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Epitopes for human monoclonal antibodies and serotyping antisera against the O-specific polysaccharide of *Pseudomonas aeruginosa* O11

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Abstract

Epitopes for *Pseudomonas aeruginosa* O11-specific human monoclonal antibodies (mAbs) and O11 serotyping antisera have been characterized. These mAbs recognized the O-polysaccharide portion of the lipopolysaccharide. The structure of the O-polysaccharide of O11 has been reported to be comprised of trisaccharide repeating-units as follows: →3)-α-L-Fuc*p*NAc-(1→3)-β-D-Fuc*p*NAc-(1→2)-β-D-Glc*p*-(1→. (Fuc*p*NAc, 2-acetamido-2,6-dideoxygalactopyranoside.) Data from inhibition studies of binding in enzyme-linked immunosorbent assays and cell-agglutination assays, using monosaccharides and periodate-oxidized O-polysaccharide showed that the glucose residue, especially the C-3–C-6 segment and the β-anomeric configuration, in the polysaccharide is essential for the epitopes of all anti-O11 mAbs; however, the detailed epitope specificities were different from one another. Furthermore, epitopes for serotyping antisera of O11 seemed to be similar to those for the human mAbs.

1. Introduction

Pseudomonas aeruginosa is one of the causative factors of opportunistic infection. *P. aeruginosa* is sometimes refractory to chemotherapy, and so immunotherapy by active or passive immunization has been considered as an alternative. Many researchers have prepared human monoclonal antibodies (mAbs) against *P. aerug-*

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inosa for therapeutic use, and the O-serotype-specific mAbs have been shown to have potent protective activities [1–6]. We have also been screening human mAbs against *P. aeruginosa* using peripheral blood lymphocytes derived from healthy adult volunteers as a lymphocyte source [7]. We found that serotype O11-specific human mAbs were obtained with a significantly higher frequency than the other serotype-specific mAbs (unpublished results). In epidemiological studies, serotype O11 (corresponding to Homma type E, Fisher type 2 [8]) is one of the most common serotypes in clinical specimens, and strains of serotype O11 have often been associated with outbreaks of infection in hospitals, drug-abuse related sepsis, and folliculitis [9]. The complete structure for the O11 O-polysaccharide had been reported [10–12]. The polysaccharide contains the enantiomers *N*-acetyl-D- and -L-fucosamine (2-acetamido-2,6-dideoxygalactose), which have not been found in mammalian sources, and D-glucose, in equal molar ratio. We are interested in the antigenicity of the serotype O11 O-polysaccharide and have here characterized the epitopes for five serotype O11-specific human mAbs and O11 serotyping antisera.

2. Experimental

Human mAbs.—Human mAbs were prepared using peripheral blood lymphocytes derived from healthy adult volunteers by the following two methods. The first is Epstein–Barr virus (EBV)-transformation, followed by cell fusion with mouse myeloma P3 × 63-Ag.8.653 according to previous work [13]. The second is the direct cell-fusion method as previously described [14]. Briefly pokeweed mitogen-stimulated peripheral blood lymphocytes were fused with human-mouse heteromyeloma SHM-D33 using polyethylene glycol. The serotype O11-specific mAbs used in this study are summarized in Table 1. An anti-LPS-core mAb FK-2E7, which reacted with ~ 90% of the serotype O11 strains and other serotype strains, has been previously described [15]. The hybridomas were adapted in serum-free medium Celgrosser H (Sumitomo Pharmaceuticals, Osaka, Japan). The mAbs were purified by gel-filtration chromatography on Superose 6 (Pharmacia, Uppsala, Sweden) for IgM and Protein A-Cellulofine (Seikagaku Kogyo Corp., Tokyo, Japan) chromatography for IgG from the concentrated culture supernatant.

Antisera—Serotyping antisera were purchased from Difco (Detroit, MI, USA) and Denka Seiken (Tokyo, Japan). The typing schema of the antisera were according to the international antigenic typing scheme [8] (type 11) and Homma's classification [16] (type E), respectively.

Bacterial strains.—Serotype standard strains, Fisher type 2 (ATCC 27313) and Habs O11 (ATCC 33358) were obtained from the American Type Culture Collection. These strains are classified into serotype O11 and chemotype 26 as reported by Liu et al. [8] and Stanislavsky et al. [17], respectively. The O-polysaccharide of chemotype 26 [10–12] comprises a trisaccharide repeating unit of: → 3)-α-L-Fuc*p*NAc(1 → 3)-β-D-Fuc*p*NAc(1 → 2)-β-D-Glc*p*(1 → (**1**). (Fuc*p*NAc: 2-

acetamido-2,6-dideoxygalactopyraoside; *N*-acetylfucosamine). A standard strain of Homma type E (IID 1130) was obtained from the Institute of Medical Science, University of Tokyo (Tokyo, Japan).

