IMMUNOCHEMICAL STUDIES OF CONJUGATES OF ISOMALTOSYL OLIGOSACCHARIDES TO LIPID

I. Antigenicity of the Glycolipids and the Production of Specific Antibodies in Rabbits*

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The antigenicity of lipids is still ill-defined, and there is very little evidence that any pure lipid by itself will act as an antigen. To produce an immune response they must be complexed with or coupled to carriers (1–6). Only glycosphingolipids and phosphatides have been found to function as haptens when mixed with carriers such as serum albumin (7), methylated bovine serum albumin (8), or other auxillary lipids (9–11), or when the glycolipid was incorporated into liposomes (12). Antibodies to glycolipids are usually directed primarily against the carbohydrate moiety (1, 7, 13, 14), and there is little evidence that antibodies can be formed to the free lipids even though free fatty acids were reported to cause inflammatory delayed-type allergic skin reactions (15, 16).

The purpose of this investigation was to synthesize and study the antigenicity and immunological properties of a series of glycolipid antigens prepared by coupling isomaltose oligosaccharides—varying in size from isomaltose to isomaltoheptaose—to a lipid carrier, stearylamine, and to compare the antigenicities of the different stearyl-isomaltosyl oligosaccharides thus obtained, either when injected alone or when incorporated into liposomes. Isomaltose oligosaccharides were chosen because they are the predominant structural units of $\alpha 1 \rightarrow 6$ dextran (17). Antibodies raised to them should cross-react with dextrans, and the heterogeneity of the response and the sizes of their antibody combining sites could be studied by quantitative precipitin and precipitin inhibition assays and could be compared with antidextran (18–22).

The stearyl-isomaltosyl oligosaccharides (stearyl-IM3 to IM7) when incorporated into liposomes were agglutinated in the presence of specific antibodies and were lysed if complement was also present. When injected into rabbits they were antigenic, either emulsified directly with complete Freund's adjuvant (CFA)[1] or incorporated into

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CFA, complete Freund's adjuvant; DNP-Cap-PE, DNP-ε-aminocaproylphosphatidyl-ethanolamine; γglo, gammaglobulin; IM2, isomaltose (D-Glcα1→6D-Glc); IM3, isomaltotriose; IM4, isomaltotetraose; IM5, isomaltopentaose; IM6, isomaltohexaose; IM7, isomaltoheptaose; PBS, phosphate-buffered saline (0.01 M phosphate, 0.8 percent sodium chloride, pH 7.2); SDS, sodium dodecyl sulfate; SRBC, sheep erythrocytes; TCA, trichloroacetic acid.

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liposomes plus CFA. The antisera obtained reacted with α1→6 dextrans and are mainly IgG and IgM, as characterized by sodium dodecyl sulfate (SDS) gel electrophoresis. The purified antibodies examined by isoelectric focusing were seen to be pauciclonal.

Materials and Methods

Chemicals. Stearylamine and dicetylphosphate were purchased from K and K Laboratories, Inc., Plainview, N. Y. Sphingomyelin and cholesterol were obtained from Sigma Chemical Co., St. Louis, Mo. Sodium cyanoborohydride was purchased from Aldrich Chemical Co. Inc., Milwaukee, Wis. B512 dextran from *Leuconostoc mesenteroides* (N279 of 4×10^6–10×10^6 mol wt) was obtained from Commercial Solvents Co., Terre Haute, Ind. (19, 23). Isomaltose (IM2), isomaltotriose (IM3), isomaltotetraose (IM4), isomaltopentaose (IM5), isomaltohexaose (IM6), and isomaltoheptaose (IM7) were described previously (24–27).

Antisera. Rabbit anti-isomaltotrionic bovine serum albumin (BSA) and anti-isomaltohexaonic BSA conjugates that gave precipitin reactions with α1→6 dextrans were available (28, 29).

Coupling of Isomaltose Oligosaccharides to Stearylamine. The method was essentially that introduced by Gray (30, 31) for coupling oligosaccharides to BSA using cyanoborohydride as the reducing agent. Briefly, oligosaccharides and a fivefold molar excess of stearylamine and sodium cyanoborohydride were dissolved in a solvent consisting either of chloroform:ethanol:water or tetrahydrofuran:water. The various oligosaccharides required a different ratio of organic and aqueous solvents, as described in the text. The reaction mixture was kept at 37°C, and the degree of completeness of the reaction was judged using paper and thin-layer chromatography to follow the disappearance of free oligosaccharide.

Paper and Thin-layer Chromatography. Descending paper chromatography was carried out at room temperature using Scheicher and Schuell paper, grade 589 Green Ribbon C (Scheicher and Schuell, Inc., Keene, N. H.). The solvent systems used (18) were: (*a*) butanol-pyridine-water (6:4:3); (*b*) butanol-pyridine-water (35:39:26). Solvent *a* was used for smaller oligosaccharides (IM2–IM4) and solvent *b* was used for larger oligosaccharides (IM5–IM7). Ascending thin-layer chromatography was also carried out at room temperature using Eastman Chromatogram sheet, 1381 silica gel (Eastman Kodak Co., Rochester, N. Y.) in solvent *a* or *b*. Sugars were detected on paper and thin-layer chromatograms using alkaline silver nitrate (18).

Chemical Analysis. Total hexose was determined by the orcinol, anthrone, and phenol sulfuric acid methods (18). Specific rotation was measured in a Perkin-Elmer 141 polarimeter (Perkin-Elmer Corp., Norwalk, Conn.) using a 1 dm cell. Total N was measured by the ninhydrin method (32).

Preparation of Liposomes. Liposomes were prepared by the method of Sessa and Weissmann (33) with minor modifications and using the lipid ratio used by Yasuda et al. (34) with sphinogmyelin:cholesterol:dicetylphosphate:antigen (2:1.5:0.2:0.1). Briefly, various lipids (20 μmol total) were dissolved in chloroform in a 100-ml round bottom flask. The solution was dried in a rotary evaporator to form a uniform thin film. 1 ml of 0.01 M phosphate-buffered saline (PBS) at pH 7.2 or an isotonic solution of sodium dichromate was added, and the lipid was dispersed on a vortex mixer; glass beads were added to help dispersion. Liposomes to be used for injection were resuspended in PBS and those to be used for agglutination and complement lysis were resuspended in the dichromate solution. The turbid suspension was allowed to stand for 2 h at room temperature and then kept on ice while being sonicated for 3 min with an MSE (Measuring and Scientific Equipment Ltd., London, England) probe type sonicator at an amplitude of 8 μm. Liposomes suspended in PBS were used immediately for immunization. Liposomes suspended in dichromate solution were passed through a Sepharose 6B column (25 cm × 1 cm) to separate liposomes with trapped dichromate from the excess untrapped dichromate. After separation the liposomes were used immediately.

Agglutination of Liposomes. Liposomes incorporated with antigens were tested for agglutination by specific antisera in two ways: (*a*) by direct observation of aggregation under the microscope. Antiserum was diluted with a Takatsy microtitrator (Cooke Engineering Co., Alexandria, Va.) in a microtiter plate, 25 μl of liposome suspensions was added to each well,

and the agglutination titer was scored as the highest dilution of the antiserum that gave aggregation of liposomes as observed microscopically; (*b*) agglutination of liposomes was followed spectrophotometrically by the change in absorbance at 600 nm, recording the increase in absorption as the liposomes were agglutinated by antibodies (35).

Lysis of Liposomes by Complement. Liposomes with trapped dichromate were used; in the presence of specific antisera and complement, liposomes are lysed and release the dichromate (36). The experiment was carried out at 37°C in Lucite microdialysis chambers (37) using membranes made from dialysis tubing. One side of the chamber was filled with liposome suspension (125 μl) and the other with 125 μl veronal buffer at pH 7.2 (18). In the presence of complement and antibody, liposomes are lysed, and released dichromate diffuses through the membrane into the chamber with veronal buffer. By measuring the amount of dye in the buffer chamber at equilibrium (absorption at 360 nm), percent lysis could be determined. Liposomes treated with 1% Triton X were used to establish 100% lysis. Equilibrium was reached after 72 h of continuous rocking at 37°C.

