous studies on phase 1 flagellin gene (fliC) in strains of five Salmonella serovars demonstrated that the central region exhibits only 21-32% nucleotide sequence similarity between serovars, although there was virtual sequence identity at each end of the gene (18, 19). In S. Typhimurium, the central region has been shown to code for the major epitope of the I antigen of phase 1 flagellin, and the terminal regions of the coding sequence of the gene have been identified as being important for the polymerization and secretion of flagellin (20,21). The amino acid sequences alignment of flagellins from various Gram negative bacteria shows that the C and

N termini are well conserved (22, 11). The overall survival of the immunized mice compared to the control mice throughout the challenge experiment suggest the protective value of the flagellin among the immunized mice in comparison to the control mice. Most of the reported challenge experiments used a significantly lower challenge LD50 (15, 16). However, due to the limited number of mice approved by Institutional Animal Care and Use Committee (IACUC), it was designed the challenge experiment using an expectedly higher LD50 to capture the difference in resistance between the immunized and the control mice to the challenge. The importance of vaccination in the control of infectious diseases is unquestionable (23). In a study comparing both systemic and mucosal immune responses of mice after parenteral, intranasal, or oral vaccination with purified flagellin proteins in various forms, it was found that flagellin was capable of inducing both a high humoral response and significant mucosal response post-challenge, regardless of what adjuvant or administration route was used (24). Recombinant vaccines that incorporate an epitope into flagellin have also been investigated. Massis et al., (25) fused heterologous antigens to Salmonella flagellin. In this system of vaccination, the antigens were incorporated into the flagellin and expressed on the cell surface as the flagella is formed and does not severely impact the motility or tissue colonization ability of the organism. The results of this study suggest that a significant elevation in the immune response in mice immunized with the recombinant flagellin 2-6 weeks after the immunization schedule are due to the immune-dominant role of flagellin on Salmonella organisms. Additionally, these results are consistent with the observations reported by McSorley et al., (26) who identified that CD4+ T-cell respond to flagellin at two and four week intervals and that flagellin was the major target of the immune system during primary infection in mice. The reported results along with the results of this study are consistent with the protection of the vaccinated mice against challenge with Salmonella infection.

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