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Preparation and Immunochemical Characterization of Meningococcal Group C Polysaccharide-Tetanus Toxoid Conjugates as a New Generation of Vaccines

E. COEN BEUVERY,[1]* FRANK MIEDEMA,[1]† ROB VAN DELFT,[1] AND JOHAN HAVERKAMP[2]

Unit of Immunochemistry, Rijksinstituut voor de Volksgezondheid, 3720 BA Bilthoven,[1] and Institute for Atomic and Molecular Physics, 1098 SJ Amsterdam,[2] The Netherlands

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Neisseria meningitidis group C polysaccharide-tetanus toxoid conjugates have been prepared by using high-molecular-weight polysaccharide and purified tetanus toxoid and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide as a coupling reagent. The influence of three conditions of preparation was studied. Biochemical assays, the enzyme-linked immunosorbent assay, and isopycnic CsCl gradient ultracentrifugation have been used to characterize the conjugates. The polysaccharide-to-protein ratios of the various conjugate preparations showed differences. The ability of the group C polysaccharide component to react with specific antibodies was reduced, whereas most of the tetanus toxoid seemed to be hidden by the polysaccharide. The composition of the conjugate was not homogeneous, and at least 10% of free polysaccharide was present. Thermostability in lyophilized condition in the presence of lactose was excellent.

During the past decade polysaccharide vaccines of *Neisseria meningitidis* groups A, C, W135, Y, and Z' (29E) (13, 14, 17, 18, 20, 34) have been developed. These vaccines are composed of purified polysaccharides, which are the main component of the capsule of the bacteria. The polysaccharides induce bactericidal (protective) antibodies in humans and mice (8, 17). Groups A and C polysaccharide vaccines have been used successfully to stop epidemics (29, 33). The antibody level induced by the vaccines depends both on its quality and on the age and previous immunological experience of the vaccinee (4, 5, 16, 23, 25). The drawbacks related to age and previous immunological experience can be ascribed to the particular nature of the polysaccharides, which are thymic-independent antigens. It has been shown that this thymic-independent quality can be overcome by conjugating the polysaccharide to proteins (6, 7, 22, 30, 31) or by applying an alternative isolation procedure (2, 3, 11, 15, 38; E. C. Beuvery, F. Miedema, R. W. van Delft, J. Haverkamp, B. J. te Pas, J. S. Teppema, and R. H. Tiesjema, Infect. Immun., in press).

Polysaccharide-protein conjugates are a serious candidate vaccine for human use. Vaccines have to be prepared with reproducible characteristics. For the control of polysaccharide vaccines, physicochemical, biochemical, and immunochemical techniques have proven to be very useful (36, 37). In this communication we describe the influence of three conditions of preparation of group C polysaccharide-tetanus toxoid conjugate (CPS/TT) and the results of the characterization by enzyme-linked immunosorbent assay (ELISA). This immunochemical technique seems to be very useful for the characterization of CPS/TT.

† Present address: Centraal Laboratorium van de Bloedtransfusiedienst van het Nederlandse Rode Kruis, 1006 AP Amsterdam, The Netherlands.

MATERIALS AND METHODS

Materials. Materials were purchased from the following sources: bovine serum albumin (code 450) from Povite, Oss, The Netherlands; ethanolamine from Onderlinge Pharmaceutische Groothandel, Utrecht, The Netherlands; Tween 80 and 5-amino-2-hydroxybenzoic acid from Merck-Schuchardt, 8011 Hohenbrunn bei München, West Germany; cesium chloride from Merck, 6100 Darmstadt, West Germany; Sephadex G-200 and Sepharose CL-2B from Pharmacia Fine Chemicals, Uppsala, Sweden; horseradish peroxidase (type VI) and *N*-acetylneuraminic acid (Neu5Ac) from Sigma Chemical Co., St. Louis, Mo.; and complete and incomplete Freund adjuvant from Difco Laboratories, Detroit, Mich.

Polysaccharide and tetanus toxoid. Purified group C polysaccharide (CPS) was kindly provided by Rudy Tiesjema from the Laboratory of Vaccine Production in the Rijksinstituut voor de Volksgezondheid, Bilthoven, The Netherlands. Tetanus toxoid (TT) was purified bulk material used for vaccine production (400 Lf/mg of protein) and was supplied by Jaap Nagel from the Unit of Immunochemistry of the Rijksinstituut.

