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Immunological properties of Tg carbohydrates: enhancement of Tg immunoreaction by removal of sialic acid

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SUMMARY

Immunoreaction of desialylated and native thyroglobulin was compared in sera of patients with anti-thyroglobulin antibodies by radioimmunoassay with ^{125}I-labelled thyroglobulin.

Two human thyroglobulins were iodinated both *in vivo* and by lactoperoxidase. After desialylation enhancement of immunoreaction was observed in several sera ranging from marginal to more than 100%. The effect was not due to iodination since it was reproducible with desialylated thyroglobulin labelled *in vivo*. In one serum (B.P.) a marked enhancement was only seen with one thyroglobulin suggesting that desialylation may unmask isoantigens of thyroglobulin.

Glycopeptides prepared from human thyroglobulin inhibited the immunoreaction between native thyroglobulin and autoantisera. The results indicate that sialic acid masks antigenic determinants in human thyroglobulin and that carbohydrates might be the determinants involved in the process of autoimmunization.

INTRODUCTION

The exact role of sialic acid as an antigenic determinant has not yet been elucidated. In glycoproteins of the red blood cell membrane sialic acid contributes to the antigenic structure of the blood group substances M and N (Springer & Ansell, 1958; Yokoyama & Trams, 1962). Lymphoid (Simmonds *et al.*, 1971) and leukaemia cells (Bagshawe & Currie, 1968), on the other hand, become more immunogenic and antigenic following removal of sialic acid from their surface, thus suggesting that sialic acid masks antigenic determinants on the cell surface or renders the cell more resistant to the immune system of the recipient.

Few investigations have so far been carried out on the antigenic role of sialic acid in complex glycoproteins.

Using heteroantisera to native glycoproteins such as fetuin (Bergman, Levine & Spiro, 1962), ceruloplasmin (Morel, Van Den Hamer & Scheinberg, 1966) and gonadotropin (Mori, 1969; Van Hall, Vaitukaitis & Ross, 1971) an immunological identity has been found between native and desialylated antigens.

In previous experiments (Salabè *et al.*, 1974b,c) it was demonstrated by means of immunoprecipitation that rabbit anti-bovine thyroglobulin sera react identically with native or desialylated thyroglobulin, whereas rabbit anti-desialylated bovine thyroglobulin precipitates a larger amount of desialylated than native thyroglobulin.

The presence of antibodies specific for desialylated gamma globulin has recently been reported in serum from a patient with rheumatoid arthritis (Zinneman, Levi & Seal 1968). In the present investigations the immunoreaction between native and desialylated human thyroglobulin was compared in sera from patients with anti-thyroglobulin antibodies.

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The ABC (Salabè *et al.*, 1974a; Salabè, Fontana & Andreoli, 1972), i.e. the amount of ^{125}I-labelled thyroglobulin bound/ml of serum, was used to evaluate immunoreaction since with this technique it is possible to measure the soluble immunocomplexes undetectable with immunoprecipitation. In addition, a new technique based on polyacrylamide gel electrophoresis was used to separate free antigen from the immune complexes.

In some sera an enhancement of the immunoreaction was seen following removal of sialic acid, thus suggesting the presence of antibodies specific for desialylated thyroglobulin in the serum of thyroid patients. Furthermore, inhibition carried out with glycopeptides purified from human thyroglobulin showed that carbohydrates might be involved in the antigenic structure of thyroglobulin.

MATERIALS

0·02 M phosphate, 0·1 KCl, pH 7·4 buffer was used in all the experiments. Thyroglobulin haemagglutination test kits were obtained from Wellcome, Beckenham (England); *Vibrio cholerae* Neuraminidase from Behringwerke-AG Marburg/Lahn (FRG); Sephadex G-25 superfine from Pharmacia (Uppsala, Sweden); Pronase (*Streptomices griseus*) B grade from Calbiochem (San Diego, California, U.S.A.). Carrier-free ^{125}I from Sorin, SpA, Saluggia (Italy); Lactoperoxidase with a specific activity of 122 i.u./mg purity (ratio 412/280 nm = 0·644), from Calbiochem. Glucose-glucose oxidase from Behringwerke-AG.