Preparation of LPS and O-polysaccharide.—LPS was extracted from bacterial cells by the hot phenol–water extraction method reported by Westphal and Jann [18]. LPS was recovered from the phenol layer. After dialysis of the phenol layer against deionized water, the insoluble material was removed by the centrifugation at 3000 rpm for 15 min. The resulting soluble material was used for LPS preparation. The LPS was hydrolyzed with 1% AcOH at 100°C for 90 min, and the resulting soluble material was fractionated using Sephadex G-50 column chromatography [19]. The polysaccharide fraction excluded from the column was used as the O-polysaccharide preparation. The rough (R)-core oligosaccharide fraction was further purified by Biogel P-6 chromatography as reported by Koval and Meadow [20].

Periodate oxidization of polysaccharide.—The O-polysaccharide preparation (20 mg) was oxidized with 0.1 M $NaIO_4$ in NaOAc buffer (pH 5.0) for 4 days at 4°C in the dark. After decomposition of excess $NaIO_4$ by adding 1,2-ethanediol, the sample was reduced with 1 M $NaBH_4$ for 8 h at 37°C. The resulting material was dialyzed against deionized water, lyophilized, and used as the periodate-oxidized O-polysaccharide preparation (12 mg).

Enzyme-linked immunosorbent assay (ELISA).—The methods for ELISA and competitive ELISA have been previously described [14]. Briefly, bacterial cells were fixed on a 96-well microtiter plate with 1% glutaraldehyde. Alkaline phosphatase-conjugated goat anti-human IgG or IgM antibodies (KPL, Gaithersburg, MD), or alkaline phosphatase-conjugated goat anti-rabbit IgG (H + L) antibodies (ICN, Costa Mesa, CA), and sodium *p*-nitrophenylphosphate were used as a second antibody and a substrate, respectively. For competitive ELISA, equal volumes, of the mAb solution at a concentration giving half maximum absorbance (10 to 50 ng/mL) in phosphate-buffered saline containing 0.05% Tween 20, and various concentrations of saccharide solution were mixed, and then subject to assay.

Western blot analysis.—SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli [21]. LPS on the gel was detected by the silver staining method [22]. Electrophoretic transfer to a polyvinylidene fluoride filter and immunoblotting were carried out as previously described [15]. The blot was incubated with alkaline phosphatase-conjugated human IgG or IgM antibodies, and then made visible with bromochloroindoyl phosphate–nitroblue tetrazolium.

Bacterial cell agglutination.—The viable bacterial cell suspension ((50 μL), 0.2 of A_{600}), serial dilution of mAb solution (50 μL), and serial dilution of saccharide solution (50 μL) were mixed, and then incubated at 4°C overnight. Agglutination activity was expressed as the minimum concentration of mAb required for agglutination.

Other materials and methods.—Monosaccharides and methyl glycosides were purchased from Nacalai Tesque (Kyoto, Japan) and Sigma (St. Louis, MO). The IgG subclass was determined by using a Human IgG 1-4 EIA Combi Kit (Binding

Site Ltd., Birmingham, UK). Other materials and methods were described in previous work [14,15].

3. Results and discussion

We have established five human–mouse heterohybridoma cell lines stably producting *P. aeruginosa* serotype O11-specific human mAbs, three IgM, and two IgG2 clones (Table 1). From the results of binding to cells of ~ 200 *P. aeruginosa* strains from various sources tested in ELISA, the mAbs reacted to all strains of serotype O11, but did not react to any strains of the other serotypes tested (data not shown). In Western blot analysis using the LPS preparation derived from the Habs O11 strain (Fig. 1), ladder or smear bands in the higher molecular-weight region were detected by staining with the O11-specific mAbs. However, lower molecular-weight bands, which were attributed to the R-type LPS carrying no or only a short O-polysaccharide chain, were not stained with the mAbs. Furthermore, in competitive ELISA, the O-polysaccharide preparation, as well as the LPS preparation, inhibited the binding of the mAbs to Habs O11 cells, but the R-core oligosaccharide preparation did not (Fig. 2). These results indicated that these mAbs recognized the O-polysaccharide portion of the serotype O11 LPS and O11-specific epitope(s).

