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ORIGINAL ARTICLE

The molecular adjuvant mC3d enhances the immunogenicity of FimA from type I fimbriae of *Salmonella enterica* serovar Enteritidis

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Background: The fimbriae of *Salmonella enterica* serovar Enteritidis are used for colonization and invasion into host cells, and have drawn considerable interest because fimbriae can serve as potential immunogens against many pathogenic bacteria that colonize on epithelial surfaces. The purpose of the study is to use a molecular adjuvant, C3d, to enhance the immunogenicity of FimA proteins against *Salmonella enterica* serovar Enteritidis.
Methods: FimA of type I fimbriae from *Salmonella enteritidis* and FimA with one copy of m*C3d*, two copies of m$C3d_2$ and three copies of m$C3d_3$ were cloned into the expression vector pCold-TF. Soluble fusion proteins of FimA with different copy of *mC3d* were induced by IPTG and expressed into *Escherichia coli* BL21 (DE3). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed that the recombinant proteins from pCold-TF-fimA, TF-fimA-*mC3d*, TF-fimA-$mC3d_2$, TF-fimA-$mC3d_3$ were 70 kDa, 100 kDa, 130 kDa and 160 kDa, respectively. The fusion protein was recognized by rabbit anti-fimbriae polyclonal antibodies, and then visualized by goat anti-rabbit polyclonal antibodies with a chrome appearance by enzyme-subtract interaction. The recombinant proteins were purified by Ni-TED (tris-carboxymethyl ethylene diamine), immobilized metal ion affinity chromatography (IMAC). Balb/c mice were subcutaneously immunized with the purified proteins and the immune response was monitored by an enzyme-linked immunosorbent assay (ELISA) for FimA-specific antibody. The immunized mice were challenged with a 10-fold LD_{50} dose (i.e., 100 CFU) of *Salmonella enterica* serovar Enteritidis standard strain (SD-2) 1 week after the second immunization.

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Results: The immunized mice with the fusion proteins FimA-mC3d$_2$ and FimA-mC3d$_3$ had increased levels of ELISA titer of antibody that were 2 and 4 logs, respectively, more immunogenic than the TF-FimA protein alone. The challenge results showed that immune protection rate in the mice immunized with 10 μg of FimA, FimA-mC3d$_2$, and FimA-mC3d$_3$ were 50%, 75% and 100%, respectively.
Conclusion: We conclude that mC3d can be expressed in a prokaryotic vector and enhance the immune response of the recombinant protein. FimA-mC3d$_3$ is potentially a subunit vaccine against *S. enterica* serovar Enteritidis infection.

Introduction

The prevalence of *Salmonella enterica* serovar Enteritidis has dramatically increased worldwide; it is reportedly the most common serotype in the United States. Approximately 1000 people die of the infection each year.[1] Fimbriae are used for colonization and invasion into host cells. They have drawn considerable interest because the fimbriae are potential immunogens against many pathogenic bacteria that colonize epithelial surfaces.[2] The main fimbriae of *S. enterica* serovar Enteritidis are SEF14, SEF17, and SEF21, which are composed of SefA, AgfA, and FimA fimbrial proteins, respectively.[3] Because of fimbriae structure and localization, they are excellent targets for the host immunological system. Fimbria-based vaccines are hypothesized to protect the host against the adherence of pathogens by blocking the organisms from attaching to the intestinal mucosa.[4] Scientific interest has recently focused on effector molecules generated by the innate immune response and on their role in shaping acquired immunity.[5] A recombinant protein consisting of an antigen fused to C3d may elicit a more robust immune response than the antigen alone. Dempsey et al.[6] demonstrated that a recombinant protein containing three copies of C3d attached to the carboxy terminus of hen egg lysozyme (HEL) could elicit a primary immune response is 10,000-fold greater with the modified HEL protein, compared to the unmodified HEL protein. Similar results were achieved by conjugating C3d to viral, bacterial, parasitic, and cellular (i.e., self) antigens.[7,8] Three repeats of C3d conjugated to a soluble trimeric form of the HIV-1 Env was more effective at inducing neutralizing antibodies to primary isolates than plasmids encoding for non-C3d conjugated Env glycoproteins.[9,10] Purified E2 proteins fused to murine and bovine homolog C3d3 were 10,000 more immunogenic than E2 alone or anti-E2 antibodies neutralized virus infection.[8,10] This immunization mode demonstrated the ability of C3d to enhance secondary humoral (i.e., IgG) immune responses and the maturation of antibody avidity, and the ability of the antibody to neutralize infection and stimulate B-cell proliferation.[11,12] In previous studies, mC3d conjugated to prokaryote antigen was expressed on a cell culture, whereas in the present study mC3d conjugated to prokaryote antigen was clones into a pCold-TF expression vector and successfully expressed in *Escherichia coli* BL21(DE3). We therefore used the active C3d as a molecular adjuvant to enhance the immunogenicity of FimA proteins against *Salmonella enterica* serovar Enteritidis. This will help in the design of more efficient vaccines against *Salmonella enterica* serovar Enteritidis.