METHODS

Thyroglobulins. Two human thyroglobulins were used. One was from a single thyroid nodule (M.A.) which at scintiscan appeared to concentrate iodine at the same rate as the surrounding tissue. Histologically the nodule had the appearance of a microfollicular adenoma. The other thyroglobulin (A.L.) was extracted from the normal thyroid lobe in a patient submitted to total thyroidectomy due to a capsulated thyroid follicular carcinoma. Specimens taken from different parts of the normal lobe showed a normal histological pattern. The patient received 0·5 mCi of carrier free ^{125}I orally 18 hr before surgery.

Extraction of soluble proteins was carried out at 5°C in phosphate buffer (0·02 M, pH 7·4, KCl 0·1 M, 2 ml/g tissue) with gentle shaking for 24 hr. Thyroglobulins were purified from the soluble extract by Sephadex G-200 chromatography (A.L.) or by salting out from 1·4–1·8 M $(NH_4)_2SO_4$ (M.A.). Analytical ultracentrifugation (Beckman model E) showed in both preparations a main symmetrical peak with a sedimentation rate of 19S and a small peak (< 5%) with a sedimentation of 27S. The iodine content measured by the Zak (Zak *et al.*, 1952) method was 0·12% (M.A.) and 0·2% (A.L.). Specific activity of *in vivo* labelled Tg was 0·2 μCi/mg.

Aliquots of these preparations were digested with *Vibrio cholerae* Neuraminidase according to the technique described by Tarutani (Tarutani & Shulman, 1971a, b). Sialic acid content estimated by the thiobarbituric acid method (Warren, 1959)

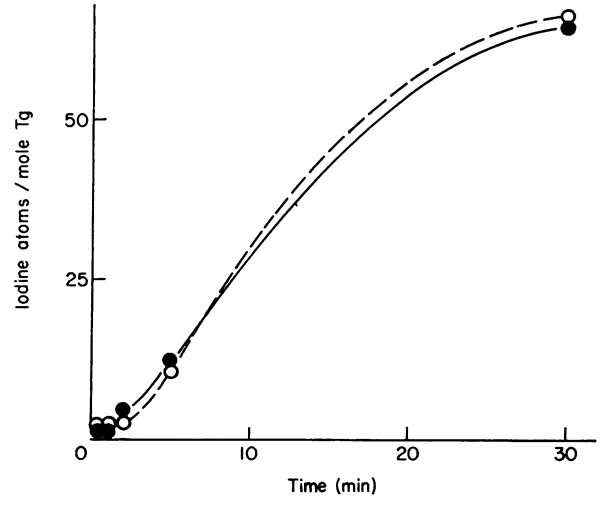

FIG. 1. Kinetics of lactoperoxidase iodination was performed with the glucose–glucose oxidase H_2O_2 generating system, followed for 30 min in presence of 2×10^{-4} iodide/ml. The iodine incorporated evaluated by paper chromatography showed no significant differences between native (●) and desialylated (○) thyroglobulin.

was found to be respectively 0·5% in M.A. and 1·07% in A.L. Following neuraminidase digestion the sialic acid was undetectable in both thyroglobulins. The sedimentation rate of the two preparations remained unchanged after neuraminidase digestion. Deiodination of *in vivo* labelled thyroglobulin during neuraminidase digestion was 14%.