The structure of the serotype O11 O-polysaccharide has been reported [10–12], along with the component trisaccharide repeating-unit (**1**). To define the epitope for each human mAb, we carried out a binding-inhibition study using monosaccharides and a chemically modified O-polysaccharide. Figs. 2 shows the binding activity of the periodate-oxidized O-polysaccharide during competitive ELISA. Periodate oxidation selectively cleaved the C-3–C-4 bond of the 2-substituted glucose residue. All of the mAbs showed little binding activity to the periodate-oxidized polysaccharide as compared with the native one.

In the inhibition study using monosaccharides (Table 2), glucose and methyl β-glucopyranoside were found to be potent inhibitors of all O11-specific mAbs. In contrast, binding of an anti-LPS-core mAb FK-2E7 was not inhibited by any monosaccharides and methyl glycosides tested, even at a concentration of 500 mM

Table 1
Human mAbs against *P. aeruginosa* serotype O11

Clone	Class/ light chain	Establish method [a]	Productivity ($\mu g/10^6$ cells day)
HI-223	IgM(λ)	EBV/fusion	30
SS-003	IgM(κ)	EBV/fusion	10
YK-3D4	IgM(λ)	fusion	8
MK-2F4	IgG2(λ)	fusion	10
OM-2D2	IgG2(κ)	fusion	4

[a] EBV/fusion: EB virus transformation followed by cell fusion with P3X63Ag 8.653; fusion: cell fusion of pokeweed mitogen-activated human peripheral blood lymphocytes with SHM-D33.

Fig. 1. Western blot analysis of *P. aeruginosa* serotype O11-specific human mAbs using LPS preparation derived from the Habs O11 strain. LPS preparation (3 μg/lane) was applied to a 15% polyacrylamide gel. Each lane showed silver staining (A), and immunoblotting with HI-223 (B), SS-003 (C), YK-3D4 (D), MK-2F4 (E), and OM-2D2 (F).

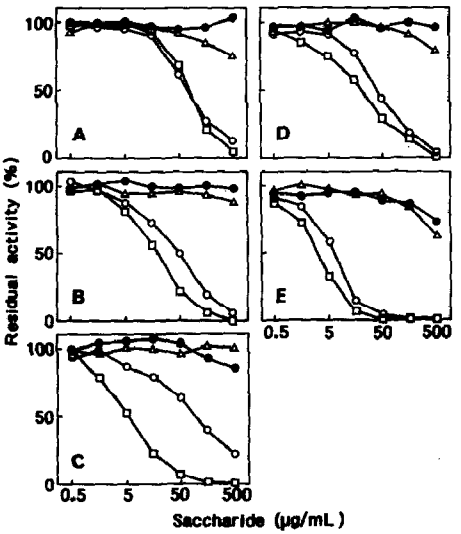

Fig. 2. Binding of *P. aeruginosa* serotype O11-specific human mAbs to LPS (□), native (○) and periodate oxidized (●) O-polysaccharide, and R-core oligosaccharide (△) preparations derived from the Habs O11 strain in competitive ELISA. The mAbs were HI-223 (A), SS-003 (B), YK-3D4 (C), MK-2F4 (D), and OM-2D2 (E). The cells of the Habs O11 strain were used as a coated antigen in ELISA.

Table 2
Competition of monosaccharides and methyl glycosides with the binding of serotype O11-specific human mAbs to *P. aeruginosa* Habs O11 cells in ELISA

Sugar	Sugar concentration giving 50% inhibition (mM) [a]				
	HI-223	SS-003	YK-3D4	MK-2F4	OM-2D2
Glc	28	270	> 500 (29%)	78	230
Me α-Glc*p*	> 500 (24%)	> 500	> 500	94	> 500 (28%)
Me β-Glc*p*	8.8	160	14	90	160
GlcNAc	52	> 500 (31%)	> 500	56	190
Gal	> 500	> 500	> 500	> 500	> 500
GalNAc	> 500	> 500	> 500	> 500	> 500
Man	86	> 500 (26%)	> 500	110	370
Me α-Man*p*	210	> 500	> 500	> 500 (11%)	> 500 (13%)
Me β-Man*p*	25	460	> 500 (47%)	200	> 500 (47%)
ManNAc	6.0	> 500 (33%)	> 500	145	> 500 (43%)
Qui	54	110	> 500	> 500	> 500
Rha	> 500 (36%)	> 500	> 500	> 500	> 500
D-Fuc	> 500 (42%)	> 500	> 500	> 500	> 500
L-Fuc	17	> 500	> 500	> 500	> 500 (45%)
Me α-L-Fuc*p*	110	260	> 500	> 500	> 500
Me β-L-Fuc*p*	120	120	> 500	> 500	> 500

[a] Values in parentheses indicate inhibition percent at a competitor concentration of 500 mM, in the case when the inhibition was less than 50% even at 500 mM. For sugars, which showed less than 10% inhibition even at a concentration of 500 mM, inhibition values are not presented. Qui: D-guinovose, Rha: L-rhamnose, Fuc: fucose.

