Carbohydrate influences the immunogenic and antigenic characteristics of the ZP3 macromolecule (M_r 55 000) of the pig zona pellucida

A. G. Sacco, E. C. Yurewicz and M. G. Subramanian

Wayne State University School of Medicine, Department of Obstetrics and Gynecology, C. S. Mott Center for Human Growth and Development, 275 East Hancock Avenue, Detroit, Michigan 48201, U.S.A.

Summary. The glycoprotein molecule ZP3 of the pig zona pellucida (M_r 55 000) was deglycosylated by trifluoromethane–sulphonic acid. While gas chromatography established that deglycosylation resulted in >91% carbohydrate removal, deglycosylated ZP3 (DG-ZP3) still retained significant immunogenic potential as shown by its ability to elicit antibody production in the rabbit. Several lines of evidence, however, demonstrated that deglycosylation produced significant modifications in the ZP3 macromolecule which profoundly altered its antigenicity: (1) ^{125}I -radiolabelling procedures using chloramine-T consistently resulted in ^{125}I -labelled ZP3 with a higher specific activity than ^{125}I -labelled DG-ZP3; (2) in competitive binding radioimmunoassays (RIAs) using an ^{125}I -labelled-ZP3 vs anti-ZP3 system, unlabelled DG-ZP3 successfully displaced ZP3 in a dose-related manner; (3) in direct binding RIAs comparing the reaction of labelled ZP3 and DG-ZP3 *versus* 5 different zona antisera, higher titres were consistently achieved with ^{125}I -labelled ZP3; (4) as compared to ZP3, 2-dimensional immunoelectrophoresis using DG-ZP3 as antigen yielded significantly modified precipitin arc patterns; (5) with electrophoretic blotting procedures, antisera to ZP3 or DG-ZP3 cross-reacted with the heterologous antigen; (6) treatment of pig zonae with anti-DG-ZP3 serum produced a dense precipitation layer on the zona surface. When evaluated collectively, these results provide grounds for an important role for protein and carbohydrate in establishing the immunological characteristics of the M_r 55 000 macromolecule of the pig zona pellucida.

Introduction

The zona pellucida is a biochemically and antigenically complex extracellular glycoprotein matrix which surrounds the mammalian oocyte and preimplantation embryo. It is currently being evaluated as a potential immunocontraceptive target antigen since antibodies raised against this structure can totally inhibit fertility *in vitro* and *in vivo* (Sacco, 1981; Dunbar, 1983). The pig zona pellucida has been studied the most extensively for potential immunocontraceptive development and consists primarily of four families of charged heterogeneous glycoproteins having apparent molecular weights (M_r) of 82 000 (ZP1), 61 000 (ZP2), 55 000 (ZP3), and 21 000 (ZP4) (Subramanian, Yurewicz & Sacco, 1981; Sacco, Yurewicz, Subramanian & DeMayo, 1981; Dunbar, Lui & Sammons, 1981; Hedrick & Wardrip, 1981). The 55K component (ZP3) is the most predominant, has been isolated by chromatographic (Yurewicz, Sacco & Subramanian, 1983, 1985) and electroelution (Dunbar & Raynor, 1980; Betancourt, Rodriguez, Serrano, Wardrip & Hedrick, 1984) procedures and represents the primary candidate amongst the various zona components for consideration as a target antigen for contraceptive vaccine development. The purpose of the present

investigation was to determine the influence of carbohydrate on the antigenic and immunogenic characteristics of this macromolecule through experiments utilizing chemically (trifluoromethane-sulphonic acid, TFMSA) deglycosylated ZP3. Such information is essential for efforts to characterize this macromolecule biochemically and immunologically.