Thyroglobulin iodination. Iodination was carried out with lactoperoxidase and glucose–glucose oxidase as the H_2O_2 generating system according to Pommier *et al.* (1973). The incubation mixture contained 2×10^{-9} moles of KI, 200 μCi of ^{125}I carrier free, 1×10^{-9} moles of Tg, 750 μg of glucose, 2 μg of lactoperoxidase and 2 μg of glucose oxidase per ml of solution. After 1 hr at room temperature the reaction was stopped by dialysis. The fraction of the total ^{125}I bound to protein was determined prior to dialysis by paper chromatography in n-BuOH/CH_3–COOH/H_2O (8:2:2) on a 5 μl aliquot. The radioactivity which remained at the origin of the chromatogram was essentially protein bound and was calculated as a fraction of the total radioactivity on the chromatogram. The kinetics of iodine incorporation of native and desialylated Tg followed for up to 30 min in the presence of 2×10^{-4} moles of iodine (Fig. 1) revealed no differences between the two thyroglobulin preparations. The specific activity of the labelled thyroglobulins was 0·2–0·5 mCi/mg, 0·5–1 atom of iodine/mole of Tg.

After dialysis the labelled thyroglobulins (native and desialylated) were purified by sucrose gradient centrifugation in order to isolate only the 19S component (Fig. 2). The solution containing the 19S components was brought to 0·1% with bovine albumin to avoid the non-specific absorption of radioactivity in the radioimmunoassay.

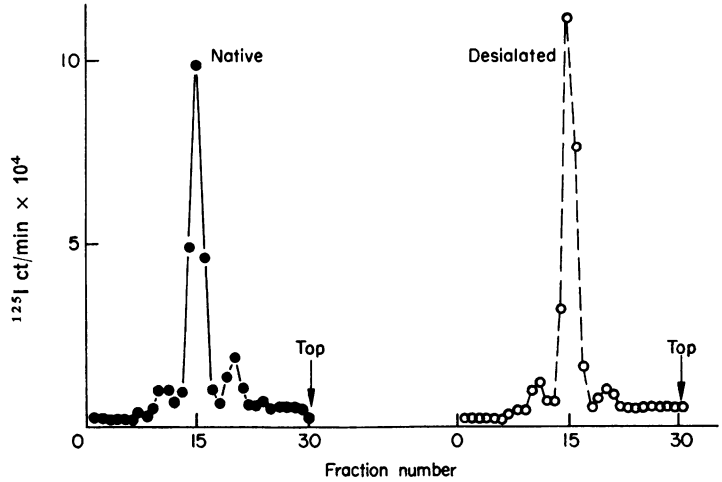

FIG. 2. Sucrose gradient centrifugation pattern of lactoperoxidase-labelled thyroglobulin (200 μCi/mg). Samples (0·5 mg/ml) were applied on a 10–40% linear sucrose gradient and run at 26,000 rpm in a Spinco Mod. L2 for 24 hr at 4°C in a rotor SW27. At the end of the centrifugation 0·5 ml fractions were collected and counted in a scintillation counter. The peak of thyroglobulin was localized in the ultracentrifugation pattern by a reference run simultaneously in a separate tube of the same rotor. Fractions of 19 S peak were pooled, dialysed, brought to 0·1% with bovine serum albumin and used as antigens.

Purification of glycopeptides. Glycopeptides from thyroglobulin were prepared according to the procedure described by Spiro (1965). 2 g of a human Tg (from a colloid goitre) were purified by salting out with $(NH_4)_2SO_4$ and digested with Pronase. This thyroglobulin preparation contained 0·04% of iodine and 1·1% of sialic acid. The thyroglobulin solution (6·8%) was digested in 0·2 M phosphate buffer pH 7·8 at 37°C in the presence of 0·0015 M $CaCl_2$ with 1% (w/w) of Pronase for 120 hr. At 48 and 72 hr a further 0·5% of Pronase was added to the incubation mixture. The digest was dialysed against several changes of NaCl 0·15 M, until the O.D. at 280 nm of the diffusate showed no further absorbancy.

The glycopeptides were then purified on a Sephadex superfine G-25 column (3·4× 50 cm) in 0·1 M phosphate buffer, pH 7·0.

A single symmetrical peak with an elution volume of 170 ml was observed. Neutral sugars, and proteins of native thyroglobulin and glycopeptides were measured respectively by the anthrone reaction (Roe, 1955) and by the biuret (Gornall, Bardowill & David, 1949) method. More than 95% of the protein was digested by Pronase, whereas 56% of the sugars, measured as neutral sugars, were still present (Table 1).

