Article

Similar Ability of FbaA with M Protein to Elicit Protective Immunity Against Group A Streptococcus Challenge in Mice

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Group A streptococcus (GAS), an important human pathogen, can cause various kinds of infections including superficial infections and potentially lethal infections, and the search for an effective vaccine to prevent GAS infections has been ongoing for many years. This paper compares the immunogenicity and immunoprotection of FbaA (an Fn-binding protein expressed on the surface of GAS) with that of M protein, the best immunogen of GAS. Assay for immune response showed that FbaA, similar to M protein, could induce protein-specific high IgG titer in BALB/c mice. Furthermore, following GAS challenge, the mice immunized with FbaA showed the same protective rate as those with M protein. These results indicate that FbaA is similar in ability to M protein in inducing protective immunity against GAS challenge in mice. *Cellular & Molecular Immunology*. 2009;6(1):73-79.

Key Words: group A streptococcus, immunogenicity, FbaA, M protein, immunoprotection

Introduction

Group A streptococcus (GAS) is an important human pathogen that can cause various kinds of infections including superficial infections of the throat or skin to highly invasive and potentially lethal infections such as necrotizing fasciitis and streptococcal toxic shock syndrome (1). Furthermore, some infections can result in severe morbidity, for example, untreated streptococcal pharyngitis may lead to acute rheumatic fever and chronic rheumatic heart disease (2). Cleary et al. proposed firstly that GAS invades cells, shielding the bacterium from antibiotics and the immune system (3). In addition, Horstmann et al. also confirmed the acquisition of complement regulatory protein factor H (FH) by GAS contributes to the bacterium's capacity to evade phagocytosis by polymorphonuclear leukocytes (PMNs) (4). The search for an effective vaccine to prevent GAS infections has been ongoing for decades. One of the major vaccine candidates is the surface M protein, which is a primary virulence factor of GAS (5). Unfortunately, M protein is restricted as a vaccine candidate because it possesses multiple serotypes and often causes tissue cross-reactions. A novel protein, FbaA, an Fn-binding protein expressed on the surface of GAS, was reported by Terao (6). Pandiripally et al. confirmed the functions of FbaA in promoting entry of GAS into the cytoplasm of human epithelial cells through binding human complement regulatory proteins FH and factor H-like protein (FHL-1) (7). Here FbaA was expressed, purified, and compared with M protein in immunogenicity and immunoprotection. Our results revealed that FbaA had a similar capability with M protein of inducing protein-specific IgG; furthermore, in mice, FbaA could elicit the same protective rate against GAS challenge as M protein.

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Materials and Methods

Protein, plasmid and mice

pGEX2T/fbaA (fbaA obtained from SSI-9, a serotype M1 GAS isolate), rabbit anti-FbaA-specific serum were kindly provided by Dr. Cue. M protein and pcDNA3.1a(+)/fbaA were conserved in our lab (pcDNA3.1a(+) was purchased from Invitrogen). Specific pathogen-free female BALB/c mice, aged 4-8 weeks, were obtained from Beijing Laboratory Animal Center (Beijing), housed and manipulated according to the Care and Use of Laboratory Animals (Beijing), and the mice were confirmed seronegative to GAS before inclusion in the studies.

Expression and purification of the FbaA protein

Expression of the FbaA protein (amino acid residues 38 to 324) was performed in pGEX2T/fbaA-transformed *E. coli* BL21 cells following induction with IPTG (0.1 mM, Merck).

GST-tagged FbaA protein (GST-FbaA) was purified by glutathione sepharose 4B, and then using thrombin according to the manufacturer's instructions (GE Healthcare) GST-tag was removed, finally, protein expression was confirmed by Western blot analysis.

Western blot

Western blot was done according to the method described previously (8). The bacterial lysates were separated in 12% SDS-PAGE gel and then transferred onto a nitrocellulose membrane. After transfer, the nitrocellulose membrane was kept in 5% nonfat milk powder solution for 2 h, followed by incubation at room temperature for another 2 h with rabbit anti-FbaA-specific serum. After three times washing with TBS-T, the nitrocellulose membrane was incubated with Alkaline Phosphatase (AP)-conjugated goat anti-rabbit immunoglobulin (Santa Cruz) at a dilution of 1:1,000 at room temperature for 1 h. The membrane was washed and visualization of the antibody-antigen complex was achieved by adding the substrate solution consisting of nitro blue tetrazolium salt and 5-bromo-4-chloro-3-indolyi phosphate (Sigma-Aldrich).

