Monoclonal antibodies with specificities for
Streptococcus pneumoniae group 9 capsular polysaccharides

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Abstract

Streptococcus pneumoniae group 9 includes four capsular polysaccharide types: 9A, 9L, 9N and 9V. We have generated four mouse monoclonal antibodies against group 9 polysaccharide using heat-treated S. pneumoniae strains of different capsular polysaccharides types as immunogens. The specificities of the monoclonal antibodies were determined by ELISA using capsular polysaccharide directly coated to the wells as antigens and by dot blotting with heat-treated bacteria. Two groups of monoclonal antibodies were found. The first group included two monoclonal antibodies which were found to be capsular type specific. The second group was monoclonal antibodies that bound to epitopes shared by two or three pneumococcal group 9 types. The monoclonal antibody 204,A-4 (IgM) was found to be specific for S. pneumoniae type 9N. The binding of the type 9V specific monoclonal antibody 206,F-5 (IgG1) was found to be dependent upon O-acetyl groups. Monoclonal antibody 205,F-3 (IgM) reacted also with type 9V, but was found to cross-react with types 9A and 9L. The binding of this monoclonal antibody to polysaccharide 9V was not dependent upon O-acetyl moieties. The fourth monoclonal antibody (214,G-5, isotype IgM) did not show any correlation between reactivity with isolated polysaccharides and dot blotting with relevant bacteria. The monoclonal antibody reacted with polysaccharides 9A and 9L in ELISA, but not with the homologous bacteria. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

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1. Introduction

Infections caused by Streptococcus pneumoniae still remain a major cause of morbidity and mortality throughout the world, in particular among infants and elderly people. Capsular polysaccharides (PS) are essential virulence determinants and are used for classification of pneumococci into 90 types [1,2]. This organism also has a common cell wall PS of which phosphorylcholine is the immunologically dominant epitope. When mice are immunised with pneumococci one of the most prominent antibody responses is to this phosphorylcholine determinant. This may be the reason why few reports on monoclonal antibodies (mAbs) against pneumococcal capsular PS and protein antigens are published. Free capsular PS are assumed to be poor immunogens in mice although the 23-valent pneumococcal PS...
vaccine has been demonstrated to yield mAbs with specificities for types 4, 8, 19A/19F and 22F [3].

We have previously reported the production of mAbs against \textit{S. pneumoniae} proteins by immunisation with heat-treated bacteria and screening of hybridoma supernatant fluids in ELISA against the immunising pneumococcal strain and against cell wall PS [4,5]. A similar strategy has been used to produce mAbs against \textit{S. pneumoniae} group 9 capsular PS. The group 9 organisms are classified into four capsular types: 9A, 9L, 9N and 9V. The two dominating types 9N and 9V are included in the currently used 23-valent capsular PS vaccine. This vaccine is not immunogenic in children under 2 years of age. The immunogenicity of PS can be greatly improved by coupling them to a protein carrier, which allows T cell-dependent help to be induced. The success with the conjugated PS vaccines in preventing \textit{Haemophilus influenzae} disease in children younger than 2 years of age has greatly stimulated the interest in developing pneumococcal PS-protein conjugate vaccines. mAbs against group 9 PS would be useful typing reagents and also valuable tools for epitope analyses after PS coupling to a carrier protein. To date, there has been only one report on mAbs against group 9 PS and this mAb reacted with all types within this group [6]. The study here describes four mAbs with individual specificity patterns for group 9 pneumococci.

2. Materials and methods

2.1. Bacterial strains

Pneumococcal strains from human clinical isolates were typed by the capsular reaction test with rabbit antisera purchased from Statens Serum Institut, Copenhagen, Denmark. Differentiation of types within groups was carried out at Statens Serum Institut, Copenhagen. This institute also supplied us with \textit{S. pneumoniae} strains of types 9A and 9L.