Materials and Methods

Purification of ZP3 from pig zona pellucidae. Isolated pig zonae were obtained as described by Yurewicz *et al.* (1983). Frozen-thawed ovaries were homogenized in Tris buffer (50 mM-Tris, 150 mM-NaCl, 4 mM-EDTA, 2 mM-sodium citrate, 0.02% sodium azide, pH 8.0) using a commercial meat grinder. Zona-encased oocytes were collected by successive passage of the homogenate through nylon monofilament screens (Tetko, Inc., Elmsford, NY, U.S.A.) of decreasing pore size (500–100 μ m). Oocytes retained on the 100 μ m screen were collected by washing into Tris buffer and isolated zonae were obtained by homogenization of the zona-encased oocytes using a tissue grinder. Isolated zonae were collected on a 50 μ m screen and washed for 60 min in 100 ml 20 mM-Tris buffer, pH 8.0, using a magnetic stirrer. Isolated zonae were solubilized by heating at 73°C for 20 min. The solubilized zonae preparation was centrifuged for 15 min at 27 000 *g*, and the supernate was concentrated 20-fold by ultrafiltration through a PM-30 membrane (Amicon, Danvers, MA, U.S.A.). Solubilized zonae were treated with 3% sodium dodecyl sulphate (SDS) and heat (60°C, 20 min) under non-reducing conditions, and ZP3 was isolated by column chromatography using Sephacryl S-400 and hydroxylapatite resins equilibrated with buffer containing 0.1% SDS. Column fractions were monitored for ZP3 by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Residual SDS was extracted with acetone:triethylamine:acetic acid:water (85:5:5:5, by vol.) and the ZP3 was dialysed from 6 M-guanidine hydrochloride (Henderson, Oroszlan & Konigsberg, 1979; McCoy & Wong, 1981). Typically, 7–8 mg ZP3 were recovered from 4000 pig ovaries. Purity of the final ZP3 preparation was verified by two-dimensional gel electrophoresis and by the electrophoretic blotting of ZP3 isolated by two-dimensional PAGE to nitrocellulose paper. For the blotting procedure, after treatment of ZP3 with a rabbit antiserum to solubilized zonae, the failure of this antiserum to detect other zona components in the ZP3 preparation was interpreted as one criterion of sample purity. Two-dimensional gel electrophoresis for the zona system was conducted as described previously (Sacco *et al.*, 1981).

Deglycosylation of ZP3 with trifluoromethanesulphonic acid (TFMSA). Deglycosylation of ZP3 was achieved by chemical treatment with TFMSA (Edge, Faltgnek, Hof, Reichert & Weber, 1981). Treatment was conducted with 4 mg ZP3/ml of TFMSA:anisole (2:1, v/v) at 4°C for 8 h followed by neutralization with aqueous pyridine, extraction with diethyl ether and dialysis against 25 mM-pyridinium acetate buffer, pH 5.0. Recovery of protein was 74%. Protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951), using bovine serum albumin as standard.

Sugar analysis. Sugar analyses of intact and deglycosylated ZP3 were performed by gas chromatography (Bhatti, Chambers & Clamp, 1970). Hydrolysis was conducted in methanol-0.5 N-HCl in the presence of 5 nmol perseitol internal standard at 80°C for 7 h followed by neutralization with silver carbonate and re-*N*-acetylation with acetic anhydride. Accurate quantitation of the *N*-acetylglucosamine content of deglycosylated-ZP3 was hindered by the resistance of the *N*-acetylglucosamine-asparagine linkage to hydrolysis with methanolic HCl. The *N*-acetylglucosamine content of deglycosylated ZP3 was therefore determined after hydrolysis in 4 N-HCl for 4 h at 100°C in the presence of 5 nmol arabitol as the internal standard. The 4 N-HCl hydrolysates were prepared for gas chromatography by lyophilization, acetylation of amino sugars in 0.05 ml pyridine-acetic anhydride (1:1, v/v) and incubation in methanol-0.5 N-HCl for 1 h at 65°C (Baenziger & Kornfeld, 1974).

Methyl glycosides were trimethylsilylated (23°C, 45 min) using a commercial reagent (Sylon HTP, Supelco, Bellefonte, PA, U.S.A.). The trimethylsilyl methyl glycosides were separated and quantitated by gas chromatography on a DB-5 bonded-phase fused silica capillary column (30 m × 0.32 mm) using a temperature programme and a flame ionization detector.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in slab gels using the buffer system of Laemmli (1970). Samples were preincubated at 100°C for 2 min in 125 mM-Tris-Cl buffer, pH 6.8, containing 4% SDS and 10% glycerol. Separated proteins were visualized with Coomassie blue stain. Gels were calibrated using a mixture of reduced and alkylated protein standards: myosin (M_r 200 000), *Escherichia coli* β -galactosidase (M_r 117 000), phosphorylase (M_r 94 000), bovine serum albumin (M_r 68 000), glutamate dehydrogenase (M_r 54 000), glyceraldehyde 3-phosphate dehydrogenase (M_r 37 000), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 22 000) and lysozyme (M_r 15 000).

