INFECTION AND IMMUNITY, Nov. 1983, p. 842–844
0019-9567/83/110842-03$02.00/0

Vol. 42, No. 2

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

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Received 24 June 1983/Accepted 24 August 1983

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 ^1H 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 injection. 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-

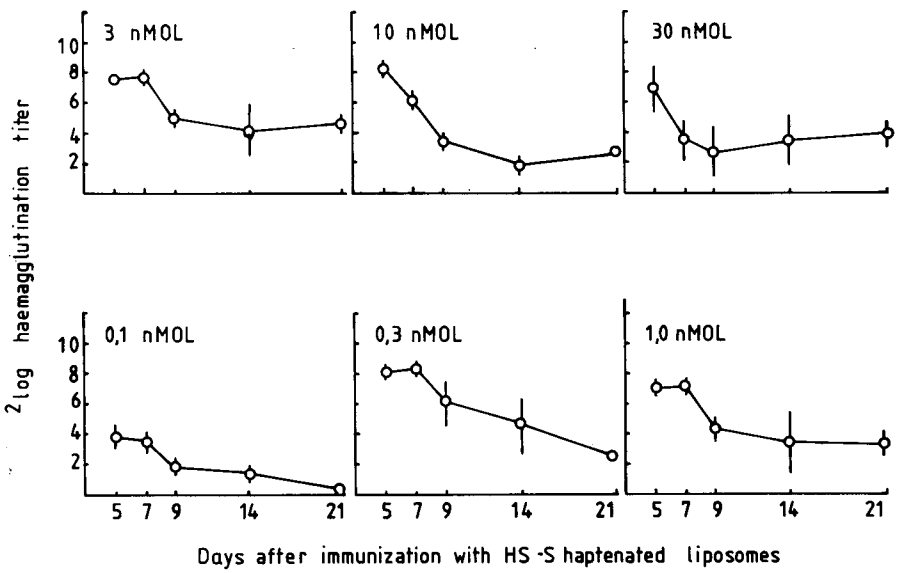

FIG. 1. Serum antibody titers after immunization with graded amounts of HS – S liposomes (see text for details). Groups of six mice were bled as indicated, and the serum anti-S3 antibody titer was determined with a hemagglutination assay.

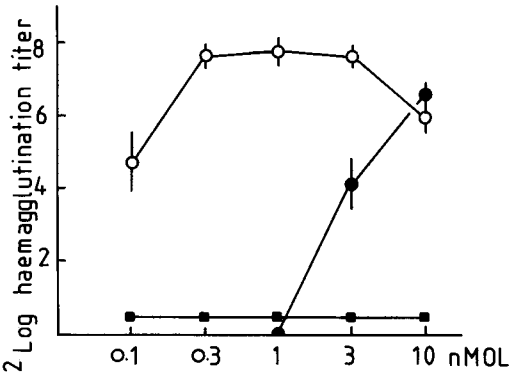

FIG. 2. Serum antibody titers at day 5 after immunization with graded amounts of HS − S liposomes (○), free HS − S (●), or HS (■).

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 progess suggest the possibility of developing semisynthetic polysaccharide vaccines. This model can be extended to other infective microorganisms with polysaccharide antigens.

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