Immunoreaction. The antibody titre of the human sera containing autoantibodies was evaluated by a radioimmunological technique set up in our laboratory to measure the sera capacity for thyroglobulin.

The native or desialylated [^{125}I]Tg (labelled *in vivo* or by lactoperoxidase as described above) were added in trace amounts (10,000 ct/min, 20 ng) in a duplicate series of glass tubes to solutions containing increasing quantities of native or desialylated Tg (from 50 ng to 0·2 mg). 0·01 ml of the sera followed by 0·2 ml of goat or rabbit sera against human gammaglobulin were then added in order to precipitate the soluble immunocomplexes. Before addition of the anti-gammaglobulin serum,

TABLE 1. Protein and neutral sugar content in Tg-glycopeptides

	Protein (mg)	Neutral sugars (mg/mg Tg)
Native thyroglobulin	1	0·063*
Glycopeptides	0·043	0·035
Residue	4·3%	56%

* Standardized on neutral sugars of Tg as determined by anthrone reaction.

the mixture was incubated for 30 min at 37°C, then for 16 hr at 4°C, after which the mixture was further incubated for 30 min at 37°C and for 16 hr at 4°C. Bound Tg was separated from free Tg by centrifugation (5000 *g* for 10 min). The results are expressed as:

$$\frac{\text{precipitated ct/min} - \text{non-specific precipitate}}{\text{total ct/min} - \text{background}}.$$

The serum thyroglobulin capacity is calculated from a saturation curve where the amount precipitated *v.* amount added, approaches a plateau.

The non-specific precipitate was obtained by incubating native or desialylated Tg with 0·01 ml of normal human serum. The per cent non-specific precipitate was 6 ± 3 for native and 8 ± 4 for desialylated Tg. In this radioimmunoassay, since 30% is the highest limit of the coefficient of variation (Salabè *et al.*, 1974a) only the greater differences between native and desialylated thyroglobulin were considered significant. Inhibition of the immunoreaction by glycopeptides was carried out by incubating [^{125}I] Tg with a fixed amount of glycopeptides before adding the human serum.

The separation of free from bound antigen was performed not only with the antigen binding capacity method but also by polyacrilamide gel electrophoresis (Ornstein, 1964). The immunocomplexes have lower electrophoretic mobility than free thyroglobulin. The binding is evaluated from the differences in electrophoretic mobility between free and bound thyroglobulin. Before electrophoresis the serum was incubated for 30 min at 37°C with ^{125}I-labelled antigens. The incubation mixture (100 μl) applied on the gel contained 5 μl of serum, 25 ng of peroxidase labelled ^{125}I-native or desialylated thyroglobulin. Controls were run simultaneously with normal serum, and/or labelled antigens.

Polyacrilamide gel electrophoresis was performed in a 4% gel at room temperature (upper buffer pH 8·9, μ 0·13; lower buffer pH 8·1, μ 0·18) for 90 min with 2·5 mA DC per tube. At the end of the migration, gels were cut with a razor blade in approximately 1 mm slices (each gel of 6 cm gave 60–65 slices). The slices were placed in the bottom of a plastic tube and counted for ^{125}I in a well type automatic scintillation counter.

Sera. A series of twenty sera was studied. The sera were from patients seen at the outpatients department. All sera contained anti-Tg antibodies assessed by the tanned red cell method and/or antigen binding capacity. The titres ranged between 1:5 and $1:2{\cdot}5 \times 10^6$. The sera were from thyroid patients with simple nodular or diffuse goitre or myxoedema or hyperthyroidism. The diagnosis of thyroiditis was established when a firm and lobulated swelling of the thyroid was associated with high TRC or ABC and the difference between PBI and T4 was higher than 2 μg%. In some cases the goitre was removed and the diagnosis confirmed by histology.