Immunization

Mice, aged 7-8 weeks, were injected *i.p.* with 1 ml GAS (M1 type) (5×10^8 CFU ml^{-1}) at weeks 0 and 2. Mice, aged 4-5 weeks, were divided into four groups with each of 20. The first three groups were immunized subcutaneously with purified FbaA + Freund's adjuvant (FA, Sigma), M protein + FA, PBS at weeks 0, 2, and 4, respectively; and the fourth one was immunized *i.m.* with pcDNA3.1a(+)/fbaA at weeks 0 and 2, and then boosted subcutaneously with FbaA + FA at the third immunization at week 4. FA used at the first immunization was complete FA, while FA used at the subsequent booster immunizations was incomplete FA. Half of the mice in each group were sacrificed to detect humoral immune response and cell-mediated immune response, and the remaining mice were challenged with GAS on day 10 after the third immunization.

ELISA

The microtiter plate was coated with 10 μg/ml of the purified FbaA or M protein respectively in carbonate-buffered saline and incubated for 16 h at 4°C. The plate was then washed with PBS, and blocked with PBS that contained 3% bovine serum albumin (Sigma). After incubation, 100 μl antiserum at different dilutions were added to the appropriate wells and incubated for 1 h at 37°C. The plate was washed and incubated for 1 h with AP-conjugated goat anti-mouse immunoglobulin (Santa Cruz) at 37°C. The reaction was initiated by adding 4-nitrophenyl phosphate (Amresco) substrate solution, and the absorbance at 405 nm was determined using a bichromatic microplate reader.

Evaluation of the anti-FbaA and anti-M IgG levels of mice exposed to GAS

Mice were injected with 1 ml GAS (M1 type) subcutaneously with 5×10^8 CFU ml^{-1} at weeks 0 and 2. On day 10, after the final injection, serum was collected and detected for specific anti-sera of FbaA protein and M protein by ELISA. Positive was indicated by fold increase in absorbance at 405 nm between sample and negative control.

Humoral immune response to FbaA protein and M protein in BALB/c mice

Sera were obtained in preimmunization and postimmunization (weeks 1, 3, 5) of the mice immunized with FbaA, M protein, pcDNA3.1a(+)/fbaA prime and FbaA boost, or PBS, and ELISA was used to measure specific antibodies. The IgG titers were expressed as the reciprocal of the last sample dilution giving an absorbance of at least two fold that of the preimmune sample and with an OD ≥ 0.15.

Flow cytometric analysis of spleen lymphocytes

To evaluate cell immune responses, the phenotype of $CD4^+$ and $CD8^+$ cells were assessed by flow cytometry (FCM). The murine splenocytes were harvested and mononuclear cells were isolated after the animals were sacrificed. Cells (1×10^6) of each sample were stained using rat monoclonal anti-mouse CD8 and CD4 IgG (eBioscience) according to standard protocols. FCM analysis was conducted on a FACSCalibur (Becton Dickinson, USA), and the data were collected and analyzed with CellQuest software (Becton Dickinson).

Challenge with GAS

Mice immunized with FbaA, M protein, pcDNA3.1a(+)/fbaA prime and FbaA boost, or PBS were injected *i.p.* with 1 ml bacterial suspension (equivalent to 2×10^8 CFU) and monitored survival daily for 15 days.

Statistical analysis

Statistical analysis was performed using the SPSS 10.0 software. Results were considered statistically significant if a probability of less than 0.05 was obtained.

Results

Expression and purification of the FbaA protein

GST-FbaA was expressed in *E. coli* at high levels by approximately 30% of total bacterial protein (Figure 1A, Lane 3). Because FbaA contains a proline-rich repeat domain (PRR) (the rates of praline 23.4%) and it has been shown that proline-rich proteins migrate more slowly in SDS-PAGE, the molecular size of GST-FbaA was determined to be 82.7 kDa. The purification of GST-FbaA and FbaA, GST-tag removed by thrombin, resulted in highly pure products (> 90%), which migrated in accordance with their theoretical molecular weight in SDS-PAGE (Figure 1A, Lanes 4, 5). Protein expression was approved by Western blot analysis with FbaA-specific serum (Figure 1B).