Materials and methods

Bacterial growth and DNA extraction

The *Salmonella enterica* serovar Enteritidis were cultured overnight at 37°C with vigorous agitation. The DNA then was extracted. The FimA gene was amplified by an upper primer with the NdeI restriction enzyme site: 5′-CGC CATATG AAA CAT AAA TTA ATG ACC TCT A-3′ and a lower primer with the NotI and BamHI restriction enzymes site: 5′-TCG GCG GCC GCG GAT CCT TCG TAT TTC ATG ATA AAG GTG-3′. Polymerase chain reaction (PCR) was performed in a volume of 25 μL that contained 2.5 μL of the DNA template, 1.5 μL of 2.5 mM dNTP mixture, 2.5 μL of 10 × PCR buffer, 1 μL upper primer, 0.5 μL lower primer, 0.5 μL Ex Taq polymerase, and 16.5 μL sterilized distilled water. The PCR conditions were an initial denaturing at 94°C for 4 minutes, followed by 30 cycles at 94°C for 1 minute. The product was annealed at 56°C for 1 minute and extended at 72°C for 1 minute. The PCR product was finally extended at 72°C for 10 minutes. The 550 bp of the FimA gene was excised from the gel and purified using DNA purification kit. The purified product was then cloned into the PMD18-T simple vector and transformed into *Escherischia coli* DH5a-competent cells. Recombinant plasmids were extracted and analyzed by restriction enzyme digestion and sequencing.

Construction of recombinant protein

The recombinant plasmids PMD18-T-FimA and the expression vector pCold TF were digested with NdeI and BamHI restriction enzymes. The FimA gene and pCold TF expression vector both recovered from the gel in the agarose gel DNA purification kit. Purified FimA DNA was cloned into pCold-TF expression vector. The recombinant FimA-pCold-TF was transformed into the *E. coli* DH5a-competent cells; the positive clones were selected and determined by restriction enzyme analysis with NdeI and BamHI.

The clones of C3d into the pUC plasmid were generated according to the strategy of Dempsey et al[6] and were a gift

from Drs Leonard J Bello and Lingshu Wang of the University of Pennsylvania (Philadelphia, PA). Each C3d was connected with a linker sequence encoding glycine/serine repeats, GS (G4S)2 GS, in which potential proteolytic cleavage sites were mutated by BamH1 and BglII fusion to mutate an arginine to a glycine. The plasmids were digested with BamHI and BglII to release the BLc3d, BLc3d2, and BLc3d3 fragments. The clones positive for pCold-TF-fimA were similarly digested with BamHI. The digested products were recovered from the gel by using an agarose gel DNA purification kit. The BLc3d, BLc3d2, and BLc3d3 fragments were then respectively cloned into the pCold-TF-fimA. The recombinant protein was transformed into *E. coli* DH5a-competent cells, and the positive clones were selected and determined by PCR and restriction enzyme analysis with NdeI and BamHI.