Radioimmunoassays. Radiolabelling of ZP3 and deglycosylated-ZP3 with Na¹²⁵I by the chloramine-T procedure (Greenwood, Hunter & Glover, 1963), was carried out as described elsewhere (Palm, Sacco, Syner & Subramanian, 1979). The competitive binding RIA and the direct binding RIA for antibody titration were similar to those detailed by Palm *et al.* (1979), Subramanian *et al.* (1981) and Sacco *et al.* (1981).

Production of antisera. Five different rabbit antisera produced against various preparations of pig zonae pellucidae proteins were used. All antisera were produced using a multi-site, intradermal injection regimen (Vaitukaitis, Robbins, Nieschlag & Ross, 1971), in conjunction with Freund's adjuvant. Details regarding production and characterizations of antisera to isolated zonae (IZP), purified pig zona antigen (PPZA), and heat-solubilized isolated zonae (SIZP) have been described previously (Sacco & Palm, 1977; Subramanian *et al.*, 1981; Sacco, Yurewicz & Zhang, 1983). For this investigation, two additional antisera were prepared in rabbits against ZP3 and deglycosylated ZP3. Male New Zealand white rabbits were each given 3 injections 1 week apart of 50 μ g ZP3 or deglycosylated-ZP3 (total 150 μ g/rabbit). Freund's complete adjuvant was used for the first injection and incomplete adjuvant for all other injections. Antibody activity was monitored by RIA and, once peak titres were achieved, a single booster immunization consisting of 100 μ g ZP3 or 50 μ g deglycosylated-ZP3 was given.

Two-dimensional immunoelectrophoresis. This was performed essentially as described by Sacco *et al.* (1983). A gel strip (22 × 80 mm) of 0.7% agarose in 20 mM-sodium barbital buffer, pH 8.6 was poured onto the end of a glass plate (80 × 100 mm) for first-dimension electrophoresis. A 4 mm diameter sample well was cut into one end of the strip. The sample was applied (15–25 μ g ZP3 or deglycosylated ZP3) and electrophoresis was carried out for 3 h at 20°C and 90 V using a Bio-Rad model 1415 electrophoresis cell. A 1% agarose gel in 20 mM-barbital buffer containing 3% antibody and 1% Triton X-100 (Bjerrum, 1977) was then poured over the remaining exposed glass surface of the plate. Second dimension electrophoresis was conducted at 20°C at 35 V for 20 h. Plates were washed, dried and stained as described by Weeke (1973a, b).

Electrophoretic blotting. SDS-PAGE was performed as described above to resolve ZP3 and deglycosylated-ZP3. A procedure modified from those reported by Towbin, Staehelin & Gordon (1979) and Hawkes, Niday & Gordon (1982) was used for the electrophoretic transfer of SDS-PAGE-resolved zona proteins to nitrocellulose paper and their detection by antibody. Electrophoretic transfers were conducted in 20 mM-Tris-HCl, 20% methanol, pH 7.5 buffer at 60 V for 3 h using a Bio-Rad Trans Blot Cell. After transfer, nitrocellulose sheets were incubated in blocking solution (50 mM-Tris-HCl, 200 mM-NaCl, 3% BSA, 10% goat serum, pH 7.4) for 1 h and rinsed for 30 min in 5 changes of saline (50 mM-Tris-HCl, 200 mM-NaCl, pH 7.4). Sheets were then incubated in primary antibody (1:200 dilution in blocking solution) for 2 h at 23°C followed by a 30-min saline wash. Sheets were next incubated in secondary antibody (peroxidase-labelled goat

anti-rabbit IgG; Cooperbiomedical, Inc., Malvern, PA, U.S.A.) for 2 h at 23°C (1 : 1000 dilution in blocking solution). After a final 30-min saline wash, sheets were developed using 4-chloro-1-naphthol (Sigma, St Louis, MO, U.S.A.).

Antibody treatment of pig zona pellucida. Zona-encased pig eggs were obtained by mincing frozen-thawed ovaries with scissors in 0.1 M-phosphate-buffered saline (PBS) and recovering the released oocytes from the buffer with a micropipette. Eggs were washed in three changes of PBS and exposed to various antisera or control sera for 15 min at 23°C. After another wash in three changes of PBS, the treated eggs were transferred to depression slides and observed by phase-contrast and bright-field microscopy.