2.2. Polysaccharides

Pneumococcal capsular PS which are components of the licensed 23-valent vaccine were obtained from American Type Culture Collection. Capsular PS of types 9A and 9L were isolated and purified by chromatography on DEAE-cellulose followed by Sepharose CL4B [7]. Purity was further confirmed by one- and two-dimensional NMR at 500 MHz [8]. Purified cell wall PS from \textit{S. pneumoniae} was a gift from Dr. Jørgen Henrichsen, Statens Serum Institut, Copenhagen.

2.3. Alkaline treatment of PS

The PS 9V O-deacylated sample was prepared by treatment of the PS with ammonia (pH 12, 18 h, 4°C). Ammonia was evaporated under a stream of N$_2$. Distilled water was added to obtain the original volume.

2.4. Production of mAbs

Heat-treated (30 min at 56°C), sonicated pneumococcal strains of capsular types 9A, 9L, 9N and 9V were used as immunogens. Six-week-old female BALB/c mice were immunised with bacterial suspensions containing 50 µg protein in 0.25 ml saline with 0.25 ml Freund’s incomplete adjuvant, followed by a booster injection 2 weeks later with the same mixture. The hybridoma cell line 204,A-4 was made in a fusion performed 9 weeks after the primary injections with the type 9N strain. Three days before fusion the mouse was injected intraperitoneally with the type 9N suspension containing 50 µg protein in 0.3 ml saline. Hybridoma cell lines 205,F-3 and 206,F-5 were from one fusion performed 5 months after the primary injections with the type 9V strain. Four days before fusion the mouse was given intraperitoneally a suspension of the 9V strain containing 50 µg protein in 0.3 ml saline. The hybridoma cell line 214,G-5 was from one fusion performed 3 weeks after the primary injections with a type 9L strain. Three days before fusion the mouse was given intraperitoneally 50 µg protein of the 9L strain in 0.3 ml saline. Spleen cells were fused with NSO myeloma cells by standard methods. Mouse splenocytes were used as feeder cells.

Hybridoma supernatant fluids were screened in ELISA (see below) against capsular PS homologous to the immunising pneumococcal strain and against cell wall PS. Those which reacted only with capsular PS were expanded and tested against different group
9 PS. Hybridoma cells were cloned by limiting dilution with Hybridoma Enhancing Supplement (Sigma Chemical Company, St. Louis, MO, USA) instead of feeder cells. Isotyping of mAbs in hybridoma culture fluids was performed in ELISA with capsular PS as coating antigen (see below) using a kit from Zymed Laboratories Inc., South San Francisco, CA, USA.

2.5. Protein assay

For determination of protein concentrations, aliquots of the bacteria were dissolved in 0.5 M NaOH. The method described by Lowry et al. [9] was used with BSA as standard.

2.6. ELISA

Flat-bottomed microtitre plates (MaxiSorp®, Nunc A/S, Roskilde, Denmark) were coated overnight at 4°C with PS (2 μg ml⁻¹) in PBS (pH 7.4) with 0.02% sodium azide, 50 μl per well. Hybridoma culture supernatants were added in 50-μl aliquots and incubated for 2 h at 37°C. Alkaline phosphatase-conjugated goat anti mouse-Ig (Sigma Chemical Company, St. Louis, MO, USA) was used at a dilution of 1/2000. p-Nitrophenyl-phosphate was used as the substrate with 1 mg ml⁻¹ in 10% diethanolamine buffer (pH 9.8) containing 5 mM MgCl₂. The washes between each step were performed with PBS plus Tween 20 (0.05%). The antibody dilutions were prepared in PBS containing 3% BSA.

2.7. Dot blot assay

Heat-treated bacteria (30 min at 56°C) from the stationary phase of growth were spotted to nitrocellulose strips as previously described [3]. The primary antibodies were used as diluted hybridoma supernatant fluids and bound mAbs were detected with peroxidase-conjugated rabbit anti-mouse Ig (Dako A/S, Glostrup, Denmark, dilution 1/1000). The immunostaining was performed with 3-amino-9-ethylcarbazole and H₂O₂ in sodium acetate buffer (pH 5.0).