Passive Hemagglutination. Sheep erythrocytes (SRBC) were coated with dextran according to the method of Ghanta et al. (38) using partially periodate oxidized dextran B512 (39). Briefly, 100 μl of a 10 mg/ml solution of oxidized B512 was added to 1 ml of 10% SRBC in borate buffer at pH 7.2. The cells were stirred for 5 h at 37°C and then washed three times with PBS and resuspended in PBS to be used for hemagglutination. Hemagglutination assays were carried out using a Takatsy microtitrator with 0.025-ml loops and a 2% suspension of B512-sheep erythrocytes (B512-SRBC).

Immunization Schedule. New Zealand white female rabbits were used. After preimmunization bleedings (designated by subscript x), rabbits were immunized; 0.5 μmol of the stearyl-isomaltosyl oligosaccharide was used for each injection. The antigen was either suspended in 0.5 ml of saline or was incorporated in 0.5 ml of liposomes (20 μmol of total lipid). The antigens were emulsified in 0.5 ml of CFA (Difco Laboratories, Detroit, Mich.) immediately before use; 0.25 ml was injected into each of the four footpads weekly for 3 wk. A blood sample was taken on the 7th d after the last injection and tested for antidextran with B512-SRBC. If the titer remained low, another set of injections was given; if the titer was high, the rabbit was bled three times a week, 50 ml of blood being taken each time, and the three bleedings were pooled. 0.25% phenol and 0.01% merthiolate were added, and the serum was stored at 4°C. The rabbit was allowed to rest for 1 wk, and a second set of three bleedings was taken; blood samples were drawn as above until the titer dropped. As many as five courses totaling 15 injections were given (Table IV).

Purification of Antidextran. A small amount of antibody was purified for gel analysis using enzymatic digestion of dextran-antidextran precipitates (40). Approximately 10 μg of antibody N was precipitated by B512 dextran as for a quantitative precipitin assay; the precipitate was left at 4°C for 1 wk and washed five times with 1-ml portions of chilled saline. The washed precipitate was then digested with dextranase (Sigma Chemical Co., EC 3.2.1.11 [41]) at a ratio of 1:5,000 (enzyme:dextran). The precipitate was digested overnight at room temperature and enzyme inactivated at 56°C for 1 h (42). The samples were used immediately for gel analysis.

SDS-Polyacrylamide Gel Electrophoresis. SDS-polyacrylamide gel electrophoresis was performed in the Bio-Rad slab gel apparatus (Bio-Rad Laboratories, Richmond, Calif.) using the Maizel gel system (43), at pH 7.2. A stacking gel of 3.5% acrylamide placed on top of a separating gel of 5% acrylamide was used. Protein markers were obtained from various sources. Purified rabbit IgG was kindly provided by Dr. B. Pernis, Columbia University, New York; rabbit IgA was provided by Dr. K. L. Knight of the University of Illinois, Chicago, Ill.; albumin was obtained from Sigma Chemical Co., and gammaglobulin (γglo) was obtained from Miles Biochemicals, Elkhart, Ind.

Isoelectric Focusing Gel. Analytical isoelectric focusing was carried out using the LKB 2117 multiphor unit. The gel was set in a thin layer of 5% polyacrylamide containing 2% ampholyte (ampholines pH 3.5–10) according to the procedures given by the manufacturer (LKB-Productor AB, Sweden). After focusing, usually 3 h after the maximum voltage (800 V) was reached, the gel was removed and fixed with 12% trichloroacetic acid (TCA) overnight using several changes. It was then soaked in methanol-acetic acid-H_2O (25:7:68) for 2 h before

staining into 0.25% Coomassie Blue in methanol-acetic acid-H_2O (45:9:46) for 30 min. Destaining was done with methanol-acetic acid-H_2O (50:7:43) and subsequently with several changes of methanol-acetic acid-H_2O (25:7:68).

Scanning of Gels. Gels stained with Coomassie Blue were scanned in a Gilford 2400S spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at A_{650}. Xerox copies of the original scans were made, and the peaks were cut out and weighed to obtain the ratio of the area under the peaks.

Results

Preparation of Stearyl-Isomaltohexaose. 97 mg of IM6 and a fivefold molar excess of stearylamine (121 mg) and sodium cyanoborohydride (30 mg) were dissolved in 20 ml of tetrahydrofuran:water (30:12). The reaction mixture was kept at 37°C for 3 wk, at which time most of the free IM6 had disappeared as judged by paper and thin-layer chromatography. The reaction (30) illustrated with IM2 is shown in Fig. 1. The solution was then taken to dryness *in vacuo* and extracted with chloroform and with water (Fig. 2). The chloroform-soluble material (38.6 mg) consisted mainly of unreacted stearylamine; the water-soluble material (32.1 mg) contained uncoupled IM6 and excess cyanoborohydride. The stearyl-IM6 (127 mg) remained insoluble in both chloroform and water and was dissolved in tetrahydrofuran (THF)-water (36:10) and

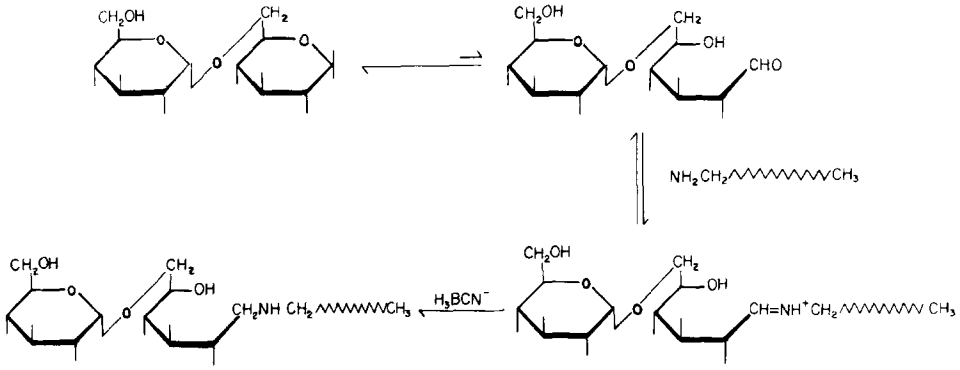

FIG. 1. Proposed mechanism (30) for coupling isomaltose oligosaccharides to stearylamine using sodium cyanoborohydride.

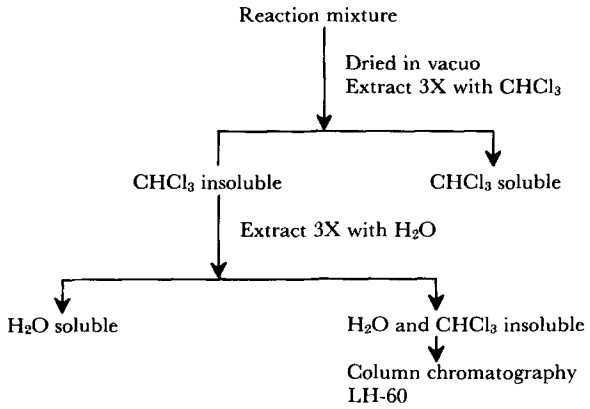

FIG. 2. Schematic diagram for purification of various stearyl-isomaltosyl oligosaccharides.

