Construction of recombinant polypeptides based on beta antigen C (Bac) protein & their usage for protection against group B streptococcal infection

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Background & objectives: Group B Streptococcus (GBS) is an important human pathogen causing serious diseases of newborn and immunocompromized adults. Approximately 50 per cent of the GBS strains carry and express the gene of Bac antigen which is capable to bind IgA. Gene encoding for the Bac antigen has been cloned and sequenced but actual IgA binding region on the protein has not been detected. The aim of the present work was to localize the region of IgA binding on Bac protein, to evaluate the role of one of the Bac protein regions MLKKIE in IgA binding, and to investigate the ability of Bac based recombinant proteins to generate protective antibodies against GBS infection.

Methods: Recombinant proteins based on beta antigen C were generated after PCR amplification of the fractions of bac gene with the following cloning of the PCR products into expression plasmids. Recombinant peptides were tested for IgA binding by immunoprecipitation and Western blot. One of the recombinant proteins expressing IgA binding was used as an antigen for immunization of mice and for GBS protection studies.

Results: Several bac gene constructs were generated. Their ability to bind IgA varied dramatically depending on the size of the construct and location of the fragment on the bac gene map. The smallest peptide expressing IgA binding was 14 kD in size. Amino acid substitutions in MLKKIE region facilitated IgA binding ability. Immunization of mice with recombinant Bac based peptide induced the appearance of anti-GBS antibody with high affinity level providing protection against GBS infection.

Interpretation & conclusion: Size dependence of Bac based recombinant peptides proved that the effective IgA binding required specific folding of the protein binding IgA. Region MLKKIE could not be considered as region, responsible for IgA binding. Generation of antibodies against Bac based recombinant peptides with high titre and affinity makes these proteins a potent candidates for generating a vaccine against GBS infection.

Key words Group B streptococcus - IgA binding - immunization - streptococcus - vaccine

Group B Streptococcus (GBS) is the major cause of newborn mortality in USA and European countries. In spite of prophylactic procedures introduced in most developed countries, newborn sepsis, pneumonia and meningitis continues to be a major medical and epidemiological problem. Most of the severe newborn infections are caused by the GBS strains with capsule serotype III. Serotypes II and Ib are more commonly found in adults. An increase was seen in GBS diseases among the adults, including the severe cases such as necrotizing fasciitis1-3. One of the GBS protein virulence factors is beta antigen C (Bac antigen)4. The bac gene is present in about 50 per cent of GBS strains and is usually expressed in serotypes II and Ib5. Gene encoding
for Bac protein has been cloned and sequenced. An amino acid region MLKKIE on the molecule of Bac protein was proposed to be responsible for IgA binding. Based on previous findings the present study was carried out to verify the IgA binding region on the Bac protein by generating recombinant peptides and to investigate the functional role of IgA binding peptides as potential vaccines against GBS infection.

**Material & Methods**

**Bacteria and plasmids:** GBS strain SU219 IIbc was obtained from collection of strains in the Department of Molecular Microbiology, Institute Experimental Medicine. This strain was used as source of bac gene for DNA amplification and cloning. The same strain was used for animal studies.

**DNA manipulations:** Routine DNA manipulations such as DNA isolation, digestion, ligation and transformation were performed according to Maniatis et al. DNA primers used for amplification and cloning of the bac gene fragments are shown in Table I. PCR fragments were cloned employing the plasmid vector pGemTEasy (Promega, USA). Plasmid vectors pQE30,31,32 (Qiagen, USA) were used for obtaining of the recombinant proteins in all possible open reading frames.

**Protein studies:** Recombinant proteins were purified by affinity chromatography employing Ni-NTA agarose columns (Qiagen, USA). Purified proteins were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and tested for IgA binding by immunoprecipitation or by Western-blot followed by detection by alkaline phosphatase labeled IgA (Sigma, USA).

Synthetic oligopeptide ITNEDKDSMLKKIEDIRKQA was kindly provided by Dr G.Vlasov, Institute of Bioorganic Chemistry, Russian Academy of Sciences, St.Petersburg.

**Immunization and GBS protection studies:** Recombinant protein p6, carrying IgA binding domain was used for immunization of mice. Animals used for immunization were unbred male mice (16-18 g). Mice were infected subcutaneously with three different doses of p6 peptide 0.3, 0.6 or 1.2 mg/kg followed by second injection of 0.15, 0.3, or 0.6 mg/kg 30 days after the first injection. On day 60 of the experiment mice were infected with two different concentrations of GBS strain SU219 IIbc (3.5x10^7 and 3.5x10^6 cfu) by intraperitoneum injection. Titre of antibodies was determined by ELISA.