(data not shown) [15]. Monosaccharides such as glucose and methyl β-glucopyranoside, which were shown to be potent inhibitors in ELISA, also inhibited bacterial-cell agglutination (Table 3). Therefore, the inhibitions in the competitive ELISA were considered to be specific for the antigen binding. These results indicated that the glucose residue of the O-polysaccharide was essential for the epitopes for all mAbs.

Table 3
Inhibition of *P. aeruginosa* O11 cell agglutination by serotype O11-specific human mAbs with monosaccharides

Clone	Minimum concentration for agglutination in presence of (μg/mL) [a]							
	none	Glc 300 mM	Me β-Glc			GlcNAc 300 mM	Gal 300 mM	Man 300 mM
			300 mM	150 mM	75 mM			
HI-223	0.63	1.25	> 10	5.0	1.25	1.25	0.63	0.63
SS-003	0.63	0.63	1.25	0.63	0.63	0.63	0.63	0.63
YK-3D4	0.63	0.63	> 10	> 10	2.5	0.63	0.63	0.63
MK-2F4	0.63	0.63	1.25	1.25	0.63	1.25	0.63	0.63
OM-2D2	0.63	0.63	1.25	0.63	0.63	1.25	0.63	0.63

[a] Two-fold serial dilution of the human mAbs were incubated with cell suspension of the Habs O11 strain and competitor solution. Agglutinating activity was expressed as the minimum concentration of mAbs required for the agglutination.

Furthermore, all of the mAbs except YK-3D4 showed binding inhibition upon addition of *N*-acetylglucosamine and sugars of the *manno* configuration, as well as *gluco* sugars. Comparison of the structures of these inhibitors shows that *N*-acetylglucosamine is a derivative of glucose, having the C-2 hydroxy group replaced by an acetamido group, and the sugars of the *manno* configuration are C-2 epimers of the *gluco* analogues. Thus, the C-2 region of the glucose residue in the O-polysaccharide does not seem to be important for the epitope. In fact, the glucose residue in the O-polysaccharide is substituted at O-2. The results indicated that the C-3–C-6 region of the glucose structure is important for an epitope for the mAbs. As the detailed specificity of each mAb in the inhibition study was different (Table 2), epitopes for the mAbs seemed to be different from each other. For example, YK-3D4 recognized the methyl β-glucopyranoside structure the most strictly. MK-2F4 was less demanding of the anomeric configuration and *gluco* configuration than another IgG clone, OM-2D2. For HI-223, *N*-acetylmannosamine was the most potent inhibitor. For SS-003, quinovose and methyl β-L-fucopyranoside were potent inhibitors. However, we were not able to explain what structure of the epitope for these mAbs reflected the monosaccharide inhibition; the D-glucose residue, especially the C-3–C-6 segment and β-anomeric linkage of the O-polysaccharide seemed to be important for the epitope for HI-223 and SS-003, as well as other O-11 specific mAbs. Inhibition by 6-deoxyhexoses and their methyl glycosides could be caused by the *N*-acetyl-D-fucosamine or *N*-acetyl-L-fucosamine residues being involved in the epitope. However, we were not able to determine the inhibitory activity of *N*-acetyl-(D- and L-)fucosamine, because sufficient amounts of these sugars were not available. Similar results to those described, which arose from using the Habs O11 strain, were observed using other strains belonging to the type O11, such as the standard strains of Fisher 2 and Homma E as an antigen.

We also used commercial serotyping antisera to the ELISA-inhibition study using the LPS preparation derived from the standard strain of Habs O11 as a coated antigen (Fig. 3). For two different serotyping antisera, similar results with the human mAb studies were obtained. The periodate-oxidized O-polysaccharide did not show an inhibitory effect, in contrast to the intact O-polysaccharide preparation. Furthermore, glucose and methyl β-glucopyranoside were potent inhibitors for both antisera, whereas *N*-acetylglucosamine, *N*-acetylmannosamine, L-fucose, and D-fucose inhibited the binding only weakly. The results indicated that the β-glucose residue in the O11 O-polysaccharide was important for the epitope not only for the mAbs but also the O11-specific polyclonal antibodies.