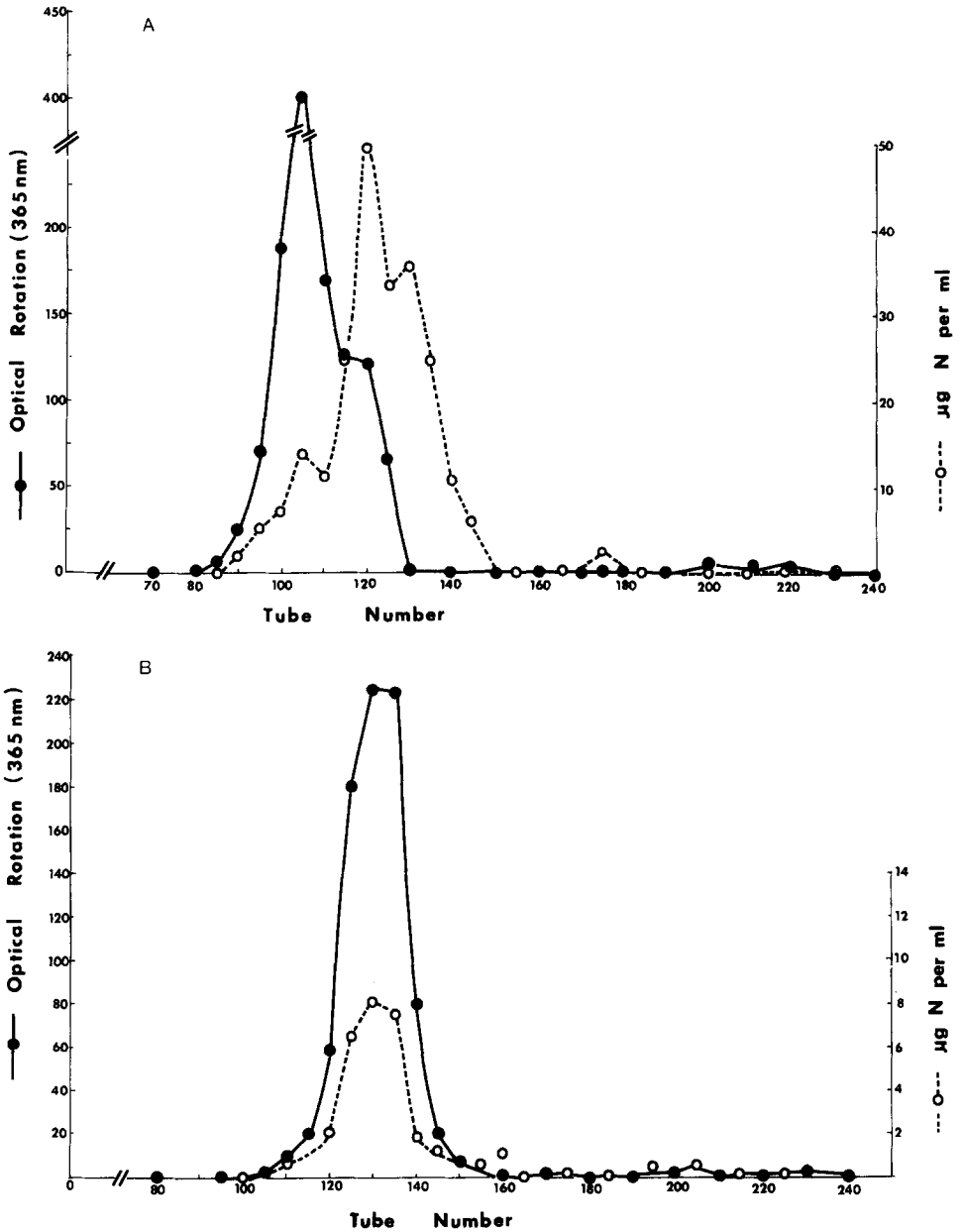

FIG. 3. Chromatography on an LH-60 (80 × 2.5 cm) column of (A) 64 mg of $CHCl_3$ and H_2O insoluble fraction of the stearyl-IM6 reaction mixture, and (B) purified stearyl-IM6.

passed through an LH-60 column (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.). The elution pattern is shown in Fig. 3A; stearylamine was determined by ninhydrin assay for total N after digestion with H_2SO_4 (32) and sugar was determined by optical rotation at 365 nm. Stearyl-IM6 was detected both by N and optical rotation; it eluted with a peak at approximately tube 120; compounds with a higher lipid:sugar ratio and free lipids were eluted at approximately tube 130.

Because the two peaks were not completely separated, the fractions containing stearyl-IM6 were pooled and passed through the column several more times to get rid of contaminating free lipid. The purified compound came out as a single peak (Fig. 3B), and the central portion of the peak was pooled and dried *in vacuo* to yield 29 mg of final product. Paper chromatography in solvent B for 36 h showed a single spot migrating more slowly than IM6 ($R_{IM6} = 0.73$). Analysis for hexose gave 61 and 63% by the orcinol and phenol sulfuric acid methods, and theory gave 65%; total N was 1.0%, and theory, 1.10%; and specific optical rotation in THF-H_2O, +97° at 589 nm and 20°C. The other stearyl-isomaltosyl oligosaccharide conjugates were purified in a similar manner, except that different solvents were used. Approximately 100 mg of IM2, IM3, IM4, IM5, and IM7 were used, and the final coupled products that were isolated weighed 89, 72, 37, 26, and 20 mg, respectively. Analytical data are summarized in Table I.

TABLE I

Characterization of Various Stearyl-Isomaltosyl Oligosaccharides

Compound	Molecular weight	Solvent for column chromatography	$[\alpha]_D$	Total N		Hexose			
				Experimental	Theory	Experimental			Theory
						Orcinol	Phenol sulfuric	Anthrone	
			degrees	%			%		%
Stearyl-IM2	573	$CHCl_3$:ETOH:H_2O 5:5:1	+38	2.2	2.4	29	28	27	28
Stearyl-IM3	735	$CHCl_3$:ETOH:H_2O 3.5:5:1	+68	1.8	1.8	43	49	45	45
Stearyl-IM4	897	$CHCl_3$:ETOH:H_2O 2.4:4:1	+90	1.6	1.5	49	55	53	54
Stearyl-IM5	1059	THF:H_2O 36:10	+68	1.3	1.2	58	60	*	61
Stearyl-IM6	1221	THF:H_2O 36:10	+97	1.1	1.1	61	63	*	65
Stearyl-IM7	1383	THF:H_2O 23:11	+111	0.8	1.0	71	67	*	69

* THF gave a very high background with anthrone.

TABLE II

Agglutination (Read Microscopically) by Rabbit Antisera to Isomaltosyl Oligosaccharides Coupled to BSA of Liposomes Containing Stearyl Isomaltose Oligosaccharides

	R-25 anti-IM3-BSA	R-25x (preimmune serum)	R-28 anti-IM3-BSA	R-28x (preimmune serum)	R-49 anti-IM6-BSA	R-49x (preimmune serum)
IM2-liposomes	2*	<2	2	<2	2	<2
IM3-liposomes	8	<2	128	2	128	<2
IM4-liposomes	16	<2	256	2	128	2
IM5-liposomes	16	<2	128	2	256	2
IM6-liposomes	16	<2	128	2	256	2
IM7-liposomes	16	<2	128	<2	128	2
Control liposomes	<2	<2	<2	<2	<2	<2

* Titer expressed as reciprocal of the highest dilution of serum that gave aggregation as read microscopically.

Agglutination of Liposomes by Antisera. Stearyl-isomaltose oligosaccharides should be readily incorporated into liposomes with the lipid portion embedded in the lipid bilayer and the oligosaccharide moiety extending outward from the surface of the liposomes, and they would be expected to be agglutinated by specific antiserum to the isomaltose oligosaccharide if the oligosaccharide moiety projected sufficiently from the liposome surface. Table II shows the titer, as observed microscopically, of anti-isomaltotrionic BSA (R-25 and R-28; 28) and anti-isomaltohexaonic BSA conjugates (R-49; 29) in agglutinating various kinds of liposomes. IM2-liposomes were only weakly agglutinated by all three sera, whereas IM3, IM4, IM5, IM6, and IM7 liposomes were agglutinated to similar titers. Of the three sera tested, R-25 was the weakest and only gave a titer of 16 with various liposomes; control preimmune sera all gave background agglutination titers of <2. The data suggest that stearyl-IM2 may not be protruding from the liposome surface sufficiently to react with the antibody.

Aggregation of liposomes could also be detected by increased absorption of light when specific antisera were added. Fig. 4 shows that stearyl-IM4 liposomes could be agglutinated by R-25 (anti-isomaltotrionic BSA conjugates) at a dilution of 1:8, measuring the agglutination by the increased OD_{600}. Control preimmune serum and control liposomes without stearyl-isomaltosyl oligosaccharides did not show any change in absorption.

