Preparation of a Semisynthetic Vaccine to *Streptococcus pneumoniae* Type 3

H. SNIPPE,[1]* J. E. G. van DAM,[1,2] A. J. van HOUTE,[1] J. M. N. WILLERS,[1] J. P. KAMERLING,[2] AND J. F. G. VLIEGENTHART[2]

Department of Immunology, Laboratory of Microbiology,[1] *and Department of Bio-Organic Chemistry, Laboratory of Organic Chemistry,*[2] *State University of Utrecht, Utrecht, The Netherlands*

A semisynthetic vaccine to *Streptococcus pneumoniae* type 3 has been developed. The hexasaccharide [→3)Glc$p$Aβ(1→4)Glc$p$β(1→]$_3$ was isolated from a partial acid hydrolysate of the capsular polysaccharide S3. It was coupled to stearylamine by reductamination with NaCNBH$_3$ and then incorporated into liposomes. These haptenated liposomes were tested for immunogenicity in mice. They induced protection to a lethal dose (25 50% lethal doses) of *S. pneumoniae* type 3 and gave rise to immunoglobulin M antibodies. No cross-protection was observed against *S. pneumoniae* type 11.

In 1974, a new approach for antigen presentation which involved incorporation of antigens into liposomal membranes was introduced by Kinsky and colleagues (10). Synthetic haptens were coupled to an amphipathic compound (e.g., phosphatidylethanolamine) and subsequently inserted noncovalently within a nonimmunogenic carrier (the liposomal lipid bilayers). These haptenated liposomes differ in two important respects from coventional hapten-carrier complexes in which haptens are covalently attached to immunogenic carriers such as proteins or polysaccharides.

For the development of a semisynthetic vaccine to *Streptococcus pneumoniae* serotype 3, an oligosaccharide served as haptenic determinant. This oligosaccharide, consisting of three β(1→3)-linked cellobiuronic acid units {[(→)-D-Glc$p$A-β(1→4)-D-Glc$p$-β(1→]$_3$}, was prepared from the bacterial capsular polysaccharide (3) (strain 2835, National Institute of Public Health, Bilthoven, The Netherlands). The latter polysaccharide consists only of repeating units of cellobiuronic acid that are β(1→3) linked to each other (4). By partial acid hydrolysis, these β(1→3) linkages are cleaved preferentially, resulting in the formation of cellobiuronic acid oligomers (7). The above-mentioned hexasaccharide (HS) was isolated by subsequent gel filtration with Sephadex G-25 superfine gel (Pharmacia Fine Chemicals, Uppsala, Sweden) and Bio-Gel P4 (−400 mesh, Bio-Rad Laboratories, Richmond, Calif.) (4, 8). The purity of the HS was checked by silica gel thin-layer chromatography with *n*-butanol–pyridine–water (6:5:5, vol/vol) and 360-MHz ^{1}H nuclear magnetic resonance spectroscopy. In addition, gas-chromatographic sugar analysis was carried out on the corresponding HS-alditol (5, 9) (GlcA: Glc:Glc-ol, 3.0:1.8:1.0). Reductamination of the hexasaccharide (2, 12) was carried out with a fivefold molar excess of stearylamine (Polysciences Inc., Warrington, Pa.) and NaCNBH$_3$ in tetrahydrofuran-water (30:12, vol/vol) as solvent. The reaction was allowed to proceed for 2 weeks at room temperature with magnetic stirring at pH 8. The excess of stearylamine was removed from the formed glycolipid (HS − S) by chloroform extraction. The lyophilized glycolipid was washed with methanol to remove the excess of NaCNBH$_3$ and was subsequently dissolved in chloroform-methanol (3:1, vol/vol). The unreacted sugar component was filtered off. The coupling product of the HS and stearylamine was investigated by gas chromatography-mass spectrometry after methanolysis and trimethylsilylation. Besides the trimethylsilylated methyl glycosides of glucose and glucuronic acid methyl ester, 2,3,4,5,6,-penta-*O*-trimethylsilyl-1-deoxy-1-*N*-stearylamino-D-glucitol was detected. In the total ion current chromatogram, small amounts of the palmityl and margaryl analogs were also observed. These compounds were due to impurities in the commercially available stearylamine.