The levels of anti-FbaA and anti-M sera in mice following exposure to GAS

ELISA plates were coated with the purified FbaA and M protein to detect specific antibodies to FbaA or M protein in

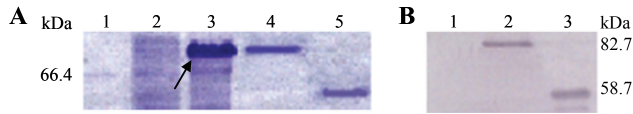


Figure 1. Expression, purification and detection of FbaA protein. (A) Analysis of the expression and purification of GST-tagged fusion FbaA protein (GST-FbaA) by SDS-PAGE. Bacterial lysates, purified GST-FbaA and FbaA protein removed GST-tag were separated on a 12% SDS-PAGE gel and stained with Coomassie blue R250. Lane 1, protein marker; Lane 2, BL21(DE3)/ pGEX-2T/*fbaA* without IPTG induction; Lane 3, BL21(DE3)/ pGEX-2T/*fbaA* with IPTG induction; Lane 4, purified GST-FbaA; Lane 5, the FbaA removed GST-tag. (B) Western blot analysis with anti-FbaA serum. After electrophoresed in SDS-PAGE, samples were transferred to a nitrocellulose membrane and detected using FbaA anti-serum. Lane 1, BL21(DE3)/pGEX-2T/*fbaA* without IPTG induction; Lane 2, purified GST-FbaA; Lane 3, The FbaA removed GST-tag.

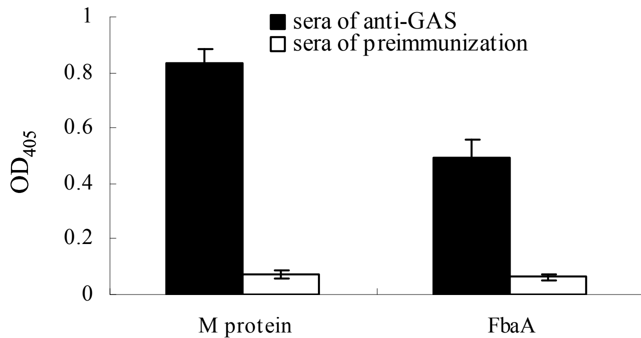
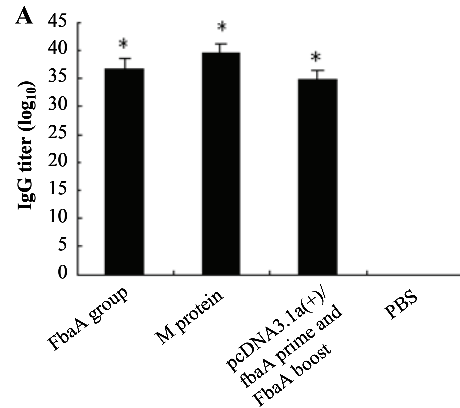


Figure 2. Mice produced anti-FbaA serum and anti-M serum following exposure to GAS. Adult (7 to 8-week-old) female BALB/c mice were injected subcutaneously with 1 ml GAS (M1 type) (5×10^8 CFU ml^{-1}) at weeks 0 and 2. Anti-sera were collected on day 10 after the final injection. The ELISA plates were coated by FbaA and M protein respectively to detect anti-FbaA serum and anti-M-protein serum of the anti-GAS sera.

the mice exposed to GAS. The positive rate of anti-FbaA antibody was 100%, as anti-M-protein antibody (Figure 2). The results suggest that mice, after exposed to GAS, will be induced to generate FbaA-specific antibody and M protein-specific antibody, indicating that both FbaA and M protein are the strong immunogenic membrane proteins of GAS.