Conjugation of polysaccharide and protein. Conjugation was performed by adding slowly 10 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) in 0.5 ml of water to 20 ml of a solution containing 16 mg of CPS and 16 mg of TT or the Sephadex G-200 fraction (7). The pH was maintained at 4.7 by adding 1.0 M HCl. After about 40 min at room temperature, the pH remained constant. After 4 h, the reaction was stopped by the addition of 4.8 mg of ethanolamine; no pH control was applied. The crude conjugate was centrifuged (30 min, 50,000 × *g*), and the supernatant was dialyzed against phosphate-buffered saline (PBS) and freed from unreacted TT on Sepharose CL-2B in PBS. The CPS/TT conjugate was eluted in the void volume. This fraction is referred to as conjugate. For the lyophilization of the conjugate, lactose was used as the menstruum (35).

CPS and TT were also treated separately with EDC. The same reaction conditions were applied. The reaction products were centrifuged (30 min, 50,000 × *g*). The supernatants were dialyzed against water and lyophilized, giving CPS* and TT*, respectively.

Chemical assays. Neu5Ac was determined by the method of Svennerholm (32) and protein by the method of Lowry (27) with Neu5Ac and bovine serum albumin, respectively, used as standards.

Isopycnic density gradient ultracentrifugation. The sample (0.4 ml in 0.01 M PBS, pH 7.2) was applied to a 12-ml cellulose nitrate tube (Beckman no. 331370) containing (from bottom to top) 0.4 ml of a saturated solution of CsCl, 3.7 ml of a CsCl solution with a density of 1.55 g ml^{-1}, 3.7 ml of a CsCl solution with a density of 1.35 g ml^{-1}, and 3.7 ml of a CsCl solution with a density of 1.20 g ml^{-1}. Centrifugation was performed in a Beckman SW41 swinging bucket rotor at 30,000 rpm at 10°C for 68 h. Fractions were collected, and the refractive index, CPS, TT, and Neu5Ac concentrations were determined for each. Before analysis, the fractions were dialyzed against PBS.

Antibodies, conjugates, and substrate used in ELISA. Antisera against CPS were raised in sheep by immunization with whole bacterial cells (1). The antibody fractions from these sera were obtained by precipitation and one wash with 18% (wt/vol) Na_2SO_4. The precipitates were dialyzed against water and were freeze-dried.

Antisera against TT were raised in sheep by three successive injections with 0.1 mg of TT. The antigen was emulsified in complete Freund adjuvant for the first injection and in incomplete Freund adjuvant for the following two injections. The interval between the injections was 1 to 2 months. The bleeding was done 10 days after the last injection. The antibody fractions from the sera were obtained as described above. Peroxidase conjugates of the antibody fractions of the antisera were prepared by the method of Nakane and Kawaoi (28) without the final reduction with $NaBH_4$ (19).

Substrate was prepared by dissolving 80 mg of 5-amino-2-hydroxybenzoic acid in 100 ml of water. The pH was adjusted to 6.0 with 1 M NaOH, and just before use 11 ml of 0.05% (wt/vol) H_2O_2 was added.

ELISA procedures. The assays were performed in polyvinyl-chloride V-shaped microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.). The buffers used were PBS with 0.01% (vol/vol) Tween 80 as the washing and diluting buffer of the sample, and PBS with 0.01% Tween 80 and 0.05% (wt/vol) bovine serum albumin as the diluting buffer of enzyme-conjugated antibodies.

(i) Homologous assays. Antibodies to CPS or TT were dissolved in PBS (15 μg ml^{-1}). Volumes of 100 μl were added to each well. The plate was incubated at room temperature overnight and washed three times with washing buffer. A 100-μl portion of the sample to be tested in dilution buffer was added. The plate was incubated at 37°C for 2 h and washed as before, and 100 μl of enzyme-conjugated sheep anti-CPS or anti-TT in dilution buffer was added. The plates were incubated at 37°C for 2 h and again washed three times. Next, 100 μl of substrate was added, the plate was incubated at room temperature for 10 min, and the reaction was stopped by adding 100 μl of 0.3 NaOH. The contents of the plate were transferred to a polystyrene Microelisa flat-bottomed plate (Dynatech M129A; Dynatech AG, Zug, Switzerland). The optical density was measured at 449 nm in a Multiskan spectrophotometer (Titertek; Flow Laboratories, Irvine, Scotland).