Lysis of Liposomes by Complement. Liposomes containing stearyl-isomaltose oligosaccharides could also be lysed by complement in the presence of specific antibodies (Table III). 60% of the stearyl-IM4 liposomes were lysed after incubating at 37°C for 72 h with complement and R-25 antiserum. Lysis was not recorded until 72 h to allow released dichromate to equilibrate between the chambers of the equilibrium dialysis cell. Stearyl-IM4 liposomes alone gave 31% spontaneous lysis after 72 h; controls of complement alone or preimmune serum gave background lysis of 24 and 32%, respectively. Liposomes that did not contain stearyl-isomaltose oligosaccharides showed no increase in lysis 72 h after adding antibody and complement. This experiment was repeated with liposomes containing other stearyl-isomaltosyl oligosaccharides; IM3, IM5, IM6, and IM7 liposomes all showed lysis but IM2-liposomes did not (data not shown), which was in agreement with the findings by agglutination,

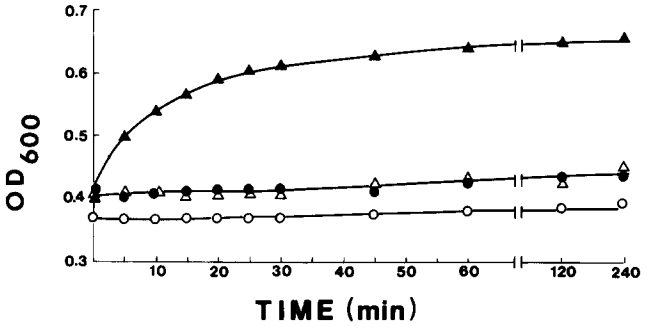

FIG. 4. Agglutination of liposomes as measured by OD_{600}. ▲, stearyl-IM4 liposomes plus R-25 (anti-isomaltotrionic-BSA; 1:8); △, stearyl-IM4 liposomes plus R-25_X (preimmune serum; undiluted); ●, control liposomes plus R-25 (1:8); ○, control liposomes plus R-25_X (undiluted).

TABLE III

Lysis of Stearyl-IM4 Liposomes by Complement and Specific Antisera to Isomaltosyl Oligosaccharides Coupled to BSA

Liposomes	Serum	Guinea pig complement	Lysis*
125 μl	*25 μl*		%
Stearyl-IM4 liposomes	R-25 anti-IM3 BSA‡	25 μl	60
Stearyl-IM4 liposomes	R-25 anti-IM3 BSA	—	29
Stearyl-IM4 liposomes	$R\text{-}25_X$ preimmune serum‡	25 μl	32
Stearyl-IM4 liposomes	—	—	24
Stearyl-IM4 liposomes	—	25 μl	24
Control liposomes	R-25 anti-IM3 BSA	25 μl	20
Control liposomes	R-25 anti-IM3 BSA	—	21
Control liposomes	$R\text{-}25_X$ preimmune serum	25 μl	32
Control liposomes	—	—	30
Control liposomes	—	25 μl	21

* Measured by dialyzable dichromate liberated on equilibrium dialysis after 72 h at 37°C. Total volume was 200 μl. Liposomes treated with 1% Triton X were used to establish 100% lysis.

‡ Undiluted serum was used.

indicating that stearyl-IM2 does not protrude from the liposome surface sufficiently to react with antibody.

Antigenicity of Stearyl-Isomaltosyl Oligosaccharides. Stearyl-IM5 and stearyl-IM6 were antigenic in rabbits when incorporated into liposomes and mixed with CFA. However, they were also antigenic when emulsified in CFA without liposomes (Table IV). Antibodies formed against stearyl-isomaltosyl oligosaccharides cross-react with $\alpha 1 \rightarrow 6$ dextrans, and the antibody level could be followed by hemagglutination of B512 dextran-coated SRBC. A few rabbits showed a high preimmune titer against B512-SRBC, even though precipitation with B512 was not measurable. Rabbits immunized by intravenous injection of stearyl-IM5 and stearyl-IM6 with or without liposomes in the absence of CFA generally did not respond; however, two rabbits (R-853 and R-865) showed a fourfold increase in titer, but the sera did not precipitate with dextran. Rabbits immunized with antigen in CFA responded after 3–6 injections, whereas those immunized without CFA did not respond, even after 15 injections, thus showing that CFA is important in enhancing the antigenicity of stearyl-isomaltosyl oligosaccharides.

Rabbit antisera to other stearyl-isomaltosyl oligosaccharides were also obtained by immunization with stearyl-IM2, -IM3, -IM4, and -IM7 in liposomes plus CFA. Their responses are summarized in Table IV. All rabbits injected with stearyl-IM4 and stearyl-IM7 gave good titers, whereas only two of three responded to stearyl-IM3 and only one of four mounted a weak response to stearyl-IM2.

Agglutination and Lysis of Liposomes by Antisera to Stearyl-Isomaltosyl Oligosaccharides. Table V gives the results of liposome agglutination assays on the various antisera to stearyl-isomaltosyl oligosaccharides. Liposomes containing stearyl-IM3, -IM4, -IM5, -IM6, and -IM7 were agglutinated as observed microscopically, whereas liposomes with stearyl-IM2 were only weakly agglutinated; these findings are comparable to those using anti-isomaltotrionic BSA and anti-isomaltohexaonic BSA conjugates antisera (Table II). Agglutination was accompanied by an increase of OD_{600} similar to Fig. 4. Lysis of liposomes was also seen with R-663 serum and

TABLE IV

Responses of Rabbits to Stearyl-Isomaltosyl Oligosaccharides

Immunogen	Rabbit number	Antigen per injection	Preimmune titer	Titer after three injections	Titer after six injections	Titer after fifteen injections
		mg				
Stearyl-IM2 liposomes + CFA	R843	0.7	8	8	16	16
	R854	0.7	16	32	64	64
	R855	0.7	64	128	256	256*
	R864	0.7	16	16	16	16
Stearyl-IM3 liposomes + CFA	R841	0.6	64	64	128	128
	R839	0.6	256	256	256	1,000*
	R859	0.6	32	32	512*	
Stearyl-IM4 liposomes + CFA	R663	0.5	32	1,000*		
	R664	0.5	64	128	1,000*	
Stearyl-IM5 liposomes + CFA	R847	0.45	32	64	512 (died)	
	R848	0.45	128	256	1,000*	
	R856	0.45	8	16	512*	
Stearyl-IM6 liposomes + CFA	R842	0.4	128	256	1,000*	
	R846	0.4	32	32	512*	
Stearyl-IM7 liposomes + CFA	R849	0.4	16	32	512*	
	R850	0.4	128	1,000*		
Stearyl-IM5 + CFA	R860	0.45	128	512	2,000*	
	R861	0.45	4	128	1,000*	
Stearyl-IM5 liposomes‡	R864	0.45	32	64	64	64
	R865	0.45	16	32	64	64
Stearyl-IM5‡	R866	0.45	4	8	8	8
	R868	0.45	8	16	8	8
Stearyl-IM6 + CFA	R851	0.4	64	512*		
	R862	0.4	4	128	2,000*	
Stearyl-IM6 liposomes‡	R852	0.4	16	32	64	64
	R853	0.4	64	64	64	64
Stearyl-IM6‡	R857	0.4	8	8	8	8
	R858	0.4	16	32	32	32

All injections with CFA were intramuscular. Titers were determined by hemagglutination of B512-SRBC.
* Time when larger bleedings were started; small test bleedings were made earlier.
‡ Intravenous.

TABLE V

Agglutination of Liposomes (Read Microscopically) by Rabbit Antisera to Stearyl-Isomaltosyl Oligosaccharides

	R-663 anti-stearyl-IM4 liposomes	$R663_x$ (preimmune serum)	R-856 anti-stearyl-IM5 liposomes	$R\text{-}856_x$	R855 anti-stearyl-IM2 liposomes*	$R\text{-}855_x$
IM2-liposomes	2	<2	2	<2	2	<2
IM3-liposomes	8	<2	8	<2	4	<2
IM4-liposomes	16	<2	16	<2	8	<2
IM5-liposomes	16	<2	32	<2	8	<2
IM6-liposomes	16	<2	32	<2	16	<2
IM7-liposomes	16	<2	32	<2	16	<2
Control liposomes	<2	<2	<2	<2	<2	<2

* All other rabbits injected with stearyl-IM2 did not agglutinate any of the liposomes listed.