Immune response to FbaA and M protein in BALB/c mice

Specific antibodies were measured in the mice immunized with M protein, FbaA, pcDNA3.1a(+)/*fbaA* prime and FbaA boost, or PBS after sera were collected at various times post-primary immunization (data not shown), and were found to be the highest in sera collected after two to three immunizing boosts. The data presented represent antibody titers in sera collected on day 10 after the last immunization (Figure 3A). Protein-specific antibody levels in sera from



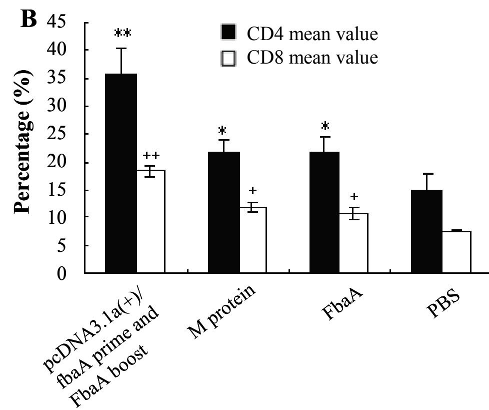


Figure 3. Immune responses in BALB/c mice. (A) Detection of the IgG titers of the four groups by ELISA. *$p < 0.05$ *vs* PBS group. (B) Analysis of the cellular immune responses in BALB/c mice by flow cytometry. **$p < 0.05$ *vs* the percentage of $CD4^+$ cells of other groups. $^{++}p < 0.05$ *vs* the percentage of $CD8^+$ cells of other groups, *$p < 0.05$ *vs* the percentage of $CD4^+$ cells of control PBS groups, $^{+}p < 0.05$ *vs* the percentage of $CD8^+$ cells of control PBS group.

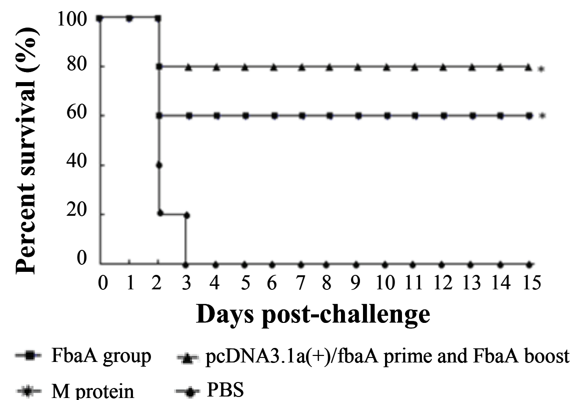


Figure 4. Survival rate of immunized and control mice following challenged *i.p.* with GAS (M1 type). Immunized and control mice were challenged *i.p.* with GAS bacterial suspension (equivalent to 2×10^8 CFU) on day 10 after the last immunization. Mice were monitored every day. *$p < 0.05$ for mice immunized with FbaA, with M protein or with pcDNA3.1a(+)/fbaA prime and FbaA boost *vs* control PBS group.

mice immunized with M protein, FbaA protein, and pcDNA3.1a(+)/fbaA prime and FbaA boost were significantly greater than those in sera from control mice given only PBS, and differences in antibody titers among the three groups of mice were not significant.

According to the FCM analysis, the percentages of $CD4^+$ or $CD8^+$ cells of splenocytes from mice immunized with pcDNA3.1a(+)/fbaA prime and FbaA boost were the highest among all the groups, with the percentages of splenic $CD4^+$ or $CD8^+$ cells from mice immunized with FbaA or M protein significantly higher than that of PBS groups. But the rates of splenic $CD4^+$ or $CD8^+$ cells from mice immunized with FbaA protein and with M protein had no significant difference (Figure 3B).

Protection against GAS challenge

Immunized and control mice were challenged *i.p.* with type M1 GAS bacterial suspension (equivalent to 2×10^8 CFU) on day 10 after the last immunization, and monitored for 15 days. The protective rate of 80% was observed for mice immunized with pcDNA3.1a(+)/fbaA prime and FbaA boost, with the protective rate for mice immunized with FbaA 60%, the same as that for the mice immunized with M protein, while no mice with PBS survived the challenge (Figure 4). Thus, mice immunized with FbaA, M protein, or pcDNA3.1a(+)/fbaA prime and FbaA boost were evoked significantly protective immune responses compared with the PBS control ($p < 0.05$).