Results

Biochemical procedures

The carbohydrate compositions of ZP3 and deglycosylated-ZP3 are presented in Table 1. The sugars fucose, mannose, galactose, *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc) and sialic acid were detected in ZP3 and comprised about 30% by weight of the glycoprotein macromolecule. Chemical deglycosylation of ZP3 with TFMSA resulted in loss of more than 91% of the original saccharide residues, leaving minimal amounts of galactose, GlcNAc, GalNAc and sialic acid bound to the protein. Fucose and mannose were no longer detectable.

Table 1. Saccharide composition of intact and deglycosylated (DG) ZP3

Saccharide	Residues/100 000 g protein*	
	ZP3	DG-ZP3
Fucose	6.9	—†
Mannose	16.9	—†
Galactose	51.9	0.6
<i>N</i> -Acetylglucosamine	56.8	5.8
<i>N</i> -Acetylgalactosamine	7.7	6.1
<i>N</i> -Acetylneuraminic acid	10.2	0.4

* As determined by Lowry *et al.* (1951).

† Not detected.

ZP3 migrated as a single diffuse band of M_r 55 000 on SDS-PAGE under reducing (not shown) and non-reducing conditions (Fig. 1). SDS-PAGE of reduced deglycosylated-ZP3 yielded bands at M_r 35 000 and 38 000 (not shown) whereas SDS-PAGE of the non-reduced form yielded bands at M_r 31 000 and 34 000 (Fig. 1). Deglycosylated-ZP3 did not produce any bands migrating at M_r 55 000, thus confirming the absence of any intact ZP3 after TFMSA treatment.

Radioimmunoassay

Radiolabelling of ZP3 and deglycosylated-ZP3 by the chloramine-T procedure under identical conditions consistently resulted in ^{125}I -labelled ZP3 with a higher specific activity than ^{125}I -labelled deglycosylated-ZP3 (25.73 ± 1.4 and $9.1 \pm 1.0 \mu\text{Ci}/\mu\text{g}$, $P < 0.001$). In competitive

Fig. 1. Gel electrophoresis of native and chemically deglycosylated (DG) ZP3. SDS-PAGE was conducted in a 15% polyacrylamide gel under non-reducing conditions. (a) ZP3, 40 μ g; (b) DG-ZP3, 4 μ g; (c) molecular weight standards. Proteins were visualized with Coomassie blue stain.

Fig. 2. Electrophoretic transfer of SDS-PAGE-resolved ZP3 (a, c) and deglycosylated (DG) ZP3 (b, d) to nitrocellulose paper followed by treatment with rabbit anti-ZP3 serum (a, b) and rabbit anti-DG-ZP3 serum (c, d). SDS-PAGE was conducted under non-reducing conditions.

binding RIAs using a rabbit anti-ZP3 against 125 I-labelled ZP3 system, unlabelled ZP3 and unlabelled deglycosylated-ZP3 successfully displaced 125 I-labelled ZP3 in a dose-related manner (Fig. 3). However, to attain the 50% inhibition of the dose-response curve, the amount of unlabelled deglycosylated-ZP3 required was significantly greater than the amount of unlabelled ZP3 required (22.0 ng vs 1.7 ng, respectively). In direct binding RIAs which compared reactions of the labelled ZP3 and deglycosylated-ZP3 against the 5 different zona antisera (Fig. 4), consistently higher titres were always achieved when ZP3 was used as the labelled antigen (Fig. 4a). Although each of the 5 antisera tested did react with labelled ZP3 and deglycosylated-ZP3 (Figs 4a & 4b) at any given dilution, all the antisera exhibited greater binding to 125 I-labelled ZP3 than to 125 I-labelled deglycosylated-ZP3 (Table 2).

Immunochemical procedures

Electrophoretic blotting experiments also demonstrated that antisera to ZP3 and deglycosylated-ZP3 were each capable of reacting with ZP3 or deglycosylated-ZP3 (Fig. 2). While the

Fig. 3. Percentage inhibition of rabbit anti-ZP3 serum bound to ^{125}I -labelled ZP3 in the presence of unlabelled ZP3 or unlabelled deglycosylated-ZP3 (DG-ZP3).

Fig. 4. Titration of various rabbit antisera to pig zona pellucidae against (a) ^{125}I -labelled ZP3 and (b) ^{125}I -labelled deglycosylated-ZP3 (DG-ZP3). Anti-DG-ZP3 = anti-deglycosylated-ZP3; anti-SIZP = anti-heat-solubilized isolated porcine zona pellucidae; anti-PPZA = anti-purified porcine zona antigen; anti-IZP = anti-isolated zona pellucidae (intact zona).