TABLE VI

Lysis of Stearyl-IM4 Liposomes by Anti-Stearyl-Isomaltosyl Oligosaccharides and Complement

Liposomes	Serum	Guinea pig complement	Lysis* R-663 anti-stearyl-IM4 liposomes	Lysis* R-856 anti-stearyl-IM5 liposomes	Lysis* R-855 anti-stearyl-IM2 liposomes
125 μl	*25 μl*	*μl*	%	%	%
Stearyl-IM4 liposomes	Immune serum‡	25	48	74	40
Stearyl-IM4 liposomes	Immune serum	—	10	25	12
Stearyl-IM4 liposomes	Preimmune serum‡	25	18	23	20
Stearyl-IM4 liposomes	—	—	8	25	15
Stearyl-IM4 liposomes	—	25	11	23	18
Control liposomes	Immune serum	25	25	24	22
Control liposomes	Immune serum	25	15	15	18
Control liposomes	Preimmune serum	25	29	15	20
Control liposomes	—	—	14	27	15
Control liposomes	—	25	18	23	17

* Measurement by dialyzable dichromate liberated on equilibrium dialysis after 72 h at 37°C. Total volume was 200 μl.

‡ Undiluted serum was used.

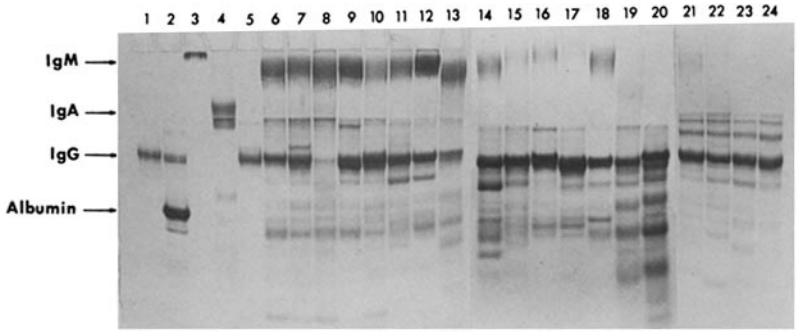

FIG. 5. SDS-polyacrylamide gel electrophoresis of various purified antibodies to stearyl-isomaltosyl oligosaccharides and isomaltosyl oligosaccharide-BSA conjugates. Lane 1, rabbit γglo; 2, crude rabbit albumin; 3, MOPC104E (mouse IgM); 4, rabbit IgA; 5, purified rabbit IgG; 6, R-855 anti-stearyl-IM2 liposomes, CFA; 7, R-839 anti-stearyl-IM3 liposomes, CFA; 8, R-859 anti-stearyl-IM3 liposomes, CFA; 9, R-663 anti-stearyl-IM4 liposomes, CFA; 10, R-664 anti-stearyl-IM4 liposomes, CFA; 11, R-848 anti-stearyl-IM5 liposomes, CFA; 12, R-856 anti-stearyl-IM5 liposomes, CFA; 13, R-842 anti-stearyl-IM6 liposomes, CFA; 14, R-846 anti-stearyl-IM6 liposomes, CFA; 15, R-849 anti-stearyl-IM7 liposomes, CFA; 16, R-850 anti-stearyl-IM7 liposomes, CFA; 17, R-860 anti-stearyl-IM5, CFA; 18, R-861 anti-stearyl-IM5, CFA; 19, R-851 anti-stearyl-IM6, CFA; 20, R-862 anti-stearyl-IM6, CFA; 21, R-25 anti-isomaltotrionic-BSA; 22, R-27 anti-isomaltotrionic-BSA; 23, R-48 anti-isomaltohexaonic-BSA; 24, R-49 anti-isomaltohexaonic-BSA.

complement (Table VI); highest lysis (48%) of stearyl-IM4 liposomes was obtained only in the presence of R-663 antiserum and complement. All other controls only gave background lysis.

SDS-Polyacrylamide Gel Electrophoresis and Isoelectric Focusing. The isotypes of various anti-stearyl-isomaltosyl oligosaccharide antibodies were studied by SDS-gel electrophoresis at pH 7.2 using rabbit IgG, IgA, γglo, albumin (contaminated with γglo),

TABLE VII

Ratio of IgM to IgG in Various Antisera as Determined from Scans of Gels Stained with Coomassie Blue

Rabbit number	Antigen	IgM:IgG
R-855	Stearyl-IM 2 liposomes + CFA	1.4:1.0
R-839	Stearyl-IM 3 liposomes + CFA	1.0:1.09
R-859	Stearyl-IM 3 liposomes + CFA	9.4:1.0
R-663	Stearyl-IM 4 liposomes + CFA	1.0:1.07
R-664	Stearyl-IM 4 liposomes + CFA	1.0:2.7
R-848	Stearyl-IM 5 liposomes + CFA	1.0:1.6
R-856	Stearyl-IM 5 liposomes + CFA	1.4:1.0
R-842	Stearyl-IM 6 liposomes + CFA	1.7:1.0
R-846	Stearyl-IM 6 liposomes + CFA	1.0:1.4
R-849	Stearyl-IM 7 liposomes + CFA	1.0:2.2
R-850	Stearyl-IM 7 liposomes + CFA	1.3:1.0
R-860	Stearyl-IM 5 + CFA	1:400
R-861	Stearyl-IM 5 + CFA	1.0:2.1
R-851	Stearyl-IM 6 + CFA	No detectable IgM
R-862	Stearyl-IM 6 + CFA	No detectable IgM

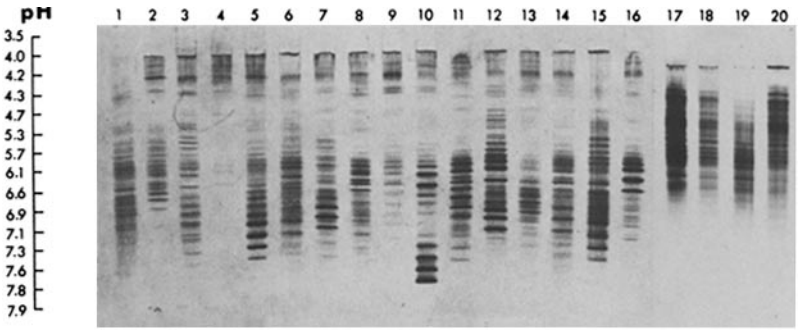

FIG. 6. Isoelectric focusing pattern of various purified antibodies to stearyl-isomaltosyl oligosaccharides and isomaltosyl oligosaccharide-BSA conjugates. Lane 1, rabbit γglo; 2, R-855; 3, R-839; 4, R-859; 5, R-663; 6, R-664; 7, R-848; 8, R-856; 9, R-842; 10, R-846; 11, R-849; 12, R-850; 13, R-860; 14, R-861; 15, R-851; 16, R-862; 17, R-25 anti-isomaltotrionic-BSA; 18, R-27 anti-isomaltotrionic-BSA; 19, R-48 anti-isomaltohexaonic-BSA; 20, R-49 anti-isomaltohexaonic-BSA.

and mouse IgM (MOPC104E) as markers. The antibodies studied were purified by precipitation with dextran (B512) and subsequent digestion with dextranase, as described in Materials and Methods. The gel (Fig. 5) shows the predominant isotypes in all sera to be IgM and IgG, with very little IgA. The other bands that migrate faster than IgG may be H and L chain dimers or H-L chains because upon reduction with mercaptoethanol most of these bands disappeared and only μ, γ, and L chains were seen. The ratio of IgM to IgG in the different antisera was calculated from scans of gels stained with Coomassie Blue (Table VII). The amounts of IgM and IgG vary from serum to serum, with R-859 containing predominantly IgM, and with R-851, R-862, and R-860 containing mostly IgG.