We have tried here to define the epitope of human mAbs and antisera against *P. aeruginosa* serotype O11. However, the epitopes for each human mAbs are different from each other, as indicated by the monosaccharide inhibition study (Table II). The glucose residue of the O-polysaccharide seemed to be essential as a part of the epitopes for all serotype O11-specific mAbs tested and typing antisera against O11; in particular the C-3–C6 segment and β-anomeric configuration of the residue were important for each epitope, as shown in Fig. 4. Interestingly, the L-FucNAc-D-FucNAc unit, which is characteristic of the O11 O-polysaccharide and

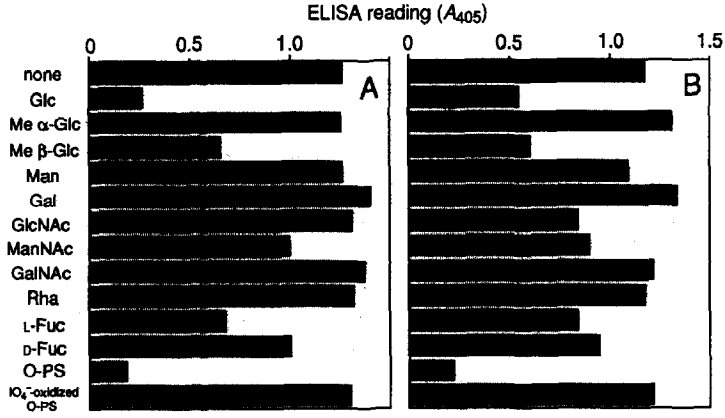

Fig. 3. Competitive ELISA of monosaccharides and polysaccharide preparations with the binding of serotype E (Homma) (A) and O11 (IATS) (B) typing sera to LPS derived from the *P. aeruginosa* Habs O11 strain. Diluted antisera were mixed with 500 mM of monosaccharides or methyl glycosides, or 500 μg/mL of O-polysaccharide (O-PS) or periodate-oxidized O-polysaccharide (IO_4^--oxidized O-PS) preparation, and then applied to ELISA.

has not been found in mammalian sources, appears to be less important for the epitope of the O11 O-polysaccharide than the glucose residue. The mAbs, as well as typing antisera, did not show binding activity to the periodate-oxidized O-polysaccharide preparation, whose glucose residue had been selectively destroyed (Figs. 2 and 3). The fucosamine disaccharide unit was also reported in the O-specific polysaccharide from a subtype of *P. aeruginosa* serotype O4 (O4 a,c) [12,17], and capsular polysaccharides of *Staphylococcus aureus*, types 5 and 8

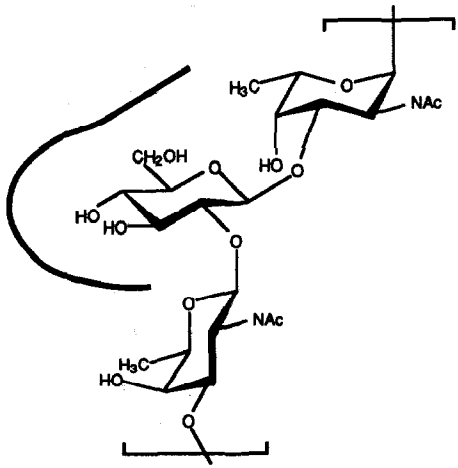

Fig. 4. Structure for the repeating unit of O-polysaccharide of *P. aeruginosa* O11 [10–12] and possible common epitope of O11-specific human mAbs and serotyping antisera.

[23,24]. However, the serotype antisera against O4 and O11 did not show significantly higher cross-reactivity to each other than among the antisera against other serotypes [8] (author's unpublished results). From one line of evidence, the fucosamine unit was likely to be less important for the antigenic determinant for serotype O11 than the glucose residue. If antibodies against the fucosamine unit can be obtained, such antibodies would be expected to cross-react to serotypes O11 and O4. In fact, a few examples of such cross-reacting human mAbs has been reported [6,25]; but their epitopes have not yet been determined.

As described in the introduction, serotype O11-specific human mAbs were obtained more frequently than the other common serotypes in clinical specimens. However, the actual reason for the higher frequency of isolation has not yet been clarified, and the glucose-containing epitopes of *P. aeruginosa* O11-specific polysaccharide are likely to be highly immunogenic.