Recombinant protein expression

Two microliters of pCold-TF-fimA, pCold-TF-fimA-BLc3d, pCold-TF-fimA-BLc3d2, and pCold-TF-fimA-BLc3d3 plasmid were used to transform 200 μL *E. coli* BL21(DE3). The *E. coli* BL21(DE3) carrying pCold-TF-fimA-BLc3d was grown on LB plates containing 100 μg/mL ampicillin at 37°C overnight. The single clone was grown in 5 mL LB broth plus ampicillin (50 μg/mL) at 37°C to OD600 of 0.4—0.5. The culture was refrigerated at 15°C and allowed to stand for 30 minutes. The culture was then split into two aliquots. One aliquot was induced by IPTG to a final concentration of 0.1 mmol/L at 15°C for 24 hours and the other aliquot was not induced (i.e., the control). Harvested cells harvested were lysed by sonication and subjected to 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1A).The protein on 8% SDS-PAGE was transferred on a nitrocellulose membrane (0.35 mA/cm^2).The fusion protein was blocked with 10% nonfat dry milk and recognized by rabbit anti-fimbriae polyclonal antibodies (1:100), and later visualized by goat anti-rabbit polyclonal antibodies (1:1500) with a chrome appearance by enzyme-subtract interaction (Fig. 1B). The recombinant proteins were separately purified by Ni-TED (tris-carboxymethyl ethylene diamine), immobilized metal ion affinity chromatography (IMAC) (Fig. 1C).

Immunization

Sixty 6-week-old healthy female BALB/c mice were purchased from the Center for Comparative Medicine, Yangzhou University (Sigma-Aldrich, Yangzhou, China). Mice were immunized subcutaneously in the right rear flank with purified FimA, FimA-mC3d, FimA-mC3d2 and FimA-mC3d3 soluble protein prepared in 100 μL phosphate-buffered saline (PBS) containing 0.1% globulin-free mouse albumin (Sigma-Aldrich).[10] The doses used for immunization were 0.1 μg, 1 μg and 10 μg for each group, whereas the control group was immunized by sterile saline. Mice were boosted intraperitoneally on day 35 with 10 μg FimA protein in incomplete Freund's adjuvant (IFA, Sigma-Aldrich). Nonlethal tail blood samples were collected 1 day before immunization and weekly after immunization by tail section. At the completion of the experiments, the mice were sedated with sodium pentobarbital and euthanatized by exsanguinations. Serum levels for FimA-specific antibody were determined for individual mice by ELISA, and titers are expressed as the geometric mean and standard error.

ELISA

Polystyrene microplates (Immulon 4 HBX; ThermoLabsystems) were coated with 100 μL of purified FimA at 0.125 μg/mL, samples were incubated with serially diluted sera from the vaccinated mice. Peroxidase-conjugated goat anti-mouse IgG HRP (1:6000 dilution) was used as the secondary antibody. The reaction was visualized with *o*-phenylenediamine (Sigma). End-point titers were calculated as the reciprocal of the last serum dilution that gave a value 2.1-fold higher than the preimmune serum. Antibody titers below the cutoff of the assay were assigned an arbitrary titer of one-half of the cutoff for calculation of the geometric mean of the titers.

Infection protection assay

One week after the second immunization, the standard strains of Salmonella enteritidis SD-2 bacteria cultures made suspensions of 100 colony-forming units (CFU) per 200 μL in normal saline were intraperitoneally injected in