The heterogeneity of the antidextran response was studied by isoelectric focusing in a pH gradient of 3.5–10 (Fig. 6). Control rabbit γglo shows substantial heterogeneity, whereas in most of the antisera restricted heterogeneity is evident from the

presence of some intense bands in the isoelectric focusing patterns ascribable to a few predominant responding clones. R-862 was most restricted with only six major bands focusing at about pH 6.5. R-846 showed two distinct groups of clones, with one focusing at about pH 6.1 and the other at pH 7.7. Another rabbit, R-859, showed only a few faint bands at ~pH 6.1, and the majority of the antibodies remained at the origin, which is in agreement with the SDS-polyacrylamide gel findings (Fig. 5) showing that this serum contains mainly IgM that stayed at the origin. For comparison, rabbit antibodies to isomaltotrionic-BSA (R-25, R-27) and isomaltohexaonic-BSA conjugates (R-48, R-49) were also studied by isoelectric focusing (Fig. 6). They are similar to the antibodies to stearyl-isomaltose oligosaccharides in having some predominant intense bands, however, most of the bands focused over a lower pH range than those obtained by immunizing with the stearyl-isomaltosyl oligosaccharides.

Discussion

The present findings show that stearylamine can be covalently attached to isomaltose oligosaccharides by reductive amination catalyzed by the cyanoborohydride anion to produce model glycolipids that are antigenic in rabbits. The reaction involves coupling of the aldehyde group at the reducing end to the amino group of stearylamine (30; Fig. 1) with the opening of the reducing sugar ring. However, as reported by Gray (31), who used sodium cyanoborohydride to couple oligosaccharides to protein and to aminoethyl polyacrylamide gels, the reaction proceeds at a very slow rate. In oligosaccharides, excess side reactions lead to the formation of tertiary amines as well, but this may be eliminated by using an excess of amino groups. Consequently, all reactions were carried out with a fivefold molar excess of stearylamine. These conditions are still not ideal because during the purification of the coupled products some unidentified compounds with a much higher lipid:sugar ratio were seen. Schwartz and Gray (45) and Wiegandt and Ziegler (46) also noted substantial amounts of unidentified amines when sodium cyanoborohydride was used for reductive amination of lactose with ammonium acetate. Despite these drawbacks, reductive amination proved an efficient way to synthesize a series of stearyl-isomaltosyl oligosaccharides.

Glycolipids are usually not antigenic. Antibodies to glycolipids may be produced (47) by injecting animals with cell membranes or subcellular particles containing the glycolipids (48, 49); by immunizing with glycolipids covalently linked to a polymer carrier (50, 51); by immunizing with a micellar suspension of purified lipid and heterologous protein carriers such as serum albumin (7, 52); and by injecting emulsions of glycolipids and CFA without carrier protein (53, 54). Liposomes have also been reported to function as carriers for various haptens. Kinsky's laboratory (55–58) showed enhancement of anti-DNP responses in guinea pigs immunized with DNP-ε-aminocaproylphosphatidyl-ethanolamine (DNP-Cap-PE) incorporated with liposomes, and van Rooijen and van Nieuwmegen (59) found higher levels of antihuman serum albumin in rabbits injected with human serum albumin entrapped in liposomes.

The purpose of this investigation was to evaluate the antigenicity of the stearyl-isomaltosyl oligosaccharides as model glycolipids using liposomes as carriers and to study the reactivities of the antibodies with various dextrans. Antibodies were titrated easily by the agglutination of liposomes containing the antigen, or optically by

recording increased light absorption as liposomes are agglutinated by antibodies (3, 5). A third method involves lysis of liposomes by complement and specific antibodies using markers (36, 60, 61) to measure the degree of lysis. In our assay a colored substance, sodium dichromate, was used as a marker, and the assay was carried out in microequilibrium dialysis cells to minimize the quantities of liposomes, antibody, and complement needed. Liposomes containing stearyl-IM2 were not agglutinated by various antibodies to $\alpha 1 \rightarrow 6$ dextran (Tables II and V), whereas those containing IM3 were agglutinated but weakly as compared to liposomes with IM4 to IM7. This indicates that with stearyl-IM2 the sugar moiety may not be protruding far enough from the liposome surface to be recognized by antibodies; at least two intact sugar rings plus an α-linkage (stearyl-IM3) must extend from the liposomes before it can bind antibodies. With the injection schedules used, stearyl-isomaltosyl oligosaccharides alone or incorporated into liposomes without CFA did not induce a significant antibody response (Table IV). Antibodies cross-reacting with dextrans were obtained when liposomes containing stearyl-isomaltosyl oligosaccharides were emulsified with CFA. Moreover, stearyl-IM5 and -IM6 by themselves gave good antibody responses when emulsified with CFA. Thus, with these glycolipids, liposomes are not required as carriers if CFA are used. Because glycolipids would be expected to form micelles in CFA, the use of liposomes may be superfluous. Indeed, antibodies to other glycolipids such as rat antigalatocerebroside (53) and rabbit anti-Forssman glycolipid (54) had been raised using only an emulsion of the glycolipid and CFA. Dancey et al. (62) and Honegger et al. (63) reported that when lipid A, a B cell mitogen, was incorporated into liposomes with antigen, it enhanced the antibody response. Westphal et al. (64) also reported that lysolecithin and other synthetic analogues can act as adjuvants. It will be of interest to determine whether lipid A and other synthetic lysolecithin analogues could substitute for CFA in enhancing the response to stearyl-isomaltosyl oligosaccharides with or without liposomes.

The haptenic nature of glycolipid was first demonstrated by Landsteiner and Simms (65), who showed that nonantigenic purified Forssman glycolipid could become antigenic when complexed noncovalently with whole serum as a carrier or "schlepper." It is now known that this carrier could be any heterologous protein, such as serum albumin (7, 52). The cellular basis of this is still poorly defined, and very little is known about T cell dependency of responses to glycolipids. However, studies of protein antigens such as BSA (66–68) coupled to fatty acids established a definite need for T cells. Coon and Hunter (66), and subsequently Dailey and Hunter (67), and Singh and Leskowitz (68) showed that coupling of dodecanoic acids to BSA modified the immunogenicity of BSA leading to selective induction of delayed-type hypersensitivity. In contrast to the above studies, coupling of haptens such as fluorescein and DNP to phosphatidylethanolamine and incorporating them into liposomes elicited humoral responses exclusively (69). Yasuda et al. (70) showed that both nude and thymectomized mice mounted a significant anti-DNP response to DNP-Cap-PE when incorporated into liposomes. Its thymus-independent character was substantiated in that liposomes containing DNP-Cap-PE sustained a DNP-specific response in cultures of primed spleen cells pretreated with antithymocyte serum and complement (71). However, it cannot be generalized that lipid-protein conjugates, such as dodecanoic acid-BSA, require T cells and that lipid-hapten conjugates, such as DNP-Cap-PE when incorporated on liposomes, are T independent,

because another lipid-hapten conjugate, mono(azobenzenearsonic acid)-tyrosyl-phosphatidylethanolamine (ABA-tyr-PE), induced both humoral and cellular immunity (57). Therefore, T cell dependency may vary from one lipid antigen to another. Because the role of T cells in antibody formation to glycolipids is not clearly defined, it would be of interest to study this with stearyl-isomaltosyl oligosaccharides with and without the use of liposomes.

The dextran response in humans immunized with native or clinical dextrans (22) was heterogeneous as evidenced by marked variations in relative binding affinity for various isomaltose oligosaccharides, as were the antibodies cross-reacting with dextran obtained by immunizing rabbits with isomaltosyl oligosaccharide-BSA conjugates (28, 29). In the latter instances this was perhaps due to the protein carrier being a part of the antigenic determinant and the consequent formation of many antigenic determinants. Most of the antibodies produced by stearyl-isomaltosyl oligosaccharides with or without liposomes were shown to be still somewhat heterogeneous, as evidenced by the ability to fractionate these antisera into more restricted antibody fractions that vary in the sizes of their antibody-combining sites[2] and by isoelectric focusing. However, a few antisera appeared to be considerably less heterogeneous. Restricted anti-DNP and anti-phosphorylcholine responses were also elicited by DNP-Cap-PE (56) and 3-[3-(4-azophenyl-phosphorylcholine)-4-hydroxyphenyl]-*N*-propionyl-phosphatidyl-ethanolamine (APPC-PPr-PE) (63) on liposomes. These antibodies also showed restricted isoelectric focusing patterns and a narrow range of binding constants, perhaps ascribable to the DNP or phosphorylcholine determinants being small, thus maintaining a relatively defined conformation on the liposomes. With the availability of this series of chemically defined glycolipids, by using inbred lines of mice and guinea pigs, one can now attack problems of the genetic control of the responses to glycolipids, their T cell dependency, and their ability to induce cellular immunity, such as delayed-type hypersensitivity.

