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CHARACTERIZATION OF ANTI-*N*-ACETYL-D-GLUCOSAMINE ANTIBODIES ELICITED THROUGH HAPTENATED LIPOSOMES

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ABSTRACT

Antiserum was raised in rabbits against *p*-aminophenyl 2-acetamido-2-deoxy-β-D-glucoside covalently coupled to phosphatidylaminoethanol-containing lecithin liposomes. The affinity-purified antibodies were found to be IgM. The properties of the antibodies were studied by ligand inhibition of quantitative precipitation, fluorescence titration with 2-acetamido-2-deoxy-D-glucose, and complement lysis of haptenated liposomes.

INTRODUCTION

Immunogenic properties of hapten-sensitized, liposomal model membranes were extensively studied by Kinsky and his co-workers, using 2,4-dinitrophenyl as the hapten[1]. Recently, we have undertaken a study of the antigenicity of carbohydrate determinants mediated through liposomes. An aminophenyl derivative of a monosaccharide was coupled to phosphatidylaminoethanol-containing, negatively charged, lecithin liposomes, and the glycosylated liposomes were found to generate a hapten-specific, humoral immune response in rabbits. Antibodies against D-galactose[2] and D-mannose[3] were raised by using this procedure. Antibodies specific for isomalto-oligosaccharides were also raised by Wood and Kabat[4] by coupling the oligosaccharides to stearylamine and incorporating the stearyl oligosaccharides into sphingomyelin liposomes. We have further demonstrated the adjuvant action of liposomes for galactosyl determinant[5]. In the present work, antibodies against 2-acetamido-2-deoxy-D-glucose have been raised by using liposomes as the carrier, and the properties of the antibodies are reported.

EXPERIMENTAL

General. — Egg lecithin (phosphatidylcholine), phosphatidylaminoethanol, and cholesterol were purchased from CSIR Centre for Biochemicals, Delhi. All the

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saccharide ligands, bovine serum albumin (BSA), and other chemicals were obtained from Sigma Chemical Co., U.S.A. Goat anti-rabbit IgG and IgM serum were received through Decruz Corporation, Bombay. Reduction of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucoside to the *p*-aminophenyl derivative was achieved by hydrogenation in the presence of powdered palladium black as the catalyst. The palladium was then removed by filtration, and the solution of *p*-aminophenyl 2-acetamido-2-deoxy-β-D-glucoside was evaporated to dryness.

Coupling of p-*aminophenyl 2-acetamido-2-deoxy-β-*D-*glucoside to liposomes (GlcNAc-liposomes).* — Liposomes were made from egg lecithin, cholesterol, dicetyl phosphate, and phosphatidylaminoethanol in the molar ratios of 7:2:1:2. *p*-Aminophenyl 2-amino-2-deoxy-β-D-glucoside was coupled to liposomes by following the method described for the preparation of galactosylated liposomes[2].

Preparation of antiserum. — Four rabbits were immunized in the hind footpads with GlcNAc-liposomes. Each animal received 1 mL of liposomes (containing 1.0 mg of sugar) emulsified with an equal volume of Freund's complete adjuvant. Three injections at 10-day intervals were given. The last two were in Freund's incomplete adjuvant. The antiserum collected at day 7 after the third injection was decomplemented for 30 min at 56°, and stored at −20°.

Preparation of glycosylated BSA ($GlcNAc_{17}$–BSA). — *p*-Aminophenyl 2-acetamido-2-deoxy-β-D-glucoside was coupled to BSA by the carbodiimide method[2,6]. Analysis[7] of the conjugated product showed 17 mol of GlcNAc per mol of BSA.

Purification of antibodies (anti-GlcNAc IgM). — Antiserum was precipitated with 33% ammonium sulfate. The precipitate was dialyzed against 0.01M sodium phosphate buffer, pH 7.4, containing 0.15M NaCl (PBS), and then loaded onto an immunoadsorbent column prepared by coupling $GlcNAc_{17}$–BSA to CNBr-activated Sepharose-4B. After the column had been washed with PBS until the absorbance at 280 nm had decreased to 0.05, bound antibodies were eluted with 3M NH_4SCN. After dialysis against PBS, the antibodies were passed through a column of Sephadex G-200 to fractionate the IgM and IgG. All of the antibodies were eluted at the IgM position. The nature of the antibodies was further checked with goat anti-rabbit IgG and IgM sera.