RESULTS

The antigen binding capacity of the human sera with native or desialylated thyroglobulin (A.L.) labelled *in vivo* is shown in Table 2. An increase of more than 30% can be observed in four out of thirteen sera.

The binding capacity of desialylated and native antigen is calculated before saturation, i.e. at a concentration of antigen where the per cent of radioactivity precipitated is high enough to calculate the differences between radioactive native or desialylated thyroglobulin precipitated. In three of the four sera showing increments by desialylation, a binding curve traced at different antigen concentrations, confirmed the effect seen with a single antigen concentration (Fig. 3).

Ten sera were also tested with the same native or desialylated thyroglobulin (A.L.) preparation labelled with lactoperoxidase. Significant increments were produced in four sera (>30%) (Table 3).

A third group of thirteen sera was examined with a different thyroglobulin (M.A.) labelled with lactoperoxidase. In Table 4 it can be seen that one serum (L.M.) confirmed the enhancement observed

TABLE 2. Enhancement of human sera immunoreaction using desialylated human thyroglobulin labelled *in vivo* (A.L.)

Patient	Tg added (μg)	Native (% ptt)	Desialylated (% ptt)	Variation (%)
1. G.S.	1·5	16·5	30·9	+87
2. Z.G.	11	11·5	13·4	+16
3. B.G.	15	19·0	22·0	+15
4. T.M.	20	21·3	21·3	0
5. M.E.	30	16·5	20·5	+24
6. N.A.	30	21·7	20·7	0
7. F.M.	200	26·4	30·7	+16
8. W.A.	1	49·0	51·0	+4
	3	23·0	25·0	+8
9. B.M.P.	1	55·9	70·4	+25
	3	23·1	33·0	+42
10. L.M.	1·5	23·5	44·3	+88
	3	11·4	34·2	+200
11. B.E.	3	58·6	77·2	+31
	10	11·9	16	+34
12. G.F.	15	53·0	55·0	+3
	50	18·2	17·1	0
13. B.V.	120	47·2	50·5	+7
	After 2 yr	39·8	44·3	+11

The incubation mixture in 0·4 ml of buffered phosphate (pH 7·4) contained 0·1 ml of serum (1:10 dilution), varying amounts of ^{125}I native or desialylated thyroglobulin labelled *in vivo* and 0·2 ml of goat anti-human gammaglobulin. The numbers refer to per cent radioactivity counted in the washed precipitate. Results are the average of duplicates, after subtraction of non-specific precipitation carried out with a normal instead of an immune serum. The variation between native and desialylated thyroglobulin was calculated according to the formula:

$$\frac{\text{Native} - \text{Desialylated}}{\text{Native}} \times 100.$$

with the previous thyroglobulin (A.L.) and one—not examined in the previous series—showed a marked increment, $> 100\%$. In nine out of fifteen sera the binding decreased from marginal 4%, to 24%.

The binding in the same sera of native and desialylated thyroglobulin with different types of iodination are compared in Table 5.

In two (G.S., B.M.P.) of the three sera a drop in immunoreactivity was observed with lactoperoxidase iodinated thyroglobulin. The effect of peroxidase on antigenic properties of thyroglobulin might be due to a slight denaturation of antigenic determinants during enzymatic iodination.

Two thyroglobulins (A.L., M.A.) both labelled by lactoperoxidase behave differently with the same sera. With thyroglobulin (A.L.) an increase in precipitation was seen in all but one sera (B.P.); with thyroglobulin (M.A.) a slight decrease was seen in five out of nine sera and a marked enhancement was observed with serum B.P. which did not show any effect with thyroglobulin A.L.

