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Abstract

BACKGROUND & OBJECTIVES: The fibronectin binding protein Sfb1 of *Streptococcus pyogenes* is a well characterised antigen which induces protection against lethal challenge with group A streptococcus (GAS) when adjuvanted with cholera toxin B-subunit (CTB). As an alternative to CTB adjuvanted intranasal immunisations we investigated the immune responses generated in mice using Sfb1 incorporated in to the skin and mucosal adjuvant SAMA4. **METHODS:** Mice (BALB/c) were vaccinated intradermally with 100 microl of either SAMA4 (adjuvant only group) or SAMA4/Sfb1 and were boosted 7 days later. Mice vaccinated with CTB based vaccines were immunised by intranasal inoculation with a mixture containing 30 microg Sfb1 and 10 microg CTB on days 1, 3, 5 and 15. At 14 days after the last booster immunisation the immune response was characterised and mice were challenged with 10(8) CFU of *S. pyogenes*. **RESULTS:** Mice vaccinated with SAMA4/Sfb1 elicited a Sfb1-specific IgG response in the sera that was significantly higher than that seen in control mice and mice immunised with the adjuvant only ($P<0.05$). No significant differences were seen for specific IgA antibodies in the sera in all groups examined. Compared with non-immunised and adjuvant only immunised controls, mice immunised with the Sfb1/SAMA4 vaccine exhibited a significant increase ($P<0.05$) in the number of Sfb1 reactive spleen cells in lymphoproliferation assays which were three fold higher than those seen for mice vaccinated with the Sfb1/CTB vaccine. Mice vaccinated with CTB/Sfb1 had the highest level of protection (80%) as where mice vaccinated with SAMA4 and SAMA4/Sfb1 displayed no protection (20% and 40%). **INTERPRETATION & CONCLUSION:** These data suggest that the SAMA4 adjuvant used in this study fails to elicit protective immunity in BALB/c mice when used to adjuvant the known protective antigen Sfb1.

Keywords

sfb1, 1, protein, binding, fibronectin, mice, streptococcal, immune, against, adjuvant, vaccine, iscom, liposome, responses, CMMB

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Immune responses of a liposome/ISCOM vaccine adjuvant against streptococcal fibronectin binding protein 1 (Sfb1) in mice

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Background & objectives: The fibronectin binding protein Sfb1 of *Streptococcus pyogenes* is a well characterised antigen which induces protection against lethal challenge with group A streptococcus (GAS) when adjuvanted with cholera toxin B-subunit (CTB). As an alternative to CTB adjuvanted intranasal immunisations we investigated the immune responses generated in mice using Sfb1 incorporated in to the skin and mucosal adjuvant SAMA4.

Methods: Mice (BALB/c) were vaccinated intradermally with 100 µl of either SAMA4 (adjuvant only group) or SAMA4/Sfb1 and were boosted 7 days later. Mice vaccinated with CTB based vaccines were immunised by intranasal inoculation with a mixture containing 30 µg Sfb1 and 10 µg CTB on days 1, 3, 5 and 15. At 14 days after the last booster immunisation the immune response was characterised and mice were challenged with 10⁸ CFU of *S. pyogenes*.

Results: Mice vaccinated with SAMA4/Sfb1 elicited a Sfb1-specific IgG response in the sera that was significantly higher than that seen in control mice and mice immunised with the adjuvant only ($P<0.05$). No significant differences were seen for specific IgA antibodies in the sera in all groups examined. Compared with non-immunised and adjuvant only immunised controls, mice immunised with the Sfb1/SAMA4 vaccine exhibited a significant increase ($P<0.05$) in the number of Sfb1 reactive spleen cells in lymphoproliferation assays which were three fold higher than those seen for mice vaccinated with the Sfb1/CTB vaccine. Mice vaccinated with CTB/Sfb1 had the highest level of protection (80%) as where mice vaccinated with SAMA4 and SAMA4/Sfb1 displayed no protection (20% and 40%).

Interpretation & conclusion: These data suggest that the SAMA4 adjuvant used in this study fails to elicit protective immunity in BALB/c mice when used to adjuvant the known protective antigen Sfb1.