Quantitative precipitation and hapten inhibition. — The quantitative, precipitin reactions of $GlcNAc_{17}$–BSA with the antiserum (50 μL) and purified IgM (70 μg) was conducted by following previous procedures[2]. Ligand (30mM) inhibition of the precipitation of $GlcNAc_{17}$–BSA (125 μg) with IgM was studied.

Fluorescence titration. — The binding of GlcNAc with IgM antibodies was followed by the method of Jolley and Glaudemans[8]. The protein solution (optical absorbance <0.1 unit at 280 nm) was excited at 280 nm, and the emission was monitored at 340 nm in an Aminco–Bowman spectrofluorimeter.

Complement lysis of haptenated liposomes. — The lysis of GlcNAc-liposomes by anti-GlcNAc IgM in the presence of complement was performed as described[5], following Six *et al.*[9].

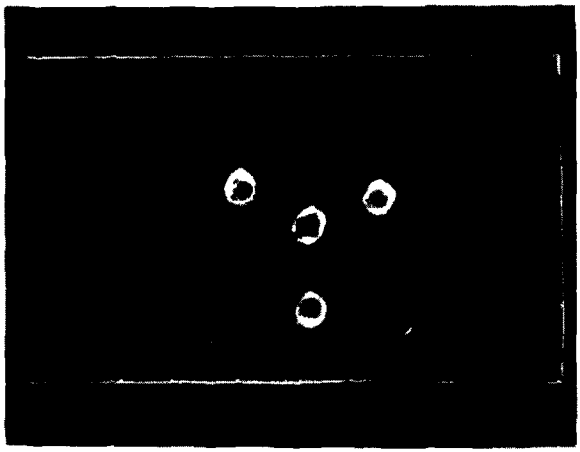

Fig. 1. Immunodiffusion of anti-GlcNAc antiserum against $GlcNAc_{17}$–BSA. [Antiserum was added in the central well. The peripheral wells had the conjugated BSA.]

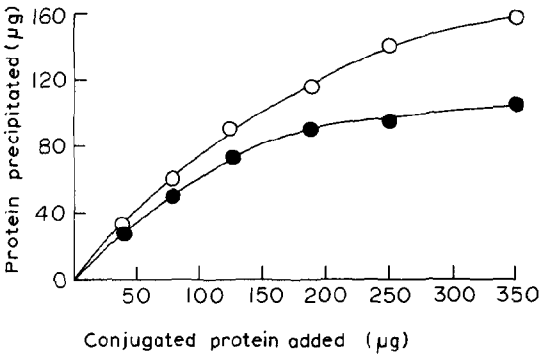

Fig. 2. Quantitative precipitin curves of the antiserum (—○—) and anti-GlcNAc IgM antibody (—●—) with $GlcNAc_{17}$–BSA.

RESULTS

Antiserum against GlcNAc-liposomes was tested against $GlcNAc_{17}$–BSA conjugate by immunodiffusion (see Fig. 1), whereby a precipitation band was observed. (Native BSA does not precipitate with the antiserum.) The quantitative, precipitin curve of the antiserum with $GlcNAc_{17}$–BSA was determined, and is shown in Fig. 2. The precipitin curve of anti-GlcNAc IgM is also shown. There seems to be little difference in the precipitation of IgM and the antiserum, as is evident from the initial portion of the curves. Various ligands were checked for their ability to inhibit the quantitative precipitation (see Table I) of $GlcNAc_{17}$–BSA with the IgM. It may be seen that GlcNAc and its *p*-aminophenyl glycoside inhibit equally well. 2-Acetamido-2-deoxy-D-galactose was also observed to be active, al-

TABLE I

PERCENT INHIBITION OF ANTI-GlcNAc IgM PRECIPITATION WITH $GlcNAc_{17}$–BSA BY LIGANDS

Ligand (30 mM)	*Inhibition (%)*
p-Aminophenyl 2-acetamido-2-deoxy-β-D-glucoside	32
p-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucoside	25
2-Acetamido-2-deoxy-D-glucose	35
D-Galactose	0
D-Glucose	0
D-Mannose	0
2-Acetamido-2-deoxy-D-galactose	15

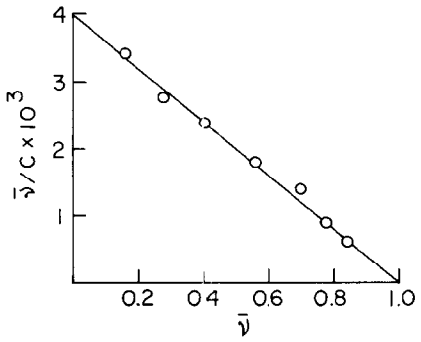

Fig. 3. Scatchard plot for the binding of anti-GlcNAc IgM with GlcNAc, as determined by fluorescence titration.