The polyacrilamide gel electrophoresis method used in two sera showed that bound thyroglobulin forms a major immunocomplex and several minor components with lower electrophoretic mobility (Fig. 4). Serum T.A. binds equal amounts of native and desialylated thyroglobulin, whereas serum

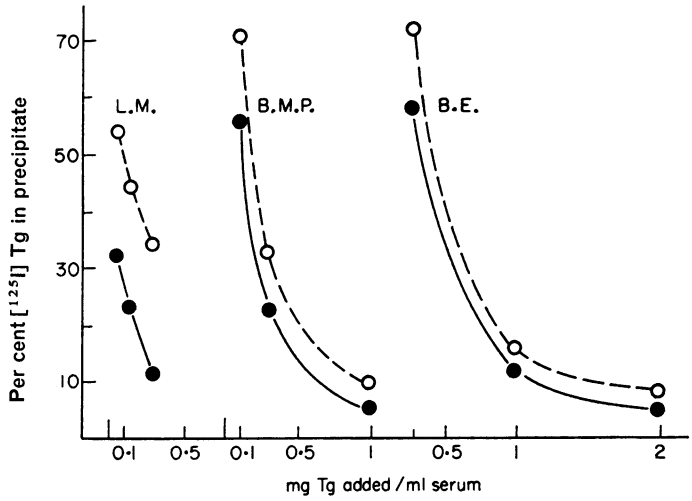

FIG. 3. Binding curves of three different sera with *in vivo* labelled thyroglobulin. ●, Native; ○, desialylated. Each immunoprecipitation was carried out in duplicate in a final volume of 0·4 ml containing 0·1 ml of 0·02 M phosphate, 0·1 M KI buffer pH 7·4, from 0·5 to 20 μg of ^{125}I *in vivo* labelled thyroglobulin and 0·1 ml of a 1:10 dilution of test serum and 0·2 ml of goat anti-human gamma-globulin serum. Initials denote subjects.

L.M. binds more desialylated than native (Table 6) confirming the data obtained with the antigen binding capacity method.

A carbohydrate-enriched fraction from human thyroglobulin was used as a blocker in the immunoreaction of native thyroglobulin with five autoantisera which had variable binding with native thyroglobulin (Fig. 5). This preparation was able to inhibit the reaction to a variable extent. Sera C.G. and B.M. with high binding to native thyroglobulin had a higher inhibition than sera W.M. and G.S. with lower binding to native thyroglobulin. It is therefore suggested that carbohydrates may play a role as antigenic determinants.

TABLE 3. Enhancement of human sera immunoreaction using desialylated human thyroglobulin (19S) labelled with lactoperoxidase (A.L.)

Patient	Tg added (μg)	Native (% ptt)	Desialylated (% ptt)	Variation
1. G.S.	1·5	19	23	+21
9. B.M.P.	1·5	47	54	+15
10. L.M.	1·5	27	50	+85
14. W.V.	0·02	25	35	+40
15. W.M. (father)	0·2	40	42	+5
16. C.M. (grandmother)	3	56	69	+23
17. B.P.	0·02	33	30	−10
18. B.E. (brother)	0·2	48	66	+37
19. C.G.	1	40	40	0
20. T.A.	3	49	64	+37

The incubation mixture in 0·4 ml of phosphate buffer (pH 7·4) contained 0·1 ml of serum (1:10 dilution), tracer amount (10,000 cpm = 25 ng) of ^{125}I native or desialylated thyroglobulin labelled with lactoperoxidase and purified by sucrose gradient centrifugation (19S) together with varying amounts of cold native or desialylated thyroglobulin and 0·2 ml of goat anti-human globulin serum. The numbers refer to percent radioactivity calculated in the precipitate as described in Table 2.

TABLE 4. Enhancement of human sera immunoreaction using desialylated human thyroglobulin (19S) labelled with lactoperoxidase (M.A.)