References

[1] S. Sawada, T. Kawamura, and Y. Masuho, *J. Gen. Microbiol.*, 133 (1987) 3581–3590.
[2] H. Suzuki, Y. Okubo, M. Moriyama, M. Sasaki, Y. Matsumoto, and T. Hozumi, *Microbiol. Immunol.*, 31 (1987) 959–966.
[3] H.J. Zweerink, M.C. Gammon, C.F. Hutchison, J.J. Jackson, D. Lombardo, K.M. Miner, J.M. Puckett, T.J. Sewell, and N.H. Sigal, *Infect. Immun.*, 56 (1988) 1873–1879.
[4] A.B. Lang, U. Bruderer, E. Fuerer, J.W. Larrick, and S.J. Cryz, Jr., in C.A.K. Borrebaeck and J.W. Larrick (Eds.), *Therapeutic Monoclonal Antibodies*, Stockton Press, New York, 1990, pp 223–234.
[5] M.S. Collins, D.K. Ladehoff, N.S. Mehton, and J.S. Noonan, *FEMS Microbiol. Immunol.*, 64 (1990) 263–268.
[6] H. O'Oka, E. Chonan, K. Mizutani, T. Fukuda, Y. Kuroiwa, Y. Ono, and S. Shigeta, *Microbiol. Immunol.*, 36 (1992) 1305–1316.
[7] H. Noguchi, S. Yokota, H. Ohtsuka, T. Kohzuki, M. Terashima, and K. Irie, Antibiot. Chemother. (Basel), 44 (1991) 172–184.
[8] P.V. Liu, H. Matsumoto, H. Kusama, and T. Bergan, Int. J. Syst. Bacteriol., 33 (1983) 256–264.
[9] T.L. Pitt, *Eur. J. Clin. Microbiol. Infect. Dis.*, 7 (1988) 238–247.
[10] D. Horton and G. Rodemeyer, *Abstr. 8th. Int. Symp. Carbohydr. Chem.*, 1976 p. 49.
[11] B.A. Dmitriev, Y.A. Knirel, N.A. Kocharova, N.K. Kochetkov, E.S. Stanislavsky, and G.M. Mashilova, *Eur. J. Biochem.*, 106 (1980) 643–651.
[12] Y.A. Knirel, E.V. Vinogradov, N.A. Kocharova, N.A. Paramonov, N.K. Kochetkov, B.A. Dmitriev, E.S. Stanislavsky, and B. Lanyi, *Acta Microbiol. Hung.*, 35 (1988) 3–24.
[13] H. Ohtsuka, K. Horigome, H. Ochi, T. Nishimoto, T. Kohzuki, M. Kato, T. Okuda and H. Noguchi, *Hybridoma*, 10 (1991) 297–307.
[14] S. Yokota, H. Ochi, H. Ohtsuka, M. Kato, and H. Noguchi, *Infect. Immun.*, 57 (1989) 1691–1696.
[15] S. Yokota, M. Terashima, J. Chiba, and H. Noguchi, *J. Gen. Microbiol.*, 138 (1992) 289–296.
[16] J.Y. Homma, *Jpn. J. Exp. Med.*, 46 (1976) 329–336.
[17] E.S. Stanislavsky, B. Lanyi, Y.A. Knirel, and B.A. Dmitriev, *Zh. Microbiol. Epidemiol. Immunol.*, 5 (1988) 10–14.
[18] O. Westphal and K. Jann, *Methods. Carbohydr. Chem.*, 5 (1965) 83–91.
[19] S.G. Wilkinson and L. Galbraith, *Eur. J. Biochem.*, 52 (1975) 331–343.
[20] S.F. Koval and P.M. Meadow, *J. Gen. Microbiol.*, 98 (1977) 387–398.
[21] U.K. Laemmli, *Nature (London)*, 227 (1970) 680–685.
[22] C.-M. Tsai and C.E. Frasch, *Anal. Biochem.*, 119 (1982) 115–119.

[23] M. Moreau, J.C. Richards, J.-M. Fournier, R.A. Byrd, W.W. Karakawa, and W.F. Vann, *Carbohydr. Res.*, 201 (1990) 285–297.
[24] A. Fattom, R. Schneerson, S.C. Szu, W.F. Vann, J. Shiloach, W.W. Karakawa, and J.B. Robbins, *Infect. Immun.*, 58 (1990) 2367–2374.
[25] A.W. Siadak and M.J. Rosok, U.K. Pat. 2 185 226 (1987).