Key words Sfb1 liposome/ISCOM vaccine adjuvant

Fibronectin binding proteins of *Streptococcus pyogenes* are believed to mediate adhesion of the pathogen to host tissue, a critical step in the initial stages of infection. Sfb1 is a well characterised fibronectin binding protein of group A streptococcus (GAS). This protein mediates bacterial attachment to host cells and internalisation of GAS into nonphagocytic cells^{1,2}. Sfb1

has also been shown to interfere with host clearance mechanisms being mediated by macrophages by binding to the Fc fragment of human immunoglobulins³.

Fibronectin binding proteins have been proposed as potential vaccine targets for preventing GAS infections⁴. Antibodies directed against such adhesins

may prevent bacterial attachment and inhibit colonisation⁵. When adjuvanted with cholera toxin B-subunit (CTB), intranasal immunisation with Sfb1 induces protection against lethal challenge with GAS⁶. Other characteristics which make Sfb1 an attractive candidate antigen are the presence of highly conserved epitopes and the fact it is expressed on the surface of GAS in 70 per cent of clinical isolates belonging to different serotypes and strains, independent of their geographic origin^{7,8}. Anti-Sfb1 antibodies do not cross-react with heart proteins and therefore may not trigger autoimmune reactions that are responsible for post streptococcal sequelae⁸.

Although ADP-ribosylating bacterial toxins such as cholera toxin and *Escherichia coli* heat-labile enterotoxin are excellent mucosal adjuvants capable of inducing both systemic and mucosal immune responses, currently none of these toxins or their derivatives have been approved for use in humans⁹. Recently these toxins have been shown to target and accumulate in olfactory nerves/epithelium and olfactory bulbs of the central nervous system which has highlighted the possibility for neuronal damage through the intranasal use of these proteins in humans^{10,11}. As an alternative to CTB adjuvanted intranasal immunisations we investigated the immune responses generated in mice using Sfb1 incorporated in to the skin and mucosal adjuvant SAMA4 (a liposome/ISCOM combination adjuvant¹²). The immune responses and protective potential were compared to those elicited by CTB/Sfb1 antigen/adjuvant combination which is known to be protective.

Material & Methods

Bacterial strains, media and growth conditions: *S. pyogenes* NS192 is a blood isolate from Northern Territory, Australia. GAS was cultured overnight without shaking at 37°C in Todd-Hewitt broth (Difco, Detroit, MI) supplemented with 1 per cent yeast extract or on 5 per cent sheep blood agar (Microdiagnostics, Australia). *E. coli* strains were routinely grown on Z agar¹³ or in Luria Bertani (LB) broth¹⁴. When required, antibiotics were added to the medium at a concentration of 100 µg/ml for ampicillin and 50 mg/ml for kanamycin. Recombinant protein expression was induced by the addition of Isopropyl-β-D-galactopyranoside (IPTG) to

a final concentration 1 mM. Liquid cultures were agitated during incubation in an orbital-shaking incubator (Paton Industries) at 180 rpm when required.

Vaccine preparation and immunisation schedule:

Fusion proteins consisting of N-terminally hexa-histidyl tagged Sfb1 were purified under denaturing conditions using Ni-NTA agarose according to manufacturers instructions (Qiagen, Chatsworth CA). Formulation of SAMA4 adjuvant and SAMA4-containing Sfb1 vaccines were performed as previously described¹². Each dose of vaccine contained 50 µg Sfb1 and 0.6 µg of QuilA (Advet, Lulea Sweden). Female BALB/c mice aged 10 wk were vaccinated intradermally with 100 µl of either SAMA4 (adjuvant only group) or SAMA4/Sfb1 and were boosted 7 days later. Mice vaccinated with CTB based vaccines were immunised by intranasal inoculation (10 µl/nare) with a mixture containing 30 µg Sfb1 and 10 µg CTB on days 1, 3, 5 and 15. For the characterisation of the immune response serum samples, spleens and lung washes were collected 14 days after the last booster immunisation. Lung washes were obtained by tracheal canulation and gentle washing with 0.7 ml of cold PBS containing 2 mM PMSF.