Liposome preparations were actively haptenated by incorporation of the glycolipid into a basic lipid mixture containing dipalmitoyl -L-α-phosphatidyl choline and cholesterol. The ratio of HS − S, dipalmotoyl L-α-phosphatidyl choline, and cholesterol was 5:85:10. Haptenated liposomes were prepared as described previously (11).

The immunogenicity of HS − S liposomes was

TABLE 1. Induction of protection to *S. pneumoniae* type 3 after immunization with HS − S liposomes, showing specificity of the reaction[a]

Group	Immunizing agent	Route[b]	Challenge organism (at 25 LD$_{50}$)	No. of survivors/ no. injected
1	HS − S liposomes (1 nmol HS − S)	i.v.	Type 3	6/6
2	HS − S liposomes (1 nmol HS − S)	i.v.	Type 11	0/6
3	Phosphate-buffered saline	i.v.	Type 3	0/6
4	Phosphate-buffered saline	i.v.	Type 11	0/6
5	S3 (0.5 µg)	i.p.	Type 3	6/6
6	S3 (0.5 µg)	i.p.	Type 11	0/6

[a] Groups of six BALB/c mice were immunized as indicated. At day 7, the mice were challenged intraperitoneally with 25 LD$_{50}$ of *S. pneumoniae* type 3 or type 11, and 2 weeks later the number of survivors was recorded.
[b] i.v., Intravenous; i.p., intraperitoneal.

studied in female BALB/c mice. Protective immunity to a lethal dose of *S. pneumoniae* type 3 (6) was determined after intraperitoneal injection of 25 50% lethal doses (LD$_{50}$ for type 3, 4 × 10^3 CFU). Immunization with 0.5 µg of S3 served as a positive control, and injections with either phosphate-buffered saline or nonhaptenated liposomes served as a negative control. Mice immunized with various amounts of HS − S liposomes (0.1 to 30 nmol of HS − S; epitope density, 5 mol%) were protected to 25 LD$_{50}$ of *S. pneumoniae* given 7 days after intravenous in-

jection. The specificity of the vaccine (HS − S liposomes) is presented in Table 1. No cross-protection was observed against *S. pneumoniae* type 11 (LD$_{50}$ for type 11, 4 × 10^5 CFU). The presence of antibodies in serum was determined by a hemagglutination assay with indicator erythrocytes optimally derivatized with S3 (1). Circulating immunoglobulin M antibodies were present in all sera starting 5 days after immunization (Fig. 1). Mice immunized with HS − S alone also developed anti-S3 immunoglobulin M antibodies, although a higher concentration (30-
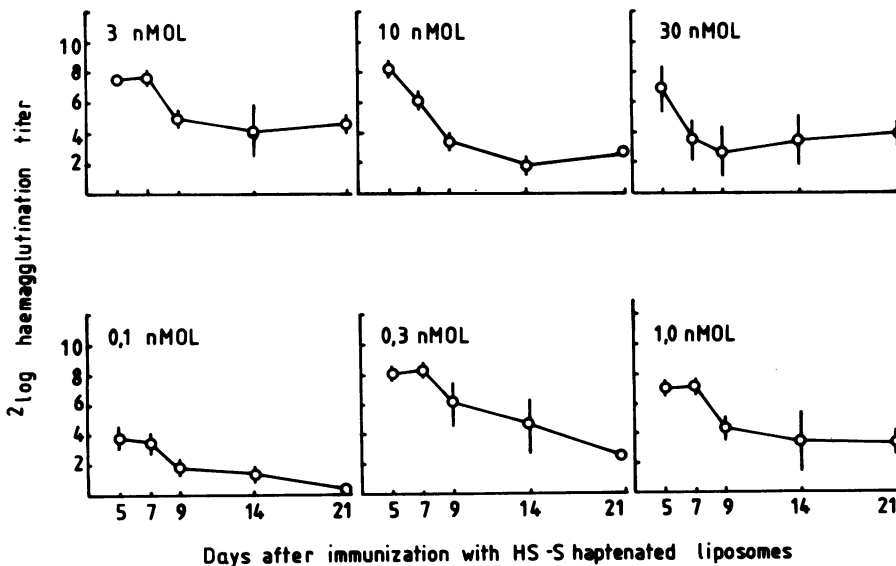