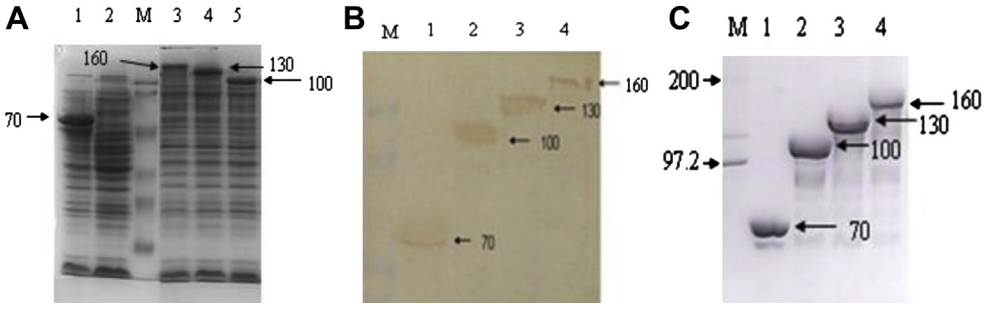

Figure 1. Recombinant protein expression. (A) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for recombinant proteins. 1. FimA. 2. Control vector. 3. FimA-mC3d3. 4. FimA-mC3d2. 5. FimA-mC3d. M. molecular marker. (B) Western blot analysis of the recombinant proteins. 1. FimA. 2. FimA-mC3d. 3. FimA-mC3d2. 4. FimA-mC3d3. M. Pre-stained low molecular weight marker. (C) SDS-PAGE analysis of purified recombinant proteins. 1. FimA. 2. FimA-mC3d. 3. FimA-mC3d2. 4. FimA-mC3d3. M. High molecular weight marker.

each group of mice. (A pretest confirmed that standard strains of *Salmonella enterica* serovar Enteritidis SD-2 poison BALB/c mice at LD50 of 10 CFU) Pathological changes in the experimental mice after the bacterial injection was observed once every 12 hours for 7 days. Clinical symptoms of dead mice in each group were dissected and their mortality was recorded. Sterile collection of liver, kidney, spleen was made in the dead mice in each group and placed in selenite cystine enrichment broth (SC). *Salmonella enterica* serovar Enteritidis in different organs was thereafter cultured for 24 hours, and 5 μL of the enrichment broth was transferred onto MacConkey agar plates to determine the number of colony-forming units. The immune protection ratio was calculated as a percentage of the number of surviving animal to the total number of animals in the experimental group.

Results

Construction of the recombinant proteins

In the present study soluble FimA protein fused to mC3d was expressed in a prokaryotic expression vector. The recombinant protein fused to mC3d was constructed as follow: the *fimA* gene was amplified by PCR using a pair of primers and the DNA template from *Salmonella enterica* serovar Enteritidis standard strain SD-2 genomic DNA. The PCR products of the *fimA* gene were digested and cloned into the expression vector pCold-TF to construct recombinant plasmid pCold-fimA, which was confirmed by restriction enzymes and sequencing. The different copies of *mC3d*, *mC3d*$_2$ and *mC3d*$_3$ were obtained through the digestion of recombinant plasmids pUC-mC3d, pUC-mC3d$_2$ and pUC-mC3d$_3$, and were cloned into the recombinant plasmid pCold-fimA to construct the recombinant plasmids (i.e., pCold-fimA-mC3d, pCold-fimA-mC3d$_2$, and pCold-fimA-mC3d$_3$). The fusion protein was optimally induced by IPTG at 15°C for 24 hours and expressed. SDS-PAGE showed that the recombinant proteins were 70 kDa, 100 kDa, 130 kDa, and 160 kDa (Fig. 1), respectively. The recombinant proteins expressing FimA were recognized by rabbit anti-fimbriae polyclonal antibodies, and then visualized by goat anti-rabbit polyclonal antibodies with a chrome appearance by enzyme-subtract interaction. The recombinant proteins were separately purified by Ni-TED (tris-carboxymethyl ethylene diamine) immobilized metal ion affinity chromatography (IMAC).

Immunization

Fig. 2 shows the changes in antibody titer in the different immune groups. When the immunization dose was 10 μg, FimA antibody levels in mice with FimA-mC3d$_2$ and FimA-mC3d$_3$ fusion protein groups were elevated to two and four times, compared with the FimA protein groups. In addition, in the 10 μg immunization dose, the antibody level in FimA-mC3d$_3$ groups was increased by four times and two times, compared to the FimA-mC3d and FimA-mC3d$_2$ groups (Fig. 3).