Enzyme linked immunosorbent assay (ELISA):

Immunoassay plates (96 flat bottom wells, Greiner) were coated by overnight incubation at 4°C with 50 µl of recombinant Sfb1 at 5 µg/ml in carbonate buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6). Unbound antigen was removed and plates were washed three times with PBS containing 0.05 per cent Tween 20 (PBS-T20). Wells were blocked with 100 µl of PBS containing 0.05 per cent v/v Tween 20 and 1 per cent w/v bovine serum albumin (Sigma, USA) for 1 h at 37°C. After washing three times with PBS containing 0.05 per cent Tween 20 (PBS-T20), plates were incubated for 2 h at 37°C with serial dilutions of individual serum or lung wash samples. The plates were again washed, and incubated for 1 h at 37°C with 50 µl of horseradish peroxidase-labelled goat anti-mouse IgG (Kirkegaard and Perry Laboratories) or IgA (Sigma) secondary antibodies diluted 1:2000 in PBS-T20. After washing, 50 µl of orthophenylenediamine substrate solution (Sigma) was added to each well and the reaction was allowed to proceed at room temperature for 15 min. The reaction was stopped by the addition of 25 µl of 2M H₂SO₄ and read at 490 nm with a SpectraMax 250 microplate

spectrophotometer (Molecular Devices). Titres for anti-Sfb1 antibodies were determined using the SoftMax program (Molecular Devices).

To determine the concentration of total IgG or IgA immunoglobulin present in lung washes, plates were coated with rat anti-mouse IgG and IgA (Pharminogen) as capture antibodies and were incubated with serial dilutions of lung wash samples or purified mouse IgG or IgA to generate standard curves. Captured immunoglobulin was then detected as described above.

Lymphocyte proliferation assays: Splenocytes were isolated and released by teasing spleens into 5 ml 1 x RPMI 1640 (Life Technologies). Cells were collected by centrifugation at 800 g for 10 min and red blood cells were lysed with 5 ml of 0.83 per cent NH_4Cl_2 , 0.1 per cent KHCO_3 and 0.01 M EDTA. Cells were washed twice in 5 ml PBS containing 1 per cent glucose and recovered by centrifugation (800 g for 5 min). Cells were then resuspended in 1 ml Complete Medium, RPMI containing 100 unit Penicillin/100 μg Streptomycin, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 0.06 mM β -Mercaptoethanol, 10 per cent fetal calf serum. An aliquot of the suspension was diluted tenfold in Trypan Blue and counted using a haemocytometer. The cell concentration was then adjusted to 5×10^6 cells/ml in Complete Medium. A 50 μl volume of the cell suspension (2.5×10^5 cells) was added in triplicate to a 96 well flat bottom cell culture plate (Interpath) containing 200 μl of complete media alone or with 25 $\mu\text{g}/\text{ml}$ of Sfb1 antigen. The plate was incubated for 72 h at 37°C in 5 per cent CO_2 in air. At the end of the incubation period, 50 μl (0.5 μCi) of ^3H thymidine (Amersham, TRK120, UK) was added to each well and the plate was incubated for a further 18 h. Cells were harvested with a PHD cell harvester (Cambridge Technology) onto glass fibre filter strips. The filters were punched out into insert vials (Pony vials, Packard, 6000286) containing 2 ml scintillant (Ultima Gold scintillation fluid, Packard, 60133). Tubes were then mixed vigorously and radioactivity were counted for 1 min with a Packard 4000 series liquid scintillation counter (model 4530).

GAS challenge procedure: *S. pyogenes* NS192 was passaged through mice 6 times to enhance virulence. For intranasal challenge, all mice were challenged with 10^8 CFU of *S. pyogenes* NS192 delivered in a 40 μl

volume, 14 days after the last boost. Mortality was recorded daily in all groups.

Results

Antigen-specific humoral immune response: Intradermal immunisation with SAMA4/Sfb1 or intranasal CTB/Sfb1 elicited a significant serum IgG antibody response to the vaccine antigen when compared to the non-vaccinated control mice ($P=0.038$ and $P=0.029$). Anti-Sfb1 IgG titres in serum were 3 times higher in mice vaccinated with CTB/Sfb1 than those seen in mice vaccinated with SAMA4/Sfb1. There was no significant increase of specific serum IgA in vaccinated animals (Fig. 1a).