FIG. 1. Serum antibody titers after immunization with graded amounts of HS − S liposomes (see text for details). Groups of six mice were bled as indicated, and the serum anti-S3 antibody titer was determined with a hemagglutination assay.
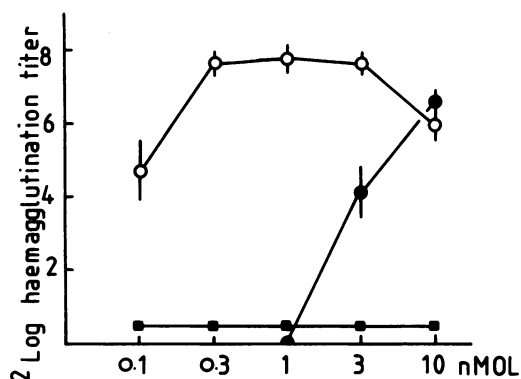
FIG. 2. Serum antibody titers at day 5 after immunization with graded amounts of HS − S liposomes (○), free HS − S (●), or HS (■).

fold increase) of free HS − S was required. The HS was not immunogenic when tested over a whole dose range (Fig. 2).

These experiments and those in progress suggest the possibility of developing semisynthetic polysaccharide vaccines. This model can be extended to other infective microorganisms with polysaccharide antigens.

LITERATURE CITED

1. Baker, P. J., P. W. Stashak, and B. Prescott. 1969. Use of erythrocytes sensitized with purified pneumococcal polysaccharide for the assay of antibody and antibody-producing cells. Appl. Microbiol. 17:422–426.
2. Borch, R. F., M. D. Bernstein, and H. D. Durst. 1971. The cyanohydridoborate anion as a selective reducing agent. J. Am. Chem. Soc. 93:2897–2904.
3. Campbell, J. H., and A. M. Pappenheimer. 1966. Quantitative studies of the specificity of anti-pneumococcal polysaccharide antibodies, type III and VIII. I. Isolation of oligosaccharides from acid and from enzymatic hydrolysates. Immunochemistry 3:195–212.
4. Hotchkiss, R. D., and W. F. Goebel. 1937. Chemo-immunological studies on the soluble specific substance of Pneumococcus. III. The structure of the aldobionic acid from the Type III polysaccharide. J. Biol. Chem. 121:195–203.
5. Kamerling, J. P., G. J. Gerwig, and J. F. G. Vliegenthart. 1975. Characterization by gas-liquid chromatography mass spectrometry and proton magnetic resonance spectroscopy of pertrimethylsilyl methyl glycosides obtained in the methanolysis of glycoproteins and glycopeptides. Biochem. J. 151:491–495.
6. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27:493–497.
7. Reeves, R. E., and W. F. Goebel. 1941. Chemo immunological studies on the soluble specific substance of pneumococcus. V. The studies of the type III polysaccharide. J. Biol. Chem. 139:511–519.
8. Snippe, H., A. J. van Houte, J. E. G. van Dam, M. J. de Reuver, M. Jansze, and J. M. N. Willers. 1983. Immunogenic properties in mice of hexasaccharide from the capsular polysaccharide of Streptococcus pneumoniae type 3. Infect. Immun. 40:856–861.
9. Tsai, C. S. 1970. Determination of the degree of polymerization of N-acetylchito oligoses by chromatographic methods. Anal. Biochem. 36:114–122.
10. Uemera, K., R. A. Nicolotti, H. R. Six, and S. C. Kinsky. 1974. Antibody formation in response to liposomal membranes sensitized with N-substituted phosphatidylethanolamine derivatives. Biochemistry 13:1572–1578.
11. Van Houte, A. J., H. Snippe, and J. M. N. Willers. 1979. Characterization of immunogenic properties of haptenated liposomal model membranes in mice. I. Thymus independency of the antigen. Immunology. 37:505–514.
12. Wood, C., and E. A. Kabat. 1981. Immunochemical studies of conjugates of isomaltosyl oligosaccharides to lipid. I. Antigenicity of the glycolipids and the production of specific antibodies in rabbits. J. Exp. Med. 154:432–449.