Patient	Tg added (μg)	Native (%ptt)	Desialylated (%)	Variation (%)
1. G.S.	1·5	11·8±2	13·8±0·7	+17
9. B.M.P.	1·5	41·1±4	34·1±1	−17
10. L.M.	0·05	15·4±2	16·8±1	+9
	1·5	6·4±0·4	10·4±0·3	+63
11. B.E.	3	71·7±2	63·9±0·7	−11
14. W.V.	0·025	11·7±0·3	13·5±0·6	+16
16. C.M.	3	58·4±4	44·3±0·7	−24
17. B.P.	0·0125	15·9±0·7	38·8±2·5	+144
	0·025	10·2±0·6	27·1±1·6	+166
18. B.E. (brother)	0·025	72·1±2·6	69·0±2	−4
20. T.A.	3	43·6±0·2	33·4±1·8	−23
21. P.E.	0·025	72·5±1·5	69·1±0·6	−5
22. P.M.	0·025	64·1±1·6	60·8±1	−5
23. T.P.	1	11·5±0·1	10·9±0·5	−5
24. C.G.	1	26·7±2·8	22·1±1	−17

^{125}I tracer labelled and cold antigens were incubated with sera and goat anti-human gamma globulin serum as described in Table 3.

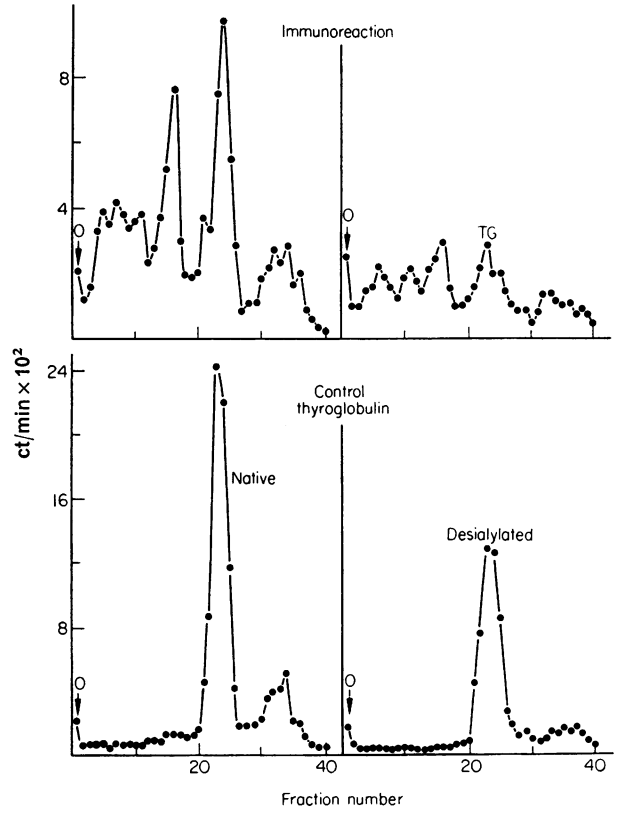

FIG. 4. Separation of immunocomplexes by polyacrilamide gel electrophoresis. The serum (T.A.) (5 μl) was incubated with 25 ng of peroxidase labelled ^{125}I native and desialylated thyroglobulin then applied on a 4% gel for 90 min with 2·5 mA DC per tube. Gels were cut in 1 mm fractions and counted. In the upper panels the electrophoretic distribution of immunocomplexes, in the lower control native and desialylated thyroglobulins.

TABLE 5. Variation in the immunoreaction of human antisera with different desialylated thyroglobulins

Patient	Tg added (μg)	Tg(A.L.) *in vivo*	Tg(A.L.) lactoperoxidase	Tg(M.A.) lactoperoxidase
1. G.S.	1·5	+87	+21	+17
9. B.M.P.	1·5	+25	+15	−17
11. B.E.	3	+31	—	−11
14. W.V.	0·02	—	+40	+16
10. L.M.	1·5	+88	+85	+64
16. C.M.	3	—	+23	−24
17. B.P.	0·025	—	−10	+166
18. B.E.	0·2 and 0·025	—	+37	−4
20. T.A.	3	—	+37	−23