Table 2. Comparison of the binding capacities of various zona antisera to ^{125}I -labelled ZP3 and deglycosylated ZP3

Antiserum (1:6400 dilution)	% labelled antigen bound	
	^{125}I -labelled ZP3	^{125}I -labelled DG-ZP3
Deglycosylated ZP3	25.0	10.7
ZP3	63.9	19.1
Solubilized zonae pellucidae	67.0	23.1
Purified pig zona antigen	68.9	18.2
Intact zonae pellucidae	29.3	0.3
* Mean $\pm$ s.e.m.	50.82 $\pm$ 9.72	14.28 $\pm$ 4.03

* $P = 0.0052$ (paired t test).

intensity of the reactions of either antiserum with deglycosylated-ZP3 appeared comparable, the reaction between anti-deglycosylated-ZP3 and ZP3 was definitely less intense than that between anti-ZP3 and ZP3.

Results from two-dimensional immunoelectrophoresis studies are summarized in Fig. 5. Reaction of ZP3 into anti-ZP3 serum resulted in the overlapping 'stacked' pattern of precipitin arcs which is characteristic of the pig zona pellucida system (Wood & Dunbar, 1981; Sacco *et al.*, 1983). In this reaction 6–7 precipitin arcs were detected (Fig. 5a). Electrophoresis of ZP3 into antiserum to deglycosylated-ZP3 serum (Fig. 5b) yielded a precipitin arc pattern similar in shape and height to that obtained when anti-ZP3 serum was used (Fig. 5a) but with significantly fewer arcs, 2–3 instead of 6–7.

Electrophoresis of deglycosylated-ZP3 into anti-ZP3 serum resulted in an arc pattern significantly altered from the characteristic zona pattern with respect to precipitin arc migration characteristics, height and number (Fig. 5c). Two distinct arc groupings were observed with a single group in close proximity to each side of the origin well. Each grouping consisted of about 5 precipitin arcs of minimal height. In addition, 3 single broad faint arcs were present on the anode side of the origin. Changing electrophoretic conditions or sample and antibody concentrations did not result in significant alteration in this arc pattern. Electrophoresis of deglycosylated-ZP3 into antiserum to deglycosylated-ZP3 (Fig. 5d) resulted in a similar double grouping pattern but only 1–2 diffuse arcs were detectable in each grouping. The 3 faint arcs present on the anode side of the origin well in Fig. 5c were not present but a single faint arc traversing the two groupings was detectable.

Antiserum treatment of porcine zonae

Treatment of zona-encased eggs with antiserum to ZP3 or deglycosylated-ZP3 resulted in the formation of a dense precipitation layer on the surface of the zona (Fig. 6). However, a distinct difference in appearance of these precipitates was noted. Treatment of zonae with anti-ZP3 serum produced a heavy but dispersed precipitate while that produced by the antiserum to deglycosylated-ZP3, although comparably heavy, was more restricted to the outer surface of the zona.

Discussion

The results from the present investigation indicate that chemical deglycosylation with TFMSA of

Fig. 5. Two-dimensional immunoelectrophoresis of ZP3 and deglycosylated (DG)-ZP3 into gels containing rabbit antiserum to ZP3 or DG-ZP3. Electrophoresis in the first dimension was conducted for 3 h at 90 V (right to left, anode at left). Electrophoresis in the second dimension was conducted for 20 h at 35 V (bottom to top, anode at top). (a) Electrophoresis of ZP3 (15 µg) into 3% anti-ZP3 serum; 6–7 precipitin arcs are observed; (b) electrophoresis of ZP3 (15 µg) into 10% anti-DG-ZP3 serum; 2–3 precipitin arcs are present; (c) electrophoresis of DG-ZP3 (25 µg) into 3% anti-ZP3 serum; arrows designate faint arcs on anode side of sample well; (d) electrophoresis of DG-ZP3 (25 µg) into 3% anti-DG-ZP3 serum; note single faint precipitin arc traversing the 2 arc groupings.

