Antibody classes & subclasses induced by mucosal immunization of mice with *Streptococcus pyogenes* M6 protein & oligodeoxynucleotides containing CpG motifs


*Istituto Superiore di Sanità, Rome, Italy*

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**Background & objectives**: Type-specific antibodies against M protein are critical for human protection as they enhance phagocytosis and are protective. An ideal vaccine for the protection against *Streptococcus pyogenes* would warrant mucosal immunity, but mucosally administered M-protein has been shown to be poorly immunogenic in animals. We used a recombinant M type 6 protein to immunize mice in the presence of synthetic oligodeoxynucleotides containing CpG motifs (immunostimulatory sequences: ISS) or cholera toxin (CT) to explore its possible usage in a mucosal vaccine.

**Methods**: Mice were immunized by intranasal (in) or intradermal (id) administration with four doses at weekly intervals of M6-protein (10 µg/mouse) with or without adjuvant (ISS, 10 µg/mouse or CT, 0.5 µg/mouse). M6 specific antibodies were measured by enzyme linked immunosorbent assay using class and subclass specific monoclonal antibodies.

**Results**: The use of ISS induced an impressive anti M-protein serum IgG response but when id administered was not detectable in the absence of adjuvant. When used in, M-protein in the presence of both ISS and CT induced anti M-protein IgA in the bronchoalveolar lavage, as well as specific IgG in the serum. IgG were able to react with serotype M6 strains of *S. pyogenes*. The level of antibodies obtained by immunizing mice in with M-protein and CT was higher in comparison to M-protein and ISS. The analysis of anti-M protein specific IgG subclasses showed high levels of IgG1, IgG2a and IgG2b, and low levels of IgG3 when ISS were used as adjuvant. Thus, in the presence of ISS, the ratio IgG2a/IgG1 and (IgG2a+IgG3)/IgG1 >1 indicated a type 1-like response obtained both in mucosally or systemically vaccinated mice.

**Interpretation & conclusion**: Our study offers a reproducible model of anti-M protein vaccination that could be applied to test new antigenic formulations to induce an anti-group A *Streptococcus* (GAS) vaccination suitable for protection against the different diseases caused by this bacterium.

**Key words** Anti GAS vaccination - M6 protein - mucosal immunity - *Streptococcus pyogenes*

M protein is considered the main determinant factor present on the surface of group A *Streptococcus* (GAS). In humans and animals only M protein type-specific antibodies have the capacity to opsonize streptococci and to override the anti-phagocytic property of M protein. The development of an efficacious and safe vaccine against GAS infections was prevented by two main factors: the high number of different, non cross-reactive serotypes and the fact that some portions of the protein (B repetitive domain) contain autoantibodies-inducing epitopes against cardiac and skeletal tissues. An ideal vaccine for the protection against GAS would warrant mucosal immunity, but mucosally administered M-protein has been shown to be poorly immunogenic in...
animals. Several adjuvants or carrier proteins such as Freund adjuvant (Fa), cholera toxin (CT), diphteria toxoid (Dt) have been used earlier to increase the antigenicity of GAS surface antigens. Because of the relative toxicity of Fa and CT, only Dt could be used in humans. It has been shown that synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG dinucleotides in particular base contexts (immuno-stimulating sequences, ISS) cause activation of B and NK cells as well as antigen presenting cells. When co-administered to experimental animals with proteins or peptides, ISS act as potent adjuvants for a type-1 immune response. A particular advantage of ISS as adjuvants is their activity both in parenteral (intramuscular, subcutaneous, sc and intradermal, id) and mucosal (intranasal, in, oral and intrarectal) administration. The aim of the present study was to define the possible use of an adjuvant that could be used without conjugation, both by parenteral and mucosal immunization, to increase the immunogenicity of M protein. We explored the potential of ISS to induce anti-M6 antibody response when used as a systemic and mucosal adjuvant.

The local and systemic immune response was studied to compare the activity of ISS to that of cholera toxin, a widely used adjuvant in experimental mucosal vaccination that induces a Th-2 type of immune response.

Material & Methods

Reagent and antigens: Single-stranded, phosphorothioated CpG motif containing ODN and control ODN (M-ODN) were synthesized (M-Medica Firenze, Italy) according to published sequences (5'-TGACTGTGAACGTTCGAGATGA-3' and 5'-TGACTGTGAAGCATCGAGATGA-3' respectively). The cholera toxin (CT) purified from *Vibrio cholerae* was purchased from Sigma (St. Louis, MO, USA). The recombinant M6 protein was kindly provided by V. Fischetti.