Both assays were also used to determine the antigenic activity of both components of CPT/TT in the fractions after ultracentrifugation. The CPS/TT preparation which was analyzed was used as a reference.

(ii) Heterologous assay. Step 1 of the heterologous assay consisted of the addition of antibodies to CPS; step 3 consisted of the addition of enzyme-conjugated antibodies to TT. The experimental conditions were the same as those of the homologous assays.

To correct the optical density for nonspecific binding in the ELISAs, the optical density obtained with the diluting buffer was taken as zero level. The difference between the optical densities obtained with the sample and diluting buffer is called the corrected optical density (abbreviated in the figures as corr. O.D.).

RESULTS

Composition of the conjugates prepared under different conditions. The influence of the three conditions of preparation of the conjugates (Table 1) was studied. The composition of the eight lots of conjugate was determined by biochemical assays. The results of these assays (Table 1) indicate that the composition of the eight lots of conjugate was variable; the ratio of Neu5Ac to protein varied from 0.67 to 1.53.

Analysis of the conjugate in both homologous ELISA systems. The eight lots of conjugate showed comparable binding to antibodies to CPS and TT in both homologous ELISA systems. For this reason, the data of only one lot (no. 1) are presented.

The binding of CPS/TT, CPS, and CPS* to antibodies to CPS is shown in Fig. 1. CPS had the highest antigenic activity, whereas the activity of CPS* was greatly reduced. The curve of CPS/TT was intermediate. The binding of CPS/TT, TT, and TT* to antibodies to TT are presented in Fig. 2. The shapes of the three curves were identical. The curves obtained with TT* and CPS/TT, however, were shifted to the

TABLE 1. Chemical composition of eight lots of CPS/TT prepared under different conditions

Conditions[a]	Conjugate			Contents (μg/ml)		Neu5Ac:protein ratio
	No.	Incubation (min)	Lot of TT (no.)	Neu5Ac	Protein	
A,C	1	240	18	150	138	1.09
A	2	240	Purified 18	150	170	0.88
B	3	30	18	150	98	1.53
	4	60	18	152	133	1.14
	5	120	18	141	129	1.09
B,C	6	240	18	151	138	1.09
C	7	240	17	100	150	0.67
	8	240	16	100	150	1.67
Mean				143	138	1.04

[a] A, Influence of gel filtration on the lot of purified TT; B, influence of incubation period after addition of the coupling reagent; C, influence of the lot of purified tetanus toxoid used for conjugation.

right. The shift amounted to a factor of about 2 (43%) for TT* and a factor of 20 (5%) for CPS/TT. At an optical density of >1.6, all curves were flattened.

CsCl density ultracentrifugation of the conjugate. CPS/TT, CPS, CPS*, TT, and TT* were analyzed by means of isopycnic density gradient ultracentrifugation in CsCl. The CPS/TT fractions were analyzed for antigenic activity in both homologous ELISA systems. The profile obtained with the conjugate is shown in Fig. 3. The recovery of the CPS component of the conjugate was almost 100%. The total activity of the TT component in the fractions was only 30%, which means that the antigenic activity of this component was reduced by a factor of about 3. The peaks in the CPS and TT curves obtained with the conjugate did not coincide, indicating its non-homogeneous composition. The densities of CPS, CPS*, TT, and TT* are also presented in Fig. 3. Treatment of CPS with coupling reagent resulted in a reduction of the density (from 1.55 to 1.38 g ml^{-1}). Such a treatment of TT, however, produced a small increase of the density (from 1.27 to 1.30 g ml^{-1}).

The conjugate fractions (nos. 7 through 10) were assayed for Neu5Ac to calculate the recovery of CPS in the fractions. These data, together with the ratios of TT to CPS, are shown in Table 2. The ratio increased with increasing fraction numbers. In fraction 7, very little except CPS could be detected; the three remaining fractions contained TT as well as CPS.

The fractions were also tested in the heterologous ELISA system (Fig. 4). The slopes of the linear parts of the curves agree with the ratio of TT to CPS presented in Table 2.