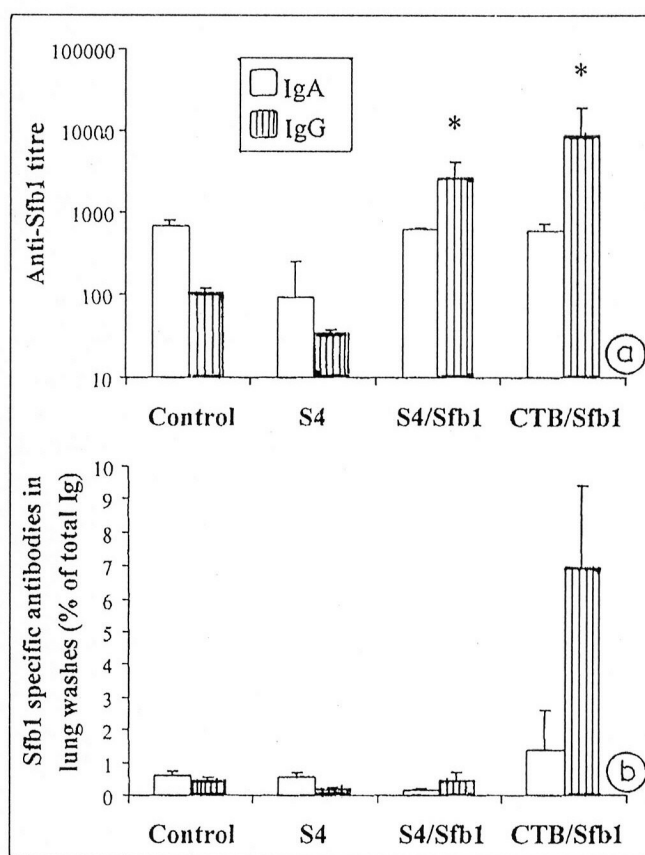


Fig. 1. The humoral immune response directed against Sfb1 at 14 days post last booster immunisation. (a) Specific IgG and IgA present in the serum of control and vaccinated mice. Results are expressed as the geometric means of 5 mice per group. SEM are indicated by vertical bars. The results are statistically significant (Student's *t* test) when compared with the values for control mice ($P < 0.05$) (*). (b) Specific antibodies in lung washes of control and vaccinated mice. Results are expressed as the per cent Sfb1-specific antibodies with respect to total immunoglobulin isotype.

Mucosal immune responses were assessed by examining the production of Sfb1-specific antibodies in the respiratory mucosal. Only mice immunised with the CTB based vaccine elicited an increased Sfb1-specific antibody response in the lung. Both IgA and IgG isotypes were seen to increase by a small amount (0.5% to 7%), however these increases may not be significant ($P=0.076$) due to the low numbers of mice per group (Fig.1b).

Antigen-specific cell-mediated immune responses: Lymphocytes isolated from the spleens of mice immunised with the SAMA4 adjuvant or SAMA4 containing Sfb1 showed significantly higher proliferative

responses when compared to untreated controls ($P=0.039$ and $P=0.01$) (Fig.2). Proliferative responses were 6.5 times higher for cells isolated from mice vaccinated with SAMA4 containing Sfb1 when compared those seen for mice vaccinated with the SAMA4 adjuvant alone. Cells isolated from mice vaccinated with CTB/Sfb1 did not show a significantly higher proliferative response (Fig.2).

Determination of protective immunity: After intranasal challenge with *S. pyogenes*, mice vaccinated with CTB/Sfb1 had the highest level of protection (80% survival). Mice vaccinated with SAMA4 and SAMA4/Sfb1 showed no protection with only 20 per cent and 40 per cent survival respectively. These were both lower than the level of protection seen in control mice (60%) (Fig.3).

Discussion

ISCOM-based adjuvants have been shown to induce both strong antibody and cellular immune responses in a variety of animal species¹⁵. ISCOMs are active after mucosal delivery and induce both local and systemic immune responses and are believed to be the most potent adjuvant for the induction of cell mediated immunity¹⁶. A licensed and commercially available ISCOM-based vaccine is currently used to protect horses from equine influenza and another ISCOM-based influenza vaccine is being tested in humans¹⁷. The liposome/ISCOM combination adjuvant, SAMA4 used in the present study has been shown to produce high specific antibodies in serum and at mucosal sites such as the lung and large intestine^{12,18}. SAMA4 has also been shown to promote good memory responses and cytotoxic T-cell activity¹². When used to adjuvant outer-membrane proteins from *Actinobacillus pleuropneumoniae*, a SAMA4 based vaccine was shown to induce protective immunity against intranasal challenge with the wild-type organism¹⁸. Although the exact mechanisms for adjuvant activity of ISCOM-based vaccines is not known, the synergism between SAMA4 and antigen components is not dependent on the antigen type and is not a particular attribute of the antigen used¹⁹.

Intradermal immunisation with SAMA4/Sfb1 resulted in a significant increase in antigen specific serum IgG.