Immunization protocols: Groups (n=6) of female BALB/c mice (6-8 wk old; Charles River, Calco, Lecco, Italy) were immunized by intradermal (id) injection of M6 protein alone (10 µg/mouse) or in combination with ISS or M-ODN at 50 µg in a total volume of 50 or 100 µl. ISS were administered in the first inoculum only. Groups (n=6) of the same animals were immunized by intranasal (in) inhalation of 10 µg/mouse of M6 protein alone or in combination with 0.5 µg/mouse of CT, 10-20 µg/mouse of ISS or M-ODN in a total volume of 66 µl (33 µl/naris). Drops of vaccine were applied to external nares of mice after a light anaesthesia with 10 µl/g intraperitoneal (ip) administration of a solution containing 5 mg/ml ketamin (Ketavet; Farmaceutici Gellini S.p.A., Latina, Italy) and 0.3 mg/ml xilazin (Rompun; Bayer S.p.A., Leverkusen, Germany).

Collection of samples: From mice immunized three times on days 0, 10 and 20; sera and bronchoalveolar lavage (BAL) were collected on day 30. Plasma was collected after a light anaesthesia by retroorbital puncture 30 days after the first inoculum. Samples were immediately frozen at -35ºC until use.

BAL fluids were obtained through the insertion of a cannula into the trachea after cervical dislocation. Lungs were then flushed with 0.6 ml of saline. The return was spun to remove cellular debris and immediately frozen at -35ºC until IgA assay.

Evaluation of the immune response: The antibody response to M6 protein in sera was determined by enzyme linked immunosorbent assay (ELISA) as previously described. Briefly, M6 protein was used at 1µg/ml in PBS (100 µl/well) to coat flat-bottomed 96-well plates (Dynatech, Chantilly, VA) overnight at +4ºC. After a postcoat of 1 h at +4ºC with PBS containing 2 per cent bovine serum albumin (BSA), sera (non-immune control and test sera) were added to appropriate wells at different dilutions in PBS with 0.5 per cent BSA containing 0.05 per cent Tween 20 (PBS-T). After overnight incubation at +4ºC and washing, plates were incubated with anti-IgG (g chain specific) goat anti-mouse antibodies conjugated with alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). After 1h incubation and washings, p-nitrophenylphosphate (Sigma, St. Louis, MO, USA) was added as a substrate. The reaction was blocked with 3 M NaOH and the absorbance evaluated at 405 nm using the Microtitre reader Victor-1420 multilabel counter (EG & G/Wallac, Turku, Finland). All tests were designed to compare in the same ELISA plate sera from mice belonging to different experimental groups.

Subclasses of M6 protein IgG positive sera were determined using rat anti-mouse IgG subclass biotinilated monoclonal antibodies (PharMingen, San Diego, CA, TELONI et al: MUCOSAL IMMUNIZATION OF MICE WITH S. PYOGENES M6 PROTEIN 127

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Subclasses of M6 protein IgG positive sera were determined using rat anti-mouse IgG subclass biotinilated monoclonal antibodies (PharMingen, San Diego, CA,
USA) and horseradish peroxidase-conjugated streptavidin (PharMingen). After the addition of o-phenylenediamine (Sigma fast; Sigma, USA) the reaction was stopped with 2N H₂SO₄ and the absorbance (ABS) was evaluated with the plate reader at 490 nm.

The anti-M6 protein IgA production in sera and BAL was evaluated using a rat anti-mouse IgA biotinilated monoclonal antibody (PharMingen, San Diego, CA) and incubated for 1 h at +37°C. After washings, horseradish peroxidase-conjugated streptavidin was added and the reaction was evaluated as subclass IgG assay. Endpoint titres were expressed as the reciprocal log₂ of the last serum or BAL dilution, which gave an optical density (OD), at 405 or 490 nm higher than the cut-off. The ABS cut-off value was determined as the double of the background and it was always higher than the mean ABS value of sera from untreated mice at the lowest dilution.

**Reactivity of anti-M protein serum:** The reactivity of the anti-M protein serum was tested by the Ouchterlony double diffusion test performed with 1 per cent agarose gel with sodium barbital buffer, pH 7.4, using the hot acidic M protein extract (according to the M serotyping procedure of Lancefield) prepared from the strains belonging to the Istituto Superiore di Sanità (ISS) collection which included Group A streptococcus M1: ISS-650, Group A streptococcus M3: ISS-651, Group A streptococcus M6: ISS-654, Group G streptococcus: ISS-3833, Group B streptococcus serotype III: ISS-2601.