Thermostability of the conjugate. CPS/TT was incubated in fluid and in lyophilized conditions at 4, 37, and 56°C for 1 month. Thereafter, the antigenic activities of both components were tested in the homologous ELISA system. From

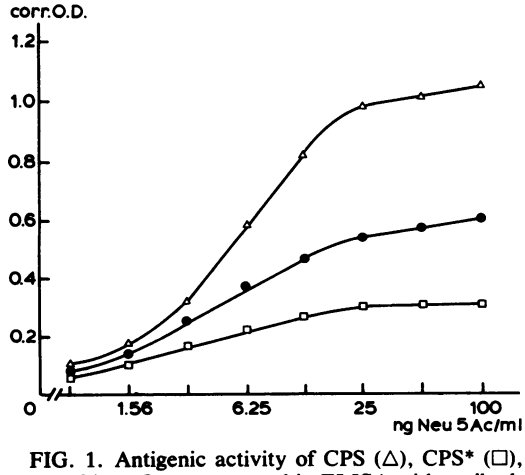

FIG. 1. Antigenic activity of CPS (△), CPS* (□), and CPS/TT (●) as measured in ELISA with antibodies to CPS.

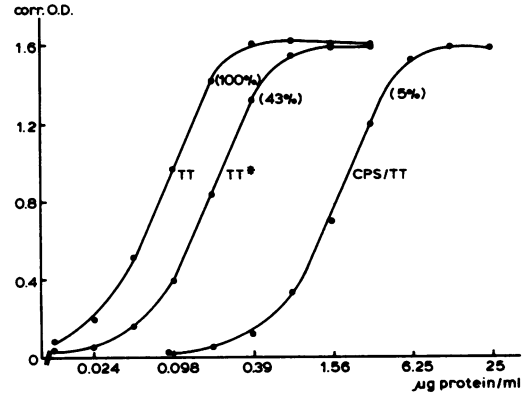

FIG. 2. Antigenic activity of TT, TT*, and CPS/TT as measured in ELISA with antibodies to TT.

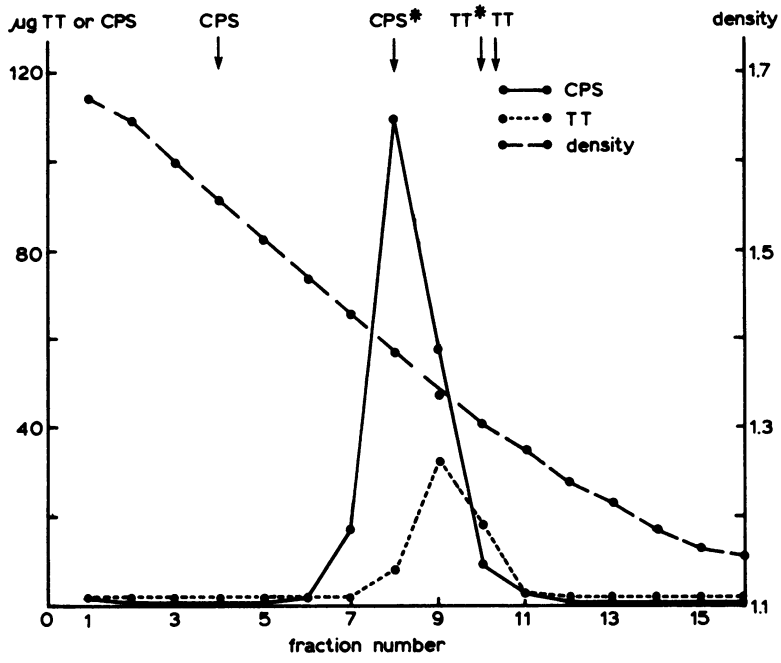

FIG. 3. Isopycnic CsCl density gradient ultracentrifugation of CPS/TT. Fractions were assayed for CPS and TT by ELISA. Also shown are the densities of CPS, CPS*, TT, and TT*.

these results (Fig. 5 and 6), it can be concluded that storage of the conjugate in fluid condition at 4 and 37°C and in lyophilized condition did not alter the antigenic activities of the two components. Storage in fluid condition at 56°C, however, resulted in a very considerable loss of the ability of both components to bind their corresponding antibodies.

DISCUSSION

Our conjugation procedure, like that described by Schneerson et al. (30, 31), consisted of cross-linking high-molecular-weight CPS and purified TT by means of the carbodiimide reaction (12, 21, 24). Both CPS and TT contain a large number of reactive groups for such a reaction (9, 10).