**Results**

Several recombinant plasmids, containing the DNA fragments of bac gene were constructed. All these constructs contained authentic or modified MLKKIE region (Table II). For modification of this region EcoRI restriction site was introduced into two primers R2 and F7 (Table I). This modification resulted in substitution of the region MLKKIE with region MLKRIQ in constructs P2, P12 and P5. All the constructs were tested for

| Table II. Construction and characteristics of recombinant peptides based on GBS bac gene sequence |
|---|---|---|---|
| Primers | Size of the insert (bp) | IgA binding activity | Size of the peptide (kD) |
| F5/R6 | p4.2(1500) | ++++ | MLKKIE 35 |
| F4/R6 | p6(840) | +++ | MLKKIE 30 |
| F1/R3 | P10(246) | + | MLKKIE 14 |
| F4/R2 | P2(562) | _ | MLKRIQ 20 |
| F4/R3 | P1(685) | + | MLKRIQ 21 |
| F4/R2 + F7/R3 | P5(685) | ++ | MLKRIQ 22 |
| F1/R2 | P12(123) | _ | MLKRIQ 7 |
expression of recombinant proteins by electrophoresis and for IgA binding by ELISA and immunoprecipitation (Fig. 1). The intensity of IgA binding correlated with the size of the peptide as well as with its location regarding region MLKKIE. The highest degree of IgA binding was demonstrated employing the products of plasmids p4.2 and p6 (35 and 30 kD). Smallest recombinant peptide P12 (7kD) did not bind IgA at all. However, the size was not the only limitation. Relatively large peptide P2 (20kD) with modified MLKKIE region at the C end also did not bind IgA and peptide P1 (21 kD) with authentic MLKKIE region was able to bind IgA poorly. Synthetic oligopeptide, containing MLKKIE region did not bind IgA in any experimental set up. Interestingly, the peptide P5 which differs from peptide P1 only by two amino acids in MLKKIE region expressed relatively high level of IgA binding both in ELISA and immunoprecipitation (Fig. 1). The smallest peptide, capable to bind IgA was P10 (14kD), but the level of binding was low and highly depended on temperature and buffer conditions.

The peptide P6 used for immunization being administrated in three doses gave the maximum rise of antibodies on day 48 from the first injection (Fig. 2) - 1:25000 for the smallest dosage and 1:100000 for the second and third dosages. Affinity of the anti-Bac antibodies was the highest (constant of dissociation was equal to 10^{13} per M) for the smallest dosage of the antigen and minimal for the highest antigen dose (10^8 per M). The second dose of antigen (0.6 mg/kg on the first injection) gave rise to the antibodies with intermediate level of affinity (10^9 per M). This dosage of the antigen was considered the best dose for mice protection studies.

GBS from strain SU219 being administrated in two different concentrations 10^7 and 10^6 cfu was highly virulent for the mice causing significant level of lethality during two first days of infection (Table III). Immunized animals demonstrated a significant level of protection which was more distinct when lower bacterial concentration (10^6 cfu) was used.

**Discussion**

Bacterial proteins capable to bind human immunoglobulins are fairly common for pathogenic bacteria. Most of these proteins such as IgG binding staphylococcal protein A or streptococcal protein G share structural organization and these proteins are often homologous on DNA or amino acid level. Bac protein from GBS does not have any homologues among bacterial proteins discovered so far. The only homologoue to Bac protein found so far was among the proteins belonging to the superfamily of eukaryotic immunoglobulins. This protein is capable to bind Fc portion of IgA which makes bacteria invisible for the host immune system. This fact seems to be vitally

![Fig.1. IgA binding by different concentrations of recombinant peptides – derivatives of Bac protein. Peptides were administrated in 3 ml drops in two times dilutions on nylon filter paper. Starting concentration was 1 mg/ml for all the peptides. Later on the filter was incubated with myeloma IgA and treated with alkaline phosphatase labeled anti-IgA IgG.](image)

![Fig.2. Immune response after immunization with recombinant Bac peptide P6.](image)

**Table III.** Protective effect of Bac antigen against GBS infection (cfu/ml in spleen)

<table>
<thead>
<tr>
<th>Time after GBS infection</th>
<th>Immunized mice (cfu)</th>
<th>Control group (cfu)</th>
</tr>
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<tr>
<td></td>
<td>s3.5x10^7</td>
<td>3.5x10^6</td>
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<tr>
<td>6 h</td>
<td>1x10^2</td>
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<tr>
<td>1 day</td>
<td>3.5x10^7</td>
<td>1x10^6</td>
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<tr>
<td>2 day</td>
<td>1.4x10^8</td>
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important for GBS colonizing rectum and vagina where IgA represents about 90 per cent of total immunoglobulins, and provides defense against infection due to specific recognition of the antigens and due to recruitment macrophages to the site of infection and binding to Fc, RI and CD89 receptors. Recent studies on the Ca2 (LLG) and Ca3 (PLAF) loops on the IgA molecule demonstrated that Bac was capable to bind with both regions interfering with loops on the IgA molecule. The requirement of the proper three-dimensional folding of the IgA binding protein explains why the smallest IgA binding peptide P10 was not able to provide efficient and stable binding of the IgA. Analysis of the region MLKKIE as potential IgA binding site clearly demonstrated that this region alone or even together with couple of amino acids on both ends was not sufficient for IgA binding. Experiments with introducing the amino acid substitutions in MLKKIE region which improved the IgA binding level can be explained by the modulatory function of this motif in the process of binding IgA.

Another set of experiments was related to the analysis of immune response to the Bac based peptides. Considering that Bac antigen is not expressed by all GBS strains we planned to use this peptide as a component of the conjugative peptide vaccine, which consists of several GBS immunogenic and protective peptides. Administration of different doses of Bac peptide to laboratory animals demonstrated that recombinant peptide used was highly immunogenic which was comparable to the effect of other streptococcal proteins candidates for vaccine production. This fact together with a good protection against GBS infection makes it possible to consider Bac protein based peptides as candidates for generating an efficient conjugative peptide vaccine against GBS infection.

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References


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