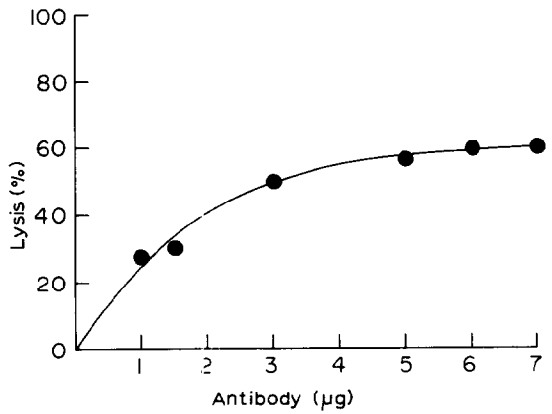

Fig. 4. Effect of the concentration of anti-GlcNAc IgM on the immune lysis of GlcNAc-liposomes. [Liposomes (20 μL) and fresh, guinea-pig serum (25 μL, as a source of complement) were taken in 1.0 mL of solution of 50mM Tris · HCl buffer, pH 7.4, supplemented with 0.15M NaCl, 0.5mM $MgCl_2$, and 0.15mM $CaCl_2$. Release of trapped marker, 4-methylumbelliferyl phosphate, from liposomes in the presence of the IgM was monitored by alkaline phosphatase (0.5 unit), fluorimetrically.]

though to a lesser extent. D-Glucose, D-galactose, and D-mannose are not effective.

Binding of GlcNAc quenches the fluorescence of anti-GlcNAc IgM to the extent of ~12%. The association constant as determined from the fluorescence-quenching data is $3.95 \times 10^3\ M^{-1}$ (see Fig. 3). A straight line indicating homogeneous binding in the IgM was obtained in Scatchard analysis.

In the range of 1.1–5.5nM, purified antibodies could lyse GlcNAc-liposomes in the presence of complement (see Fig. 4). The maximum immune lysis was ~60%. It was noted that liposomes not coupled with the sugar are not lysed in the presence of the antibody and the complement.

DISCUSSION

Recently, liposomal model membranes have been extensively used for presentation of antigens and haptens to the immune system. In the present report, antibodies specific for 2-acetamido-2-deoxy-D-glucose have been raised in rabbits by using liposomes as the immunological carriers. The IgM nature of the purified antibodies is established. It is known that DNP-coupled liposomes function as a classical, T-independent immunogen in mice[10]. Anti-DNP antibodies were IgM of restricted heterogeneity. Wood and Kabat[4] have found both IgM and IgG antibodies against isomalto-oligosaccharides raised through liposomes in rabbits. We have observed that D-galactosylated liposomes elicit IgM and IgG classes in rabbits, although the majority of the antibody population (70%) belongs[11] to IgM. The selective production of IgM antibodies suggests the T-independent nature of GlcNAc-liposomes. However, the role of T-cells cannot be precluded in the immune response of glycosylated liposomes in general. The present results do not resolve this point.

Purified antibodies were found to be quite specific for GlcNAc, as judged by quantitative-inhibition data. The ability of GalNAc to inhibit IgM/$GlcNAc_{17}$–BSA precipitation shows the role of the *N*-acetyl group in the specificity of anti-GlcNAc IgM, because D-galactose does not inhibit the foregoing precipitation. The K_a value of the IgM ($3.95 \times 10^3\ M^{-1}$) for GlcNAc compares well with the reported value ($3 \times 10^3 M^{-1}$) for antigroup A, streptococcal polysaccharide for *p*-nitrophenyl GlcNAc[12]. Our results do not reveal heterogeneity in the binding of anti-GlcNAc IgM. It may be added that a similar homogeneity was noticed in the binding of rabbit anti-pneumococcal type 27 antibodies with the polysaccharide[13].

Glycosylated liposomes offer an alternative method of producing anti-carbohydrate antibodies, the majority of which are of the IgM type. It would be of interest to study the ability of these antibodies in recognizing the specific saccharide residues in complex glycoconjugates of various tissues and cells. Cellular mechanisms involved in the liposomal immunogenicity of carbohydrate determinants could be an interesting area for further investigation.

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