**Statistical analysis:** The data were expressed as arithmetic means of at least four independent experiments ± standard error of the mean (SEM). The data were analyzed for normal distribution and the statistical significance of the difference between groups was determined by the two-tailed unpaired Student’s t-test using the Statview 4.1 program (Abacus Concepts, Berkeley, CA, USA). Differences were considered significant with $P < 0.05$.

**Results**

Mice vaccinated id with recombinant M6 protein in the absence of adjuvant or in the presence of CT or M-ODN did not develop a specific IgG response (data not shown). One the other hand, when ISS were used, mice developed a detectable anti-M6 protein specific immune response. IgG titres were very high, when ISS were used as adjuvants for a systemic (id) vaccination (Fig. 1). However, in mice vaccinated id with ISS, M6 specific IgA were not detected in BAL. The specificity of the response was determined using both ELISA (Figs 1-4) and radial immunodiffusion methods (Table).

Mice vaccinated in with recombinant M6 protein in the absence of adjuvant did not develop a specific Ig response. Intranasal M-6 vaccination in the presence of ISS induced a good systemic IgG response (Fig. 1) and mucosal IgA response (Fig. 2). When using ISS, however, mean titres of BAL anti-M-6 IgA were lower than those obtained immunizing mice in the presence of CT (Fig. 2) using the same immunization protocol. The use of ISS induced a relative higher synthesis of anti-M-6 IgG2b than IgG2a. This phenomenon, that is indicative of a Th1-like immune response, was observed when ISS were used as systemic adjuvants (Fig. 3). When ISS were used as mucosal adjuvants, IgG2a had the same mean Table.

**Table.** Specificity of M protein response by immunodiffusion and ELISA

<table>
<thead>
<tr>
<th>M antigen extract from</th>
<th>Non immune serum</th>
<th>Anti M protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAS M1 – ISS 650</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>GAS M3 – ISS 651</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>GAS M6 – ISS 654</td>
<td>negative</td>
<td>positive</td>
</tr>
<tr>
<td>GGS – ISS 3833</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>GBS serotype III - ISS 2601</td>
<td>negative</td>
<td>negative</td>
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</tbody>
</table>

**Fig. 1.** Anti M6 protein serum IgG titres. Mice were immunized with M6 in the absence (no treatment) or in the presence of ISS or CT as systemic (id) or mucosal (in) adjuvant. Data are expressed as mean log₂ reciprocal titres ± SEM of three independent experiments using six mice for each experimental group.
titre than IgG2b. However, the ratio IgG2a/IgG1 (Fig.4) as well as the ratio (IgG2a+IgG3)/IgG1 (data not shown) were >1 when ISS were used both as systemic and mucosal adjuvant, indicating that ISS induce a Th1-like immune response10. On the other hand, CT induced a relative higher synthesis of anti M-6 IgG1 and IgG2a than IgG2b (Fig.3). This pattern of M6 specific IgG subclass and the ratio IgG2a/IgG1 < 1 (Fig.4) confirmed that CT favored a Th2-like immune response.

**Discussion**

The wide range of streptococcal diseases, which include suppurative infections, toxin mediated diseases and non-suppurative sequelae makes it difficult to clearly define the relevance of the different role of specific immunity in protection or autoimmunity induction12-15.

The main GAS virulence factor, M protein, is known to have anti-opsonic activity and some portions of this protein (B repetitive domain) contains autoantibodies inducing-epitopes against cardiac and skeletal tissues. The design of a vaccine to prevent GAS mediated diseases should take into account all these problems and together with the research for suitable antigens, it should also consider the prevalent type of immune response (Th1 or Th2) raised by the antigen-adjuvant combination used.

In the present study it was confirmed that CT is a strong mucosal adjuvant16 for GAS M protein. In addition, we demonstrated that ISS showed strong systemic and mucosal adjuvant activity when co-administered with M protein. Both adjuvants did not require conjugation with the protein to induce significant antibody responses. CT induced a Th2 response, as indirectly demonstrated by the prevalent production of IgG1 over IgG2a specific anti-M protein IgG subclass. On the other hand, the IgG2a/IgG1 ratio was clearly >1 in mice receiving ISS as adjuvants, thus indicating that ISS favor an anti-M protein Th1 immune response. In addition, ISS, but not CT, were shown to strongly induce anti-M protein specific IgG response irrespective of the systemic or mucosal route of vaccination. In conclusion, our study offers a reproducible model of anti-M protein vaccination that could be applied to test new antigenic formulations to induce an anti-GAS vaccination suitable for protection against the different diseases caused by this bacterium.
References


Reprint requests: Dr Roberto Nisini, Lab Batteriologia e Micologia Medica, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161, Rome, Italy
e-mail <r.nisini@ISS.it>