The Neu5Ac-to-protein ratios of the eight lots, as determined by the biochemical assays, were variable (Table 1). The data found in both the homologous and heterologous ELISA systems suggest that the antigenic activity of both components of the lots of CPS/TT were similar. Taking into account the experimental errors of the biochemical assays, it is most likely that the ratios of CPS to TT differed only slightly and that the conditions of preparation did not influence the composition of the conjugates.

Treatment of CPS with EDC and ethanolamine resulted in a considerable reduction of its antigenic activity. Possible causes for this reduction are the presence of ethanolamine covalently bound through amide linkages, the binding of *N*-acylurea derivative of EDC (12, 21, 24), or the

TABLE 2. Results of CsCl density gradient ultracentrifugation

Fraction	TT:CPS (%)[a]	Neu5Ac recovery (%)[b]
7	<1	9
8	9	47
9	45	34
10	112	10
Conjugate	100	100

[a] Based upon the results of ELISA experiments.
[b] Based upon the results of Neu5Ac assay.

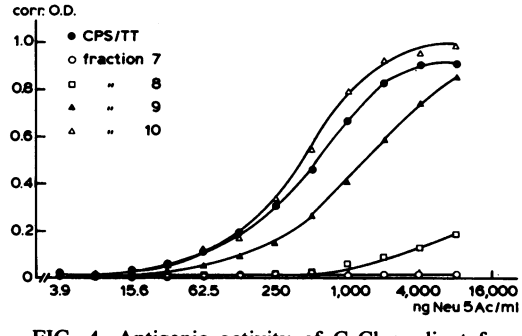

FIG. 4. Antigenic activity of CsCl gradient fractions of CPS/TT measured in heterologous ELISA.

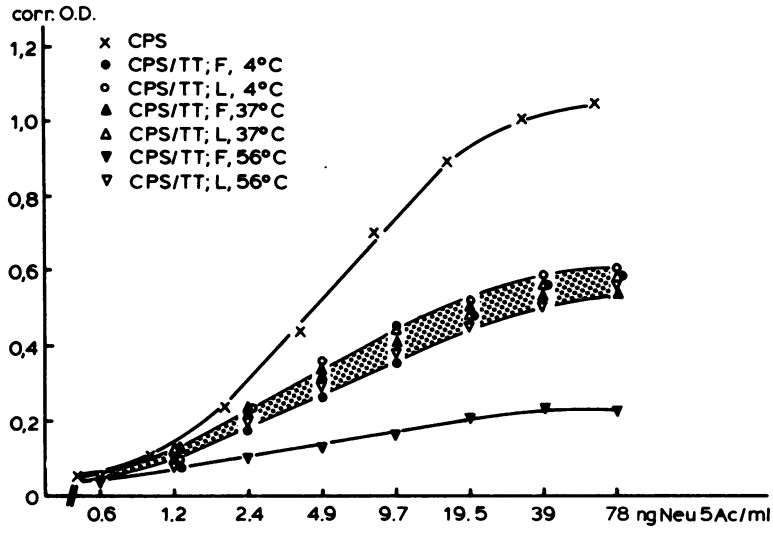

FIG. 5. Antigenic activity of the CPS component of CPS/TT after storage in fluid (F) and in lyophilized (L) conditions at different temperatures for 1 month.

ester linkages in CPS (24, 26). EDC treatment reduced the antigenic activity of TT by a factor of about 2. This reduction may be explained by a partial inactivation of TT or an increased sensitivity of the Lowry assay for TT*. The influence of the treatment on the density of TT was small.

Conjugation of CPS with TT resulted in an increased binding to antibodies to CPS in comparison with CPS*. Such an increase may be ascribed to the increased molecular weight of the conjugate.