Numbers refer to per cent change with respect to native thyroglobulin calculated using the formula:

$$\frac{\text{Native—Desialylated}}{\text{Native}} \times 100.$$

DISCUSSION

In the present investigations it was shown that at least four autoantisera contain autoantibodies specific for thyroglobulin determinants which are hidden in the native antigen by sialic acid. In view of the enhancement observed with desialylated thyroglobulin and autoantibodies it is tempting to suggest that sialic acid exerts a protective role by covering determinants of thyroglobulin which may act as autoantigens. In support of this hypothesis is the well-known example of IgM antibody in mixed cryo-

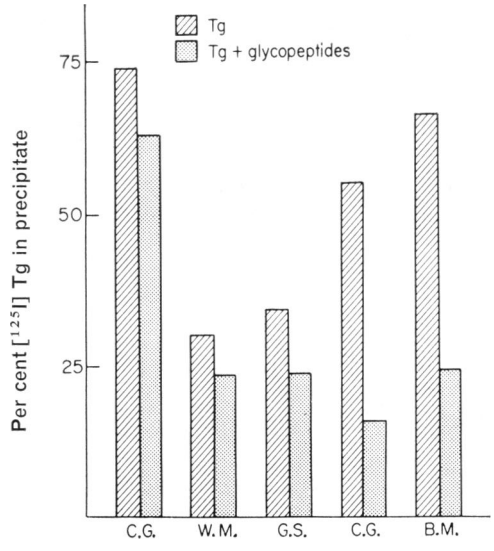

FIG. 5. Inhibition of the immunoreaction was carried out in a final volume of 0·5 ml of phosphate buffer pH 7·4. 0·007 mg of the glycopeptides preparation 0·1 ml of test serum diluted 1:10 and 0·025 μg of thyroglobulin labelled with ^{125}I by lactoperoxidase and purified by sucrose gradient ultracentrifugation and finally 0·2 ml of goat anti-human gamma globulin serum were sequentially added to the test tube. The columns express the per cent of radioactivity counted in the washed precipitate. Results are the average of duplicates after subtraction of non-specific precipitation (6±2) carried out with a normal serum. Initials refer to the subjects examined.

TABLE 6. Evaluation of immunoreaction by polyacrilamide gel electrophoresis

Serum	Thyroglobulin bound	
	Native	Desialylated
10. L.M.	6	19
20. T.A.	42	45

Numbers refer to per cent of total radioactivity measured on the gel; the non-specific varied from 10 to 13%.

The non-specific binding was the radioactivity on the control gel corresponding to the fractions where immunocomplexes were found.

A mixture containing 0·5 μg of labelled antigen (~ 10,000 ct/min) and 2·5 μl of serum was applied to each gel.

Samples were run for 90 min at 2·5 mA/gel at room temperature.

globulinaemia reacting with desialylated but not with native gammaglobulin (Zinneman Levi & Seal *et al.*, 1968). The primary event triggering off the autoimmune response could be the biosynthesis of a desialylated thyroglobulin due to a defect in the membrane bound enzyme sialyltransferase (Monaco & Robbins, 1973). The appearance of a new determinant in thyroglobulin breaks the normal lymphocyte tolerance and initiates a process of cellular and humoral autoimmunity (Salabè, 1975).

Carbohydrates such as antigenic determinants in autoimmunity of the thyroid is an attractive field of investigation taking into consideration that homologies have been found between oligosaccharides of thyroglobulin and carbohydrates of blood group substances (Ginsburg, 1972) and also that in cold agglutinins haemolytic anaemia beta-linked acetyl-D-glucosamine and beta-linked galactose are the autoantigenic determinants (Feizi *et al.*, 1971). So far our experiments on the inhibition of immunoreaction of autoantisera by a crude preparation of thyroglobulin glycopeptides points towards a possible role in autoimmune thyroiditis, of carbohydrates as antigenic determinants and stimulate further investigations to clarify the antigenic structure of oligosaccharides of thyroglobulin.

In our experiments using two different thyroglobulins, the effect of desialylation was observed only with one thyroglobulin, thus indicating the existence of isoantigens in the antigenic structure of thyroglobulin carbohydrates.

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