the pig zona glycoprotein family of M_r 55 000 (ZP3) results in dramatic alterations of the physico-chemical and immunological properties of the macromolecule. Native ZP3 is comprised of 30% carbohydrate by weight and migrates as a single characteristically diffuse band on SDS-PAGE at M_r 55 000. Treatment of ZP3 with TFMSA for 8 h at 4°C resulted in loss of ~91% of the original saccharide residues. The *N*-acetylgalactosamine and *N*-acetylglucosamine which remain associated with deglycosylated-ZP3 are presumed to represent sugars which are linked directly to the

Fig. 6. Treatment of pig zonae with rabbit anti-ZP3 serum (lower right) and anti-DG-ZP3 serum (lower left). Control egg (C) has been treated with a rabbit preimmune serum. All sera were used at full strength. Note dispersed appearance of precipitation layer on the zona of the anti-ZP3 serum-treated oocyte as compared to the more restricted appearance of the precipitation layer to the outer surface of zona of the anti-DG-ZP3 serum treated oocyte. Zona of control oocyte has no precipitation layer, $\times 570$.

polypeptide moiety, i.e. serine- and threonine-linked *N*-acetylgalactosamine and asparagine-linked *N*-acetylglucosamine, and are therefore resistant to removal with TFMSA (Edge *et al.*, 1981). SDS-PAGE of deglycosylated-ZP3 yielded a pair of polypeptides with M_r values (reduced) of 38 000 and 35 000. Such results strongly suggest that ZP3, which exhibits apparent electrophoretic homogeneity in its native state, is in fact, compositionally complex. It is concluded that ZP3 is composed of two previously unresolved glycoproteins with core polypeptides of M_r 38 000 and 35 000. Whether the two core polypeptides are structurally and/or immunologically related is as yet unresolved. The two core polypeptides may be products of unrelated genes and therefore be distinct as to amino acid sequence. Alternatively, the two core polypeptides may be products of separate but related genes and thus possess partial structural homology. Finally, the polypeptide of M_r 35 000 may be a degradation product of the core polypeptide of M_r 38 000 as a result of in-vivo or in-vitro proteolysis or of TFMSA treatment. With regard to the latter hypothesis, the observation of Yurewicz *et al.* (1984) that partial enzymic deglycosylation of ZP3 by digestion with endo- β -galactosidase results in formation of two glycoprotein products which may be resolved by two-dimensional gel electrophoresis would suggest that the presence of two polypeptides after chemical deglycosylation of ZP3 with TFMSA is not an artefact of the reaction conditions. Experiments to define further the structural relatedness of the two core polypeptides by N-terminal amino acid sequence analysis and by peptide mapping are in progress.

Electrophoretic mobility of ZP3 is also dramatically affected by carbohydrate removal as revealed by the significantly altered gel patterns produced after two-dimensional immunoelectrophoresis of deglycosylated-ZP3. The appearance of two distinct arc groupings (Figs 5c & d) may be an expression of the presence of the core polypeptides of M_r 38 000 and 35 000.

Significant physicochemical alteration of the ZP3 macromolecule by TFMSA treatment was also indicated by the impaired ability of deglycosylated-ZP3 to incorporate radioactivity by the chloramine-T iodination procedure. Since ^{125}I is incorporated into tyrosine residues in the process of iodination (Greenwood *et al.*, 1963), these results suggest that such differences in incorporation of radioactive iodine may result from a decrease in the availability of tyrosine in deglycosylated-ZP3, due possibly to conformational changes in the macromolecule brought about by carbohydrate removal or perhaps to some direct but unknown modification of the protein itself caused by the conditions necessary to achieve deglycosylation.