No influence of the increased molecular

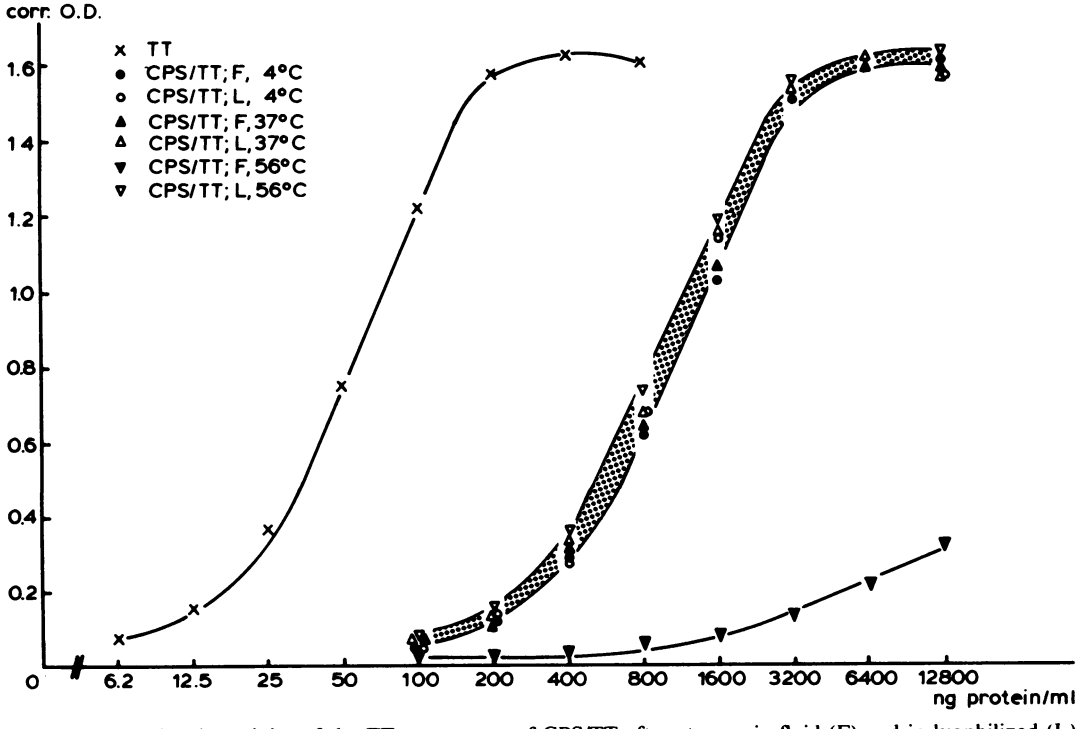

FIG. 6. Antigenic activity of the TT component of CPS/TT after storage in fluid (F) and in lyophilized (L) conditions at different temperatures for 1 month.

weight of CPT/TT, however, was seen in the ELISA system for TT. The influence of the molecular weight may have been masked by a flattening of the curve at optical densities of 1.6 and higher. The shapes of the curves were similar. The curves of TT* and CPS/TT were shifted to higher protein concentrations. From these shifts, it can be concluded that for TT* and CPS/TT, about 50 and 5%, respectively, are available for the binding of enzyme-conjugated anti-TT. By an absorption experiment with anti-TT serum, however, it was shown that the TT component of CPS/TT was on a protein base as active in the removal of antibody activity as purified TT was (6). This indicates that most of the TT component can still react with native anti-TT, although in the ELISA only 5% of it reacts with high-molecular-weight enzyme-conjugated anti-TT. One may conclude from this that only 5% of the TT component is available at the surface of the conjugate molecules.

The composition of the conjugate was not homogeneous, as was shown by isopycnic CsCl gradient ultracentrifugation. The conjugate seems to be composed of molecules with different ratios of TT to CPS. Based upon the recovery of Neu5Ac in the fractions and the results of the immunochemical analysis, one may conclude that the conjugate contained about 10% free CPS, which has only reacted with the coupling reagent. Such high-molecular-weight CPS will not be excluded in Sepharose CL-2B. The differences in the ratios of TT to CPS of the gradient fractions were confirmed in the heterologous ELISA system. The slopes of the linear parts of the curves of the fractions obtained in this system correlated well with the ratios just mentioned and also with their immunogenetic activities in mice. Fraction 7 did not induce immunoglobulin G antibodies, whereas fraction 10 showed the greatest potency, inducing immunoglobulin G antibodies to CPS as well as to TT (E. C. Beuvery, R. W. van Delft, F. Miedema, V. Kanhai, and J. Nagel, submitted for publication).

The thermostability of the conjugate can be monitored very well by ELISA. After lyophilization in the presence of lactose (32), it showed an excellent stability, whereas the stability in fluid condition at elevated temperatures was less. The same data have also been found with mice (E. C. Beuvery, F. Miedema, R. W. van Delft, J. Haverkamp, R. H. Tiesjema, and J. Nagel, J. Infect., in press).

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