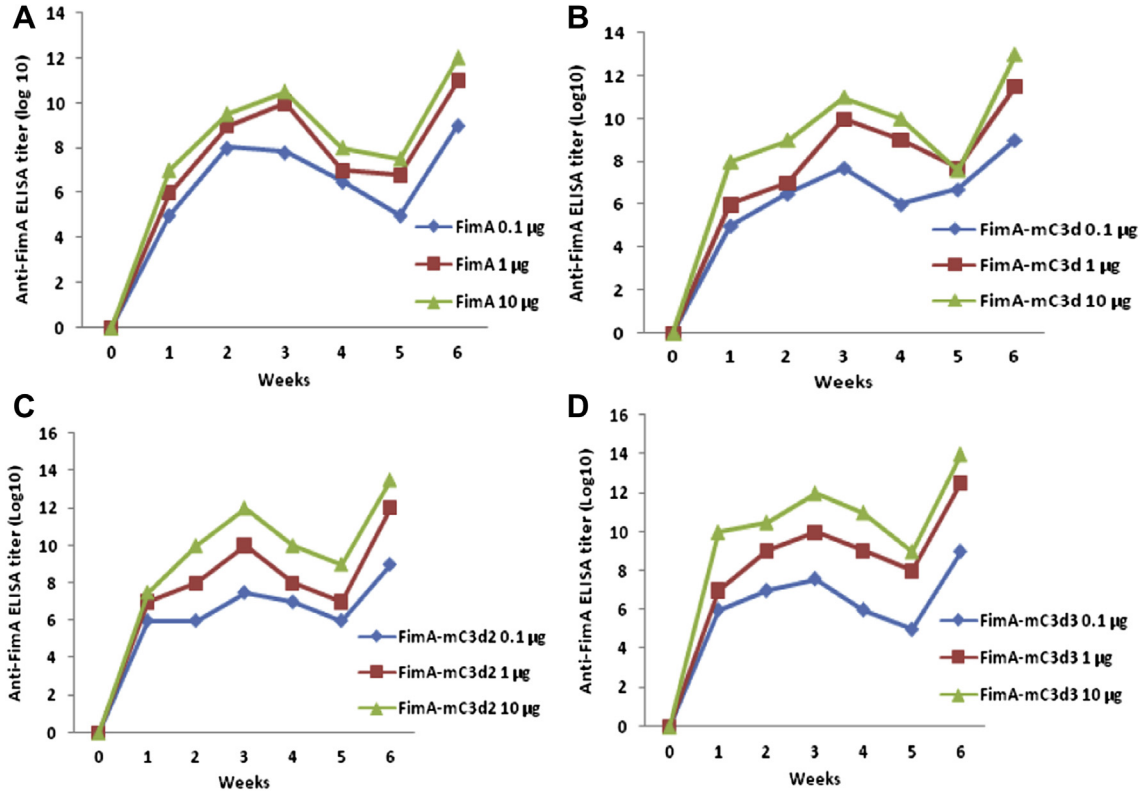

Figure 2. Change in antibody titer in the immune groups. (A) FimA; (B) FimA-mC3d; (C) FimA-mC3d$_2$; and (D) FimA-mC3d$_3$. Doses of purified protein at 0.1 μg, 1 μg, and 10 μg were used for the experimental group. Sterile saline was used for control group. Mice received an intraperitoneal boost on day 35 with 10 μg FimA protein in incomplete Freund's adjuvant (IFA; Sigma).