Despite the physicochemical alterations in the deglycosylated-ZP3 macromolecule, it still retained immunogenic potential as shown by the successful production of a rabbit antiserum to deglycosylated-ZP3. Moreover, this molecule must still possess antigenic sites in common with ZP3 as indicated by the data from competitive binding RIAs (Fig. 3), direct binding RIAs (Fig. 4), electrophoretic blots (Fig. 2) and two-dimensional immunoelectrophoresis (Fig. 5). Such reactions emphasize the important contribution of protein to antigenicity of the ZP3 macromolecule. Nonetheless, removal of 91% of the carbohydrate from ZP3 does dramatically affect the immunological properties of this macromolecule as indicated by significant reductions in both its immunogenicity and antigenicity: e.g. titres of antisera to deglycosylated-ZP3 against homologous antigen (Fig. 4b) were consistently low; titres of all antisera against ^{125}I -labelled deglycosylated-ZP3 were typically lower than when against ^{125}I -labelled ZP3 (Fig. 4); about 13 times more deglycosylated-ZP3 than ZP3 is required to displace ^{125}I -labelled ZP3 at the 50% inhibition level of the dose-response curve (Fig. 3); and the fewer numbers of precipitin arcs detected in two-dimensional immunoelectrophoresis when deglycosylated-ZP3 is used as antigen suggests a reduction or modification of available antigenic sites (Figs 5c & d). Precisely why deglycosylated-ZP3 becomes a poorer immunogen and antigen cannot be determined from the present data. Carbohydrate removal may result in actual elimination of major antigenic sites, it may produce conformational changes in the macromolecule which mask previously exposed sites or may be a combination of several factors. However, such data emphasize a significant role also for the carbohydrate moieties in establishing the immunogenic and antigenic properties of the ZP3 macromolecule. Therefore, when evaluated collectively, our findings strongly implicate both carbohydrate and protein in contributing to the immunological properties of this glycoprotein.

Treatment of intact pig zonae with rabbit antiserum to deglycosylated-ZP3 produced a dense precipitation layer restricted to the outer surface of the zona (Fig. 6). Such findings are significant from an immunocontraceptive aspect because they indicate that antiserum produced against the deglycosylated-ZP3 macromolecule is capable of reacting with ZP3 in its native conformation within the intact zona. Deglycosylated-ZP3 and the native ZP3 macromolecule therefore share similar or identical epitopes. Such data encourage the consideration of deglycosylated and/or modified forms of ZP3 as potential immunocontraceptive target antigens and their possible future synthesis. An explanation for the difference in appearance of the precipitate produced by antisera to deglycosylated-ZP3 and ZP3 is presently lacking. However, the similarity of the precipitate produced by the antiserum to deglycosylated-ZP3 in this investigation and that produced by a univalent anti-ZP3 serum (Sacco *et al.*, 1984) cannot go unremarked. In both instances, the restricted appearance of the precipitation layer may be due to the absence or reduction of antibody cross-linkages between adjacent macromolecules.

In summary, electrophoretic and RIA data demonstrated that carbohydrate removal dramatically altered the immunochemical properties of ZP3 and thus suggest a significant role for carbohydrate in contributing to the antigenicity and immunogenicity of the native macromolecule. However, since the protein backbone also contributed to its antigenicity, carbohydrate and protein are both involved in establishing the immunological characteristics of the pig zona pellucida ZP3 macromolecule.

This research was supported in part by NIH grant No. HD 10189.