Summary

Isomaltose oligosaccharides varying in size from two sugars, isomaltose (IM2), to seven sugars, isomaltoheptaose (IM7), were coupled to stearylamine by reductive amination with sodium cyanoborohydride. Each compound was purified by column chromatography to yield a series of glycolipids containing oligosaccharides differing in length. Stearyl-isomaltotriose to stearyl-IM7 could be incorporated into liposomes and could render them agglutinable by specific antibodies to $\alpha 1 \rightarrow 6$ dextran and could be lysed if complement was also added, whereas those containing stearyl-IM2 were not agglutinated or lysed, indicating that stearyl-IM2 may not be protruding from the liposome surface sufficiently to react with the antibody. Stearyl-isomaltosyl oligosaccharides by themselves or incorporated into liposomes were equally antigenic when emulsified in complete Freund's adjuvant. They elicited pauciclonal responses, and the antibodies were $\alpha 1 \rightarrow 6$ specific, cross-reacted with dextran, and gave semirestricted isoelectric focusing patterns.

[2] Wood, C., and E. A. Kabat. Immunochemical studies on conjugates of isomaltosyl oligosaccharides to lipid. III. Fractionation of rabbit antibodies to stearyl-isomaltosyl oligosaccharides and a study of their combining sites by a competitive binding assay. Manuscript submitted for publication.

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References

1. Rapport, M. M., and L. Graf. 1969. Immunochemical reactions of lipids. *Prog. Allergy.* **13:** 273.
2. Marcus, D. M., and G. A. Schwarting. 1976. Immunochemical properties of glycolipids and phospholipids. *Adv. Immunol.* **23:**203.
3. Alving, C. R. 1977. Immune reaction of lipids and lipid model membranes. *In* The Antigens. M. Sela, editor. Academic Press, Inc., New York. **4:**1.
4. Niedieck, B. 1975. On a glycolipid hapten of myelin. *Prog. Allergy.* **18:**351.
5. Arnon, R., M. Sela, E. S. Rachaman, and D. Shapiro. 1967. Cytolipin H-specific antibodies obtained with a synthetic polypeptide conjugate. *Eur. J. Biochem.* **2:**79.
6. Teitelbaum, D., R. Arnon, M. Sela, Y. Rabinsohn, and D. Shapiro. 1973. Sphingomyelin specific antibodies elicited by synthetic conjugates. *Immunochemistry.* **10:**735.
7. Koscielak, J., S. Hakomori, and R. W. Jeanloz. 1968. Glycolipid antigen and its antibody. *Immunochemistry.* **5:**441.
8. Inoue, K. I., and S. Nojima. 1967. Immunochemical studies of phospholipids. III. Production of antibody to cardiolipin. *Biochim. Biophys. Acta.* **144:**409.
9. Rapport, M. M., L. Graf, V. P. Skipski, and N. F. Alonzo. 1959. Immunochemical studies of organ and tumor lipids. VI. Isolation and properties of cytolipin H. *Cancer (Phila.).* **12:** 438.
10. Koscielak, J. 1962. Reversible inactivation of blood group A and B substances from red cell. *Nature (Lond.).* **194:**751.
11. Koscielak, J. 1963. Blood group A specific glycolipids from human erythrocytes. *Biochim. Biophys. Acta.* **78:**313.
12. Ngai, Y., and T. Ohsawa. 1974. Production of high titer antisera against sialoglycosphingolipids and their characterization using sensitized liposomes. *Jpn. J. Exp. Med.* **44:**451.
13. Rapport, M. M., L. Graf, and J. Yariv. 1961. Immunochemical studies of organ and tumor lipids. IX. Configuration of the carbohydrate residues in cytolipin H. *Arch. Biochem. Biophys.* **92:**438.
14. Zalc, B., P. Dupouey, M. J. Coulon-Marelec, and N. A. Baumann. 1979. Immunogenic properties of glycosylceramide. *Mol. Immunol.* **16:**297.
15. Anderson, B. A., L. Hellgren, and J. Vincent. 1976. Allergic delayed skin reactions from lipid fractions of trichophytin. *Sabouraudia.* **14:**237.
16. Hellgren, L., and J. Vincent. 1976. Contact sensitizing properties of some fatty acids in dermatophytes. *Sabouraudia.* **14:**243.
17. Jeanes, A., W. C. Haynes, C. A. Wilham, J. C. Rankin, E. H. Melvin, M. J. Austin, J. E. Cluskey, B. F. Fisher, H. M. Tsuchiya, and C. E. Rist. 1954. Characterization and classification of dextrans from ninety-six strains of bacteria. *J. Am. Chem. Soc.* **76:**5041.
18. Kabat, E. A. 1961. Kabat and Mayer's Experimental Immunochemistry. Charles C. Thomas Publisher. 2nd edition: Springfield, Ill. 3–905.
19. Kabat, E. A., and D. Berg. 1953 Dextran—an antigen in man. *J. Immunol.* **70:**514.
20. Maurer, P. H. 1953. Dextran, an antigen in man. *Proc. Soc. Exp. Biol. Med.* **83:**879.
21. Kabat, E. A. 1954. Some configurational requirements and dimensions of the combining site on an antibody to a naturally occurring antigen. *J. Am. Chem. Soc.* **76:**3709.
22. Kabat, E. A. 1956. Heterogeneity of the combining regions of human antidextran. *J. Immunol.* **77:**377.