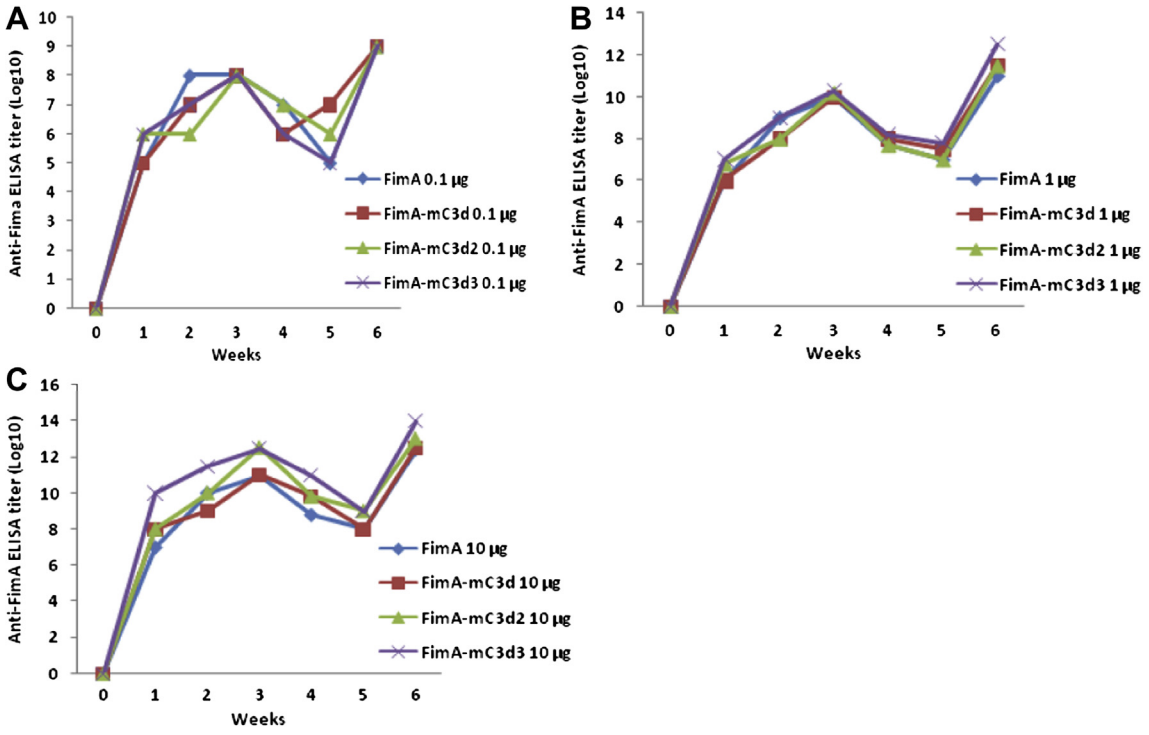

Figure 3. Change in antibody titer with the same immune dose in the immune groups. (A) 0.1 μg immune dose; (B) 1 μg immune dose; and (C) 10 μg immune dose. Doses of purified protein at 0.1 μg, 1 μg, and 10 μg were used for the experimental group. Sterile saline was used for the control group. Mice received an intraperitoneal boost on day 35 with 10 μg FimA protein in incomplete Freund's adjuvant.

Infection protection assay

Examination of the dead mice showed *Salmonella enterica* serovar Enteritidis pathological lesions. The challenge test results showed that immune protection ratio in mice immunized with 10 μg of FimA, FimA-mC3d, FimA-mC3d$_2$ and FimA-mC3d$_3$ were 50%, 50%, 75%, and 100%, respectively (Fig. 4). Therefore, we confirm that C3d can enhance the immune effects of the conjugated protein which are expressed in prokaryotic vector. Further study of C3d as a novel molecular adjuvant for a prokaryotic protein vaccine and its application in the prevention of *Salmonella* may be promising.