Fig. 1. Structures of the repeating units of S. pneumoniae group 9 PS as reported by Jones et al. [10] (type 9N), Richards et al. [12] (type 9L), Richards and Perry [13] (type 9A) and Perry et al. [11] and Rutherford et al. [7] (type 9V).
3. Results and discussion

3.1. mAb 214,G-5 reacts with PS 9A and 9L, but not with homologous bacteria

The chemical structures of the group 9 PS are shown in Fig. 1. Each group 9 PS is a high molecular mass polymer with a linear pentasaccharide repeating unit [7,10–13]. Residues are labelled A–E for convenient specification of atom numbering.

mAb 214,G-5 (IgM) was developed after immunisation with a type 9L S. pneumoniae strain. The mAb reacted in ELISA both with PS types 9A and 9L (Fig. 2). PS 9A and 9L are not included in the 23-valent pneumococcal vaccine. Residues B, C, D and E are common between PS 9A and 9L (Fig. 1) and we propose that the epitope for mAb 214,G-5 is located within these residues. The residues B, C, D and E shared between PS 9A and 9L are also found in PS 9V, but with a complex pattern of O-acetylation (Fig. 1). However, removal of the O-acetyl groups did not result in binding of the mAb to PS 9V.

Dot blotting was performed with S. pneumoniae representing all types in group 9 (six strains of type 9V, four of 9N and one strain each of 9A and 9L). Some strains outside this group were also included. As expected, all strains reacted with mAb 147,A-1 which is specific for the phosphorylcholine determinant found in all pneumococci [14]. Unexpectedly, mAb 214,G-5, which reacted in ELISA with both PS 9A and 9L, showed negative dot blot reactions with the homologous pneumococci including the 9L strain used for immunisation (Fig. 3). This was also the case when heat-treated pneumococci were used as coating antigen in ELISA. This raised the question of the specificity of the ELISA assay with PS 9A or 9L as coating antigens. Competitive inhibition was therefore performed. The antibody at a dilution of 1:32 was incubated with each group 9 PS in the concentration range of 0.001–1 mg ml$^{-1}$ overnight at room temperature. The samples were then added to ELISA plates with PS 9A or 9L as coating antigens. The negative controls (PS 9N and 9V) did not inhibit mAb binding. A dose-dependent inhibition of mAb binding in the presence of PS 9A or 9L revealed that
the ELISA binding of this mAb was antigen specific (data not shown). One possible explanation for the lack of mAb binding to pneumococci in the dot blot assay might be that parts of PS 9A and 9L essential for binding of mAb 214,G-5 are not surface exposed even in heat-treated bacteria. Antigenic processing of *S. pneumoniae* in mice exposes these capsular structures resulting in antibody production. This is the only mAb we have developed against pneumococcal PS with such an unexpected pattern of reactivity. It seems to be important to analyse mAbs against bacterial PS not only against isolated PS, but also against homologous bacteria.

Two fusions were performed after immunisation with a *S. pneumoniae* group 9A strain. Only one clone was selected and this clone produced antibodies showing the same pattern of reactivity as mAb 214,G-5 (immunising strain 9L) in ELISA with each of the PS in the 23-valent vaccine as coating antigens. Due to this similarity and the finding that this mAb was also of isotype IgM, the clone was discarded. Thus, three fusions performed with spleen cells from mice immunised with either 9A or 9L strains did not result in type-specific mAbs.