Discussion

The objective of our study was to compare the immunogenicity and the protective effects of FbaA with that of M protein in BALB/c mice. After exposed to GAS, mice, aged 7-8 weeks, could be evoked to generate high levels of M-protein-specific or FbaA-specific antibody, although OD value of FbaA-specific antibody was slightly less than that of M-specific antibody at the same testing conditions. Subsequently, mice aged 4-5 weeks, were immunized with FbaA or M protein, and consequently we achieved higher IgG titers of protein specific anti-sera, with no significant difference between the two groups. These results reveal that FbaA shows strong immunogenicity as similar as M protein in BALB/c mice.

We also detected $CD4^+$ and $CD8^+$ cells in mice immunized with FbaA, M protein, pcDNA3.1a(+)/fbaA prime and FbaA boost or PBS control to evaluate the T cell immune response in BALB/c mice by FCM analysis. The results showed that the percentages of $CD4^+$ or $CD8^+$ cells from mice immunized with pcDNA3.1a(+)/fbaA prime and FbaA boost were the highest among all the groups. But the percentages of $CD4^+$ or $CD8^+$ cells from mice immunized with FbaA protein or with M protein had no significant difference, although those of above two groups were significantly higher than that of PBS groups. The results reveal that FbaA is similar to M protein in eliciting T cell response, while pcDNA3.1a(+)/fbaA has potential capacity to elicit a robust T cell response. In the challenge experiment, mice immunized with FbaA showed the same protective rate of 60%, as those immunized with M protein, and the mice immunized with pcDNA3.1a(+)/fbaA prime and FbaA boost showed the protective rate of 80%. These data indicate that FbaA like M protein has an efficiently protective effect against GAS infections. Thus it can be concluded that humoral immune response induced by FbaA was enough to interrupt infection with GAS, and T cell activation enhanced the anti-infective process. M protein is widely known to induce a high level of protein specific antibody, which can efficiently protect animals from GAS infection. However, M protein is an unacceptable vaccine candidate and is restricted from vaccine consideration because it contains epitopes known to cross-react with tissues in several animals, including humans (9). Besides, it possesses multiplicity serotypes in which M protein exhibits a low homology. FbaA distributes broadly among streptococcal species, such as M1, 2, 4, 9, 13, 22, 28, etc. Moreover, it exhibits a high homology in different types of *S. pyogenes* (6). Recently, Terao et al. reported that the immunogenic domains of FbaA were N terminus and PRR domain (10), and our recent data (not shown) showed the strongest immunogenic domain of FbaA was coiled-coil domain (CC) of the N terminus.

FbaA belongs to Fn-binding protein and shows a high homology to FnBPA, the Fn-binding protein of *Staphylococcus aureus* (6, 11). Soluble Fn is known to mediate the binding of protein F1 (one of Fn-binding proteins) of GAS and integrin $\alpha5\beta1$, which acts as a cellular receptor in the process of the internalization of *S. pyogenes* by epithelial cells (12). It is reported that FbaA, *via* the binding of Fn, also mediates the internalization of GAS to Hep-2 cells (13). It reveals that FbaA potentially promote the entry of GAS into human epithelial cells to shield the bacterium from antibiotics and the immune system. The ability of bacterial pathogens to evade complement attack and opsonophagocytosis is often influenced or dictated by a pathogen's ability to bind complement regulatory proteins (14, 15). FbaA, the first non-M-like protein of GAS, is shown to bind these complement regulatory factors. FbaA, *via* the binding of FH and FHL-1, possibly contributes to GAS evasion of complement attack and opsonophagocytosis by PMNs and can promote the entry of GAS into human epithelial cells (7). The significant increase in antibody titer and the effective protection with immune response induced by FbaA indicate that FbaA-specific antibody, *via* the binding of surface protein FbaA of GAS, possibly blocks the entry of GAS into human epithelial cells or facilitates the human body to eliminate GAS by complement attack and opsonophagocytosis through interfering GAS binding with complement regulatory proteins or/and Fn.

In this study, all of our data reveal that FbaA protein and M protein have a similar immunogenicity in immune response in BALB/c mice. Further research is required to assess the ability of FbaA to induce tissue cross-reactions. Ongoing testing of FbaA as a potential *S. pyogenes* vaccine candidate will focus on immunogenicity, safety and efficacy in humans.

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