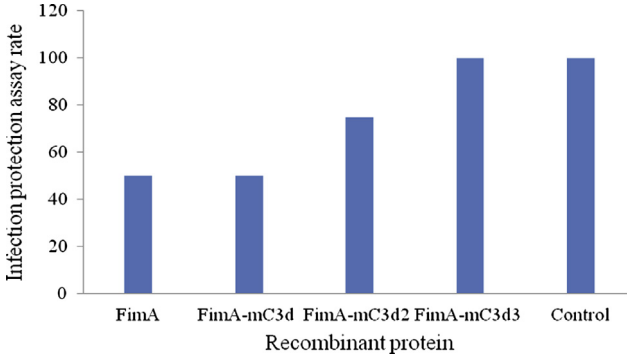

Figure 4. Immune protection ratio in mice immunized with 10 μg of FimA, FimA-mC3d, FimA-mC3d$_2$, and FimA-mC3d$_3$.

Discussion

The problem with pure recombinant or synthetic antigens used in modern day vaccines is that they generally are generally far less immunogenic than older style live or killed whole organism vaccines.[13] In the present study, we enhanced the immunogenicity of FimA protein by using the molecular adjuvant mC3d. The production of recombinant protein in *Escherichia coli* is often hampered by low expression levels and low solubility. A variety of methodologies have been developed, including protein production at low temperature and fusion protein expression using soluble protein tags.[14] The recombinant protein Fim A fused to mC3d was cloned into pCold-TF vector and expressed in *E. coli* BL21 (DE3). The protein expressed was confirmed by SDS-PAGE. The antigenicity of the recombinant proteins expressing FimA were recognized by rabbit anti-fimbriae polyclonal antibodies, and then visualized by goat anti-rabbit polyclonal antibodies with a chrome appearance by using enzyme—substrate interaction. The recombinant proteins were separately purified by Ni-TED and IMAC. The pCold-GST expression system was applied to 10 proteins that could not be expressed using conventional *E. coli* expression methodologies, Nine of these proteins were successfully obtained in the soluble fraction.[14] This suggests that the pCold-GST expression system can be utilized to improve the expression and purification of various proteins.[14]

Fimbriae are surface appendages found on many species of *Enterobacteriaceae* and they display adhesive properties.[15] Some fimbriae are considered virulence factors because they initiate adherence, which is the first step in the

colonization of host mucosal surfaces.[16,17] Information regarding the role of SEF17 and SEF21 fimbriae in serovar Enteritidis pathogenesis is lacking.[2] The factors that influence fimbrial phase variation *in vivo* and influence variability in the fimbriation of cells carrying an intact fimbrial operon are unknown; therefore, it is difficult to evaluate the role of fimbriae in pathogenesis.[2]

FimA is a potent inducer of pro-inflammatory cytokines involved in tissue destruction.[18] Immunization with FimA protects rats against homologous bacterial challenge.[19] In addition, FimA immunization confers antibody mediated protection against *Streptococcus parasanguis* endocarditis in rats.[20] Immunization with FimA protein has further been shown to reduce *Porphyromonas gingivalis*-induced alveolar bone loss in experimental rats,[21] and to confer protection against subsequent lethal infection with *P. gingivalis* in mice.[22] The purified recombinant FimA protein of *Salmonella enterica* serovar Enteritidis was detected by Kisiela et al.[3] They indicated that immunization of hens with FimA fimbrial proteins of *Salmonella Enteritidis* induces a strong humoral response. The levels of anti-FimA antibodies induced by purified recombinant proteins are similar to that level obtained with live bacteria.[3] FimA fimbrial proteins of *Salmonella enterica* serovar Enteritidis, in addition to inducing a strong humoral response, can induce cellular response.[23] In the present study we showed that, when the immunization dose was 10 μg, antibody levels in mice with FimA-mC3d_2 and FimA-mC3d_3 fusion protein groups were elevated to two and four times, compared to the FimA protein groups. The challenge test showed that the immune protection ratio in mice with FimA, FimA-mC3d, FimA-mC3d_2 and FimA-mC3d_3 were 50%, 75% and 100%, respectively. Therefore, we confirm that C3d can enhance the immune effects of the conjugated protein that are expressed in a prokaryotic vector.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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