3.2. mAb 204,A-4 is specific for *S. pneumoniae* type 9N

The mAb 204,A-4 (IgM) reacted in ELISA with different group 9 PS as coating antigens only with type 9N PS (Fig. 2). This specificity was confirmed by dot blotting with different bacteria (Fig. 3). The dot blot assay has recently been tested in 1082 isolates and comparison to the standard quellung assay showed the assay to be specific and sensitive [15]. The structure of type 9N differs from all the other three types in the configuration of C-4 in the residue C (Glc in 9N, Gal in 9A, 9V and 9L). This Glc residue in PS 9N seems thus to be essential for mAb binding. This mAb can be used as a typing reagent in the dot blot assay. Unlike the three other group 9 types which reacted with two of our mAbs, PS 9N and type 9N pneumococci were only detected by mAb 204,A-4. Therefore only one epitope expressed in PS 9N was recognised by our mAbs.
3.3. mAb 205,F-3, raised against a 9V strain, cross-reacts with types 9A and 9L

Two mAbs of different isotypes and specificities were generated in a fusion with spleen cells from a mouse immunised with a type 9V strain. mAb 205,F-3 (IgM) reacted in ELISA with 9V capsular PS, but cross-reacted with 9L at high antibody concentrations (Fig. 2). Dot blotting with the mAb showed a different cross-reaction pattern by immunostaining with \textit{S. pneumoniae} of types 9L and 9A in addition to 9V. The non-reactivity by mAb 205,F-3 in ELISA with PS 9A cannot be due to lack of binding of these PS to the microtitre plates used in our study because mAb 214,G-5 reacted with PS 9A (Fig. 2). One possible explanation for the difference seen by ELISA compared with dot blotting might be that isolated 9A PS bound to the wells in the ELISA plates has a different conformation than in heat-treated bacteria. We assume that PS 9A and 9L each have two different epitopes as seen by the differences in ELISA and dot blot reaction patterns by mAb 205,F-3 (immunising strain 9V) and mAb 214,G-5 (immunising strain 9L). The residue C (Gal) shared between the reacting types 9V, 9L and 9A is probably essential for binding of mAb 205,F-3 because in the non-reacting group 9N this residue is replaced with Glc (Fig. 1). Comparison of the structures of PS 9V and 9A shows that 9V is an O-acetylated 9A sequence, with a complex pattern of O-acetylation (Fig. 1). Removal of O-acetylated moieties from PS 9V showed some reduction in the degree of mAb binding (Fig. 2). The mAb produced by Lee et al. [6] against PS 9V conjugated to inactivated pneumolysin also showed decreased binding after removal of O-acetyl groups, but this mAb reacted with all four group 9 PS.

3.4. Binding of the type 9V specific mAb 206,F-5 is dependent upon O-acetyl groups

mAb 206,F-5 (IgG1) was shown to be type 9V specific by its reactivity in ELISA with different group 9 capsular PS (Fig. 2) and by its dot blot staining pattern with heat-treated pneumococci (Fig. 3). Type 9V is an O-acetylated 9A sequence, with a complex pattern of O-acetylation. Unlike the other PS 9V reacting mAb 205,F-3, this mAb did not cross-react with PS of type 9A. Removal of the O-acetyl groups from the 9V PS by alkaline treatment showed that the binding of mAb 206,F-5 was dependent on the O-acetyl groups. This further supports different epitopes for the two mAbs developed after immunisation with a pneumococcal type 9V strain. The 206,F-5 mAb can be used as a typing reagent in the dot blot assay.

The currently used 23-valent pneumococcal PS vaccine contains two (9N and 9V) of the four types within group 9. This vaccine is less immunogenic in young children than in adults [16]. The immunological properties can be improved by coupling the pneumococcal capsular PS to a carrier protein. It would not be possible to include all 23 different capsular PS in a conjugate vaccine. PS 9V is proposed to be part of a nonavalent conjugate vaccine for global use [17]. Our 9V-specific mAb was dependent upon the presence of O-acetyl groups. The methods used for cleaving 9V PS into oligosaccharides, activation and the coupling procedure should therefore not destroy the O-acetyl groups.

In summary, our studies with a panel of mAbs generated against different group 9 pneumococcal strains indicate that the repertoire of antibodies includes diverse specificities. The mAbs were either capsular type specific or directed against epitopes shared among two or three types.

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References

pneumococcal capsular polysaccharide types 4, 8, 22F and 19A/19F. APMIS 100, 91^94.