23. Larm, O., B. Lindberg, and S. Svensson. 1971. Studies on the length of the side chains elaborated by *Leuconostoc mesenteroides* NRRL B-512. *Carbohydr. Res.* **20:**39.
24. Jeanes, A., C. A. Wilham, R. W. Jones, H. M. Tsuchiya, and C. E. Rist. 1953. Isomaltose and isomaltotriose from enzymatic hydrolyzates of dextran. *J. Am. Chem. Soc.* **75:**5911.
25. Turvey, J. R., and W. J. Whelan. 1957. Preparation and characterization of the isomaltodextrins. *Biochem. J.* **67:**49.
26. Allen, P. Z., and E. A. Kabat. 1959. Immunochemical studies on dextrans. II. Antidextran specificities involving the $\alpha 1 \rightarrow 3$ and $\alpha 1 \rightarrow 2$ linked glucosyl residues. *J. Am. Chem. Soc.* **81:** 4382.
27. Schlossman, S. F., and E. A. Kabat. 1962. Specific fractionation of a population of antidextran molecules with combining sites of various sizes. *J. Exp. Med.* **116:**535.
28. Arakatsu, Y., G. Ashwell, and E. A. Kabat. 1966. Immunochemical studies on dextran. V. Specificity and cross-reactivity with dextrans of the antibodies formed in rabbits to isomaltonic and isomaltotrionic acids coupled to bovine serum albumin. *J. Immunol.* **97:**858.
29. Outschoorn, I. M., G. Ashwell, F. Gruezo, and E. A. Kabat. 1974. Immunochemical studies on dextrans. VIII. Specificity and cross-reactivity with dextrans of the antibodies formed in rabbits to isomaltohexaonic acid coupled to bovine serum albumin. *J. Immunol.* **113:**896.
30. Gray, G. R. 1978. Antibodies to carbohydrates: preparation of antigens by coupling carbohydrates to protein by reductive amination with cyanoborohydride. *Methods Enzymol.* **50:**115.
31. Gray, G. R. 1974. The direct coupling of oligosaccharides to proteins and derivatized gels. *Arch. Biochem. Biophys.* **163:**426.
32. Schiffman, G., E. A. Kabat, and W. Thompson. 1964. Immunochemical studies on blood groups. XXX. Cleavage of A, B, and H blood-group substances by alkali. *Biochemistry.* **3:** 113.
33. Sessa, G., and G. Weissmann. 1970. Incorporation of lysozyme into liposomes. A model for structure-linked latency. *J. Biol. Chem.* **245:**3295.
34. Yasuda, T., G. F. Dancey, and S. C. Kinsky. 1977. Immunogenicity of liposomal model membranes in mice. Dependence on phospholipid composition. *Proc. Natl. Acad. Sci. U. S. A.* **74:**1234.
35. Hamers, M. N., W. E. Donker-Koopman, D. J. Reijngoud, A. W. Schram, and J. M. Tager. 1978. An optical method for the detection and quantitation of antibodies to glycosphingolipids and other antigens. *Immunochemistry.* **15:**97.
36. Kinsky, S. C. 1972. Antibody-complement interaction with lipid model membranes. *Biochim. Biophys. Acta.* **265:**1.
37. Eisen, H. N. 1971. Equilibrium dialysis. V. Microtechnique. *In* Methods in Immunology and Immunochemistry. C. A. Williams, and M. W. Chase, editors. Academic Press, Inc., New York. **3:**393.
38. Ghanta, V. K., N. M. Hamlin, T. G. Pretlow, and R. N. Hiramoto. 1972. Use of dextran-conjugated SRBC for the detection of MOPC104E tumor cells. *J. Immunol.* **109:**810.
39. Sanderson, C. J., and D. V. Wilson. 1971. Methods for coupling protein or polysaccharide to red cells by periodate oxidation. *Immunochemistry.* **8:**163.
40. Kabat, E. A. 1954. Purification of human antidextran. *Science (Wash. D. C.).* **120:**782.
41. Sugiura, M., A. Ito, T. Ogiso, K. Kato, and H. Asano. 1973. Studies on dextranase. Purification of dextranase from *Penicillium funiculosum* and its enzymatic properties. *Biochim. Biophys. Acta.* **309:**357.
42. Sharon, J., E. A. Kabat, and S. L. Morrison. 1981. Mouse hybridomas secreting antibodies to $\alpha 1 \rightarrow 6$ linked dextran. *Mol. Immunol.* In press.
43. Maizel, J. V. 1971. Methods in Virology. Academic Press, Inc., New York. **5:**179.
44. Borsch, R. F., M. D. Bernstein, and H. D. Durst. 1971. The cyanohydridoborate anion as a selective reducing agent. *J. Am. Chem. Soc.* **93:**2897.

45. Schwartz, B. A., and G. R. Gray. 1974. Proteins containing reductive aminated disaccharides, synthesis and chemical characterization. *Arch. Biochem. Biophys.* **181:**542.
46. Wiegandt, H., and W. Ziegler. 1974. The use of reductaminated sugars for the preparation of oligosaccharide conjugates. I. Synthetic glycolipids containing glycosphingolipid derived oligosaccharides. *Hoppe-Seyler's Z. Physiol. Chem.* **355:**11.
47. Hakomori, S. I. 1972. Preparation of antisera against glycolipids. *Methods Enzymol.* **28:**232.
48. Joffe, S., M. M. Rapport, and L. Graf. 1963. Identification of an organ specific lipid hapten in brain. *Nature (Lond.).* **197:**60.
49. Naiki, M., and T. Taketomi. 1969. Chemical and immunochemical properties of glycolipids from pig spleen. *Jpn. J. Exp. Med.* **39:**549.
50. Taketomi, T., and T. Yamakawa. 1963. Immunochemical studies of lipids. (1) Preparations and immunological properties of synthetic psychosine-protein antigen. *J. Biochem.* **54:**444.
51. Taketomi, T., and T. Yamakawa. 1966. Antigenic properties of a synthetic protein complex with glycolipids and related substances. *Lipids.* **1:**31.
52. Marcus, D. M., and R. Janis. 1970. Localization of glycosphingolipids in human tissues by immunofluorescence. *J. Immunol.* **104:**1530.
53. Czlonkowska, A., and S. Leibowitz. 1974. The effect of homologous and heterologous carriers on the immunogenicity of the galactocerebroside hapten. *Immunology.* **27:**1117.
54. Uemura, K., M. Yuzawa, and T. Taketomi. 1979. Immunochemical studies of lipids. V. Effect of modified hydrophobic moiety on immunogenicity and immunologic reactivity of Forssman glycolipids. *Jpn. J. Exp. Med.* **49:**1.
55. Uemura, K., R. A. Nicolotti, H. R. Six, and S. C. Kinsky. 1974. Antibody formation in response to liposomal model membranes sensitized with N-substituted phosphatidylethanolamine derivatives. *Biochemistry.* **13:**1572.
56. Uemura, K., J. L. Clafin, J. M. Davie, and S. C. Kinsky. 1975. Immune response to liposomal model membranes: restricted IgM and IgG anti-dinitrophenyl antibodies produced in guinea pigs. *J. Immunol.* **114:**958.
57. Nicolotti, R. A., and S. C. Kinsky. 1975. Immunogenicity of liposomal model membranes sensitized with mono(*p*-azobenzenearsonic acid) tyrosyl phosphatidylethanolamine derivatives. Antibody formation and delayed hypersensitivity reaction. *Biochemistry.* **14:**2331.
58. Kochibe, N., R. A. Nicolotti, J. M. Davie, and S. C. Kinsky. 1975. Stimulation and inhibition of anti-hapten responses in guinea pigs with hybrid liposomes. *Proc. Natl. Acad. Sci. U. S. A.* **72:**4582.
59. van Rooijen, N., and R. van Nieuwmegen. 1977. Liposomes in immunology: the immune response against antigen-containing liposomes. *Immunol. Commun.* **6:**489.
60. Hesketh, T. R., S. N. Payne, and J. H. Humphrey. 1972. Complement and phospholipase C lysis of lipid membranes. *Immunology.* **23:**705.
61. Hsia, J. C., and C. T. Tan. 1978. Membrane immunoassay principle and application of spin membrane immunoassay. *Ann. N. Y. Acad. Sci.* **308:**139.
62. Dancey, C. F., T. Yasuda, and S. C. Kinsky. 1977. Enhancement of liposomal model membrane immunogenicity by incorporation of lipid A. *J. Immunol.* **119:**1868.
63. Honegger, J. L., P. C. Isakson, and S. C. Kinsky. 1980. Murine immunogenicity of N-substituted phosphatidylethanolamine derivatives in liposomes: response to the hapten phosphocholine. *J. Immunol.* **124:**669.
64. Westphal, O., H. Fisher, and P. G. Munder. 1970. Adjuvanticity of lysolecithin and synthetic analogues. *8th Int. Congr. Biochem. Abstr.* P 319.
65. Landsteiner, K., and S. Simms. 1923. Production of heterogenetic antibodies with mixtures of the binding part of the antigen and protein. *J. Exp. Med.* **38:**127.
66. Coon, J., and R. L. Hunter. 1973. Selective induction of delayed hypersensitivity by a lipid conjugated protein antigen which is localized in thymus dependent tissue. *J. Immunol.* **110:** 183.

67. Dailey, M. O., and R. L. Hunter. 1977. Induction of cell-mediated immunity to chemically modified antigens in guinea pigs. I. Characterization of the immune response to lipid-conjugated protein antigens. *J. Immunol.* **118:**957.
68. Singh, S. B., and S. Leskowitz. 1978. Some biological characteristics of lipid conjugates of protein antigen that selectively induce delayed-type hypersensitivity in mice. *Cell. Immunol.* **37:**96.
69. Nicolotti, R. A., N. Koshibe, and S. C. Kinsky. 1976. Comparative immunological properties of N-substituted phosphatidylethanolamine derivatives and liposomal model membranes. *J. Immunol.* **117:**1898.
70. Yasuda, T., G. F. Dancey, and S. C. Kinsky. 1977. Immunogenic properties of liposomal model membranes in mice. *J. Immunol.* **119:**1863.
71. Yasuda, T., T. Tadakume, C. W. Pierce, and S. C. Kinsky. 1979. Primary *in vitro* immunogenicity of liposomal model membranes in mouse spleen cell cultures. *J. Immunol.* **123:**1535.