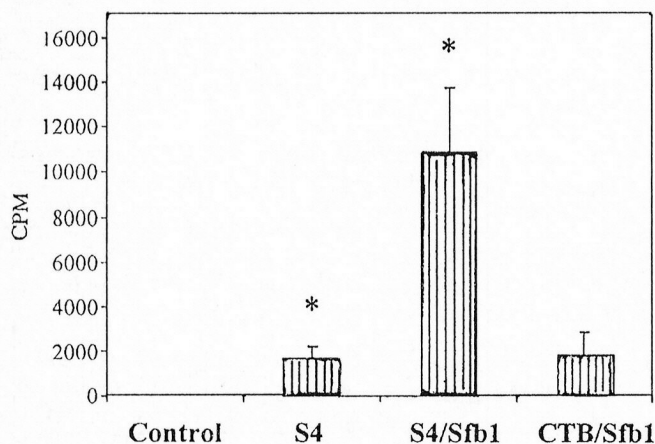


Fig.2. Sfb1-specific proliferative responses of spleen cells. The results are statistically significant (Student's t test) when compared with the values for control mice ($P<0.05$) (*). Results are expressed as the mean counts per minute (CPM) of triplicate samples for 3-4 mice per group. Standard error of the mean is indicated by vertical lines.

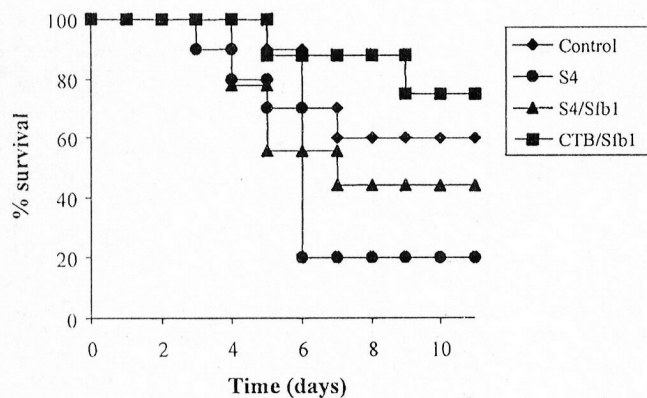


Fig.3. Survival of mice after intranasal challenge with *S. pyogenes* strain NS192. Animals (n=10) were challenged with 10^8 CFU of NS192, 14 days post last boost and mortality was recorded daily.

However, this was 3 times lower than those seen in mice vaccinated with CTB/Sfb1. SAMA4/Sfb1 vaccinated animals failed to elicit a mucosal immune response in the lung, but as expected exhibited a significant increase in the number of Sfb1-reactive spleen cells in lymphocyte proliferation assays. Mice vaccinated with CTB/Sfb1 produced an increased Sfb1-specific antibody response in the lung, where as cells isolated from the spleens of these mice failed to show a significant proliferative response. This is comparable to the results reported by Schulze *et al*²⁰ where mice were intranasally immunised with various fragments of Sfb1 using CTB as an adjuvant also failed to produce a significant increase in reactive lymphocytes isolated from spleens.

Mice vaccinated with CTB/Sfb1 have previously been shown to be protected against lethal intranasal challenge with GAS⁶. This was again demonstrated in this study where mice vaccinated with CTB/Sfb1 displayed the highest level of protection (80%). Protection in these animals have been correlated with the level of Sfb1-specific antibodies in serum and lung washes with secretory IgA believed to be critical for immunity⁶. Mice receiving SAMA4 based vaccines displayed no mucosal immunity and had the lowest levels of protection. As these mice also displayed a significant increase in the number of Sfb1-reactive spleen cells, cell mediated immunity does not appear to play a role in protective immunity against an intranasal GAS challenge. In endemic areas, the incidence of streptococcal skin and respiratory infections decrease with increasing age and this corresponds to increasing levels of anti-streptococcal serum antibodies²¹. However, mice vaccinated with SAMA4 based vaccines also displayed an increased susceptibility to GAS infection as demonstrated by having lower levels of protection than those seen for control mice. A reason why SAMA4 may exacerbate GAS infections may be due to the systemic toxic effects of QuilA (a semi-purified saponin component of the adjuvant) which has been reported by others^{19,22,23}.

These data suggests that the skin and mucosal adjuvant, SAMA4 used in this study fails to elicit protective immunity in BALB/c mice when used to adjuvant the known protective antigen Sfb1 and cannot be considered as an alternative for the mucosal adjuvant, CTB in this situation.

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