References

- Baenziger, J. & Kornfeld, S. (1974) Structure of the carbohydrate units of IgE immunoglobulin. *J. biol. Chem.* **249**, 1897–1903.
- Betancourt, M., Rodriquez, E., Serrano, H., Wardrip, N. & Hedrick, J.L. (1984) Monoclonal antibodies against the 55K glycoprotein isolated from the pig zona pellucida. *J. Cell Biol.* **99**, 395a, Abstr.
- Bhatti, T., Chambers, R.E. & Clamp, J.R. (1970) The gas chromatographic properties of biologically important N-acetylglucosamine derivatives, monosaccharides, disaccharides, trisaccharides, tetrasaccharides and pentasaccharides. *Biochem. Biophys. Acta* **222**, 339–347.
- Bjerrum, O.J. (1977) Immunochemical investigation of membrane proteins. A methodological survey with emphasis on immunoprecipitation in gels. *Biochem. Biophys. Acta* **473**, 135–195.
- Dunbar, B.S. (1983) Antibodies to zona pellucida antigens and their role in fertility. In *Immunology of Reproduction*, pp. 506–534. Eds T. Wegmann, T. Gill, C. Cumming & E. Nisbet-Brown. Oxford University Press, New York.
- Dunbar, B.S. & Raynor, B.D. (1980) Immunochemical characterization of porcine zona pellucida antigens. *Biol. Reprod.* **22**, 941–954.
- Dunbar, B.S., Lui, C. & Sammons, D.W. (1981) Identification of the three major proteins of porcine and rabbit zonae pellucidae by high resolution two-dimensional gel electrophoresis: comparison with serum, follicular fluid and ovarian cell proteins. *Biol. Reprod.* **24**, 1111–1124.
- Edge, A.S.B., Faltgnek, C.R., Hof, L., Reichert, L.E. & Weber, P. (1981) Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. *Analyt. Biochem.* **118**, 131–137.
- Greenwood, F.C., Hunter, W.M. & Glover, J.S. (1963) The preparation of ¹³¹I-labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89**, 114–123.
- Hawkes, R., Niday, E. & Gordon, J. (1982) A dot-immunobinding assay for monoclonal and other antibodies. *Analyt. Biochem.* **119**, 142–147.
- Hedrick, J.L. & Wardrip, N. (1981) Microheterogeneity in the glycoproteins of the zona pellucida is due to the carbohydrate moiety. *J. Cell Biol.* **91**, 177a, Abstr.
- Henderson, L.E., Oroszlan, S. & Konigsberg, W. (1979) A micro-method for complete removal of dodecyl sulfate from proteins by ion-pair extraction. *Analyt. Biochem.* **93**, 153–157.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, Lond.* **227**, 680–685.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265–275.
- McCoy, L.F., Jr & Wong, K.P. (1981) Renaturation of bovine erythrocyte carbonic anhydrase B denatured by acid, heat and detergent. *Biochemistry, N.Y.* **20**, 3062–3067.
- Palm, V.S., Sacco A.G., Syner, F.N. & Subramanian, M.G. (1979) Tissue specificity of porcine zona pellucida antigen(s) tested by radioimmunoassay. *Biol. Reprod.* **21**, 709–713.
- Sacco, A.G. (1981) Immun contraception: consideration of the zona pellucida as a target antigen. In *Obstetrics and Gynecology Annual*, Vol. 10, pp. 1–26. Ed. R. M. Wynn. Appleton-Century-Crofts, New York.
- Sacco, A.G. & Palm, V.S. (1977) Heteroimmunization with isolated pig zonae pellucidae. *J. Reprod. Fert.* **51**, 165–168.
- Sacco, A.G., Yurewicz, E.C., Subramanian, M.G. & DeMayo, F.J. (1981) Zona pellucida composition: species cross reactivity and contraceptive potential of antiserum to a purified pig zona antigen (PPZA). *Biol. Reprod.* **25**, 997–1008.
- Sacco, A.G., Yurewicz, E.C. & Zhang, S. (1983) Immuno electrophoretic analyses of the porcine zona pellucida. *J. Reprod. Fert.* **68**, 21–31.
- Sacco, A.G., Subramanian, M.G. & Yurewicz, E.C. (1984) Association of sperm receptor activity with a purified pig zona antigen (PPZA). *J. Reprod. Immunol.* **6**, 89–103.
- Subramanian, M.G., Yurewicz, E.C. & Sacco, A.G. (1981) Specific radioimmunoassay for the detection of a purified porcine zona pellucida antigen (PPZA). *Biol. Reprod.* **24**, 933–943.
- Towbin, H., Staehelin, T. & Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4350–4354.
- Vaitukaitis, J., Robbins, J.B., Nieschlag, E. & Ross, G.T. (1971) A method for producing specific antisera with small doses of immunogen. *J. clin. Endocr. Metab.* **33**, 988–991.
- Weeke, B. (1973a) Cross immunoelectrophoresis. *Scand. J. Immunol.* **2**, 47–56.
- Weeke, B. (1973b) General remarks on principles, equipment, reagents and procedures. *Scand. J. Immunol.* **2**, 15–35.
- Wood, D.M. & Dunbar, B.S. (1981) Direct detection of two cross-reactive antigens between porcine and rabbit zonae pellucidae by radioimmunoassay and immunoelectrophoresis. *J. exp. Zool.* **271**, 423–433.
- Yurewicz, E.C., Sacco, A.G. & Subramanian, M.G. (1983) Isolation and preliminary characterization of a purified pig zona antigen (PPZA) from porcine oocytes. *Biol. Reprod.* **29**, 511–523.
- Yurewicz, E.C., Sacco, A.G. & Subramanian, M.G. (1984) Structure of the porcine zona pellucida: isolation and properties of the major glycoprotein family. *J. Cell Biol.* **99**, 73a, Abstr.
- Yurewicz, E.C., Sacco, A.G. & Subramanian, M.G. (1985) Pathways to immun contraception: biochemical and immunological properties of glycoprotein antigens of the porcine zona pellucida. In *The Molecular and Cellular Biology of Fertilization*, (in press). Ed. J. L. Hedrick, Plenum Press, New York.

Received 29 May 1985