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Asialoagalactothyroglobulin binds to the surface of human thyroid cells at a site distinct from the 'microsomal' autoantigen

I. M. ROITT, R. PUJOL-BORRELL, T. HANAFUSA, P. J. DELVES, G. F. BOTTAZZO & L. D. KOHN* *Department of Immunology, the Middlesex Hospital Medical School, London, UK and * Section on Biochemistry of Cell Regulation, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland, USA*

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SUMMARY

Human thyroglobulin has been shown for the first time to bind to the surface of cultured human thyroid follicular cells. Binding was only observed with partially glycosylated asialoagalactothyroglobulin, not with the fully glycosylated iodoprotein. The binding site for asialoagalactothyroglobulin in the cell membrane is distinct from membrane associated microsomal/microvilli antigen. Since asialoagalactothyroglobulin bears the autoantigenic determinants of the parent molecule, its ability to bind to the thyroid cell surface suggests a possible role for this protein in the pathogenesis of human thyroiditis.

Keywords thyroglobulin thyroid cell thyroiditis thyroid microsomal antigen

INTRODUCTION

In autoimmune thyroiditis autoantibodies are found against thyroid microsomal antigen and thyroglobulin. The microsomal antigen is a poorly characterized membrane protein (Roitt *et al.*, 1964) whose distribution in the cytoplasm and on the microvilli suggests that it may be involved in the secretion of thyroglobulin into the colloid of the thyroid follicles. We have recently demonstrated that this autoantigen is also present on the cell surface at the apical pole of thyroid follicular cells (Khoury *et al.*, 1981). The presence of autoantigens on membrane surfaces makes them suitable targets for potentially damaging autoantibodies but so far it has proved difficult to demonstrate the presence of binding sites for thyroglobulin on thyroid cell membranes. Recently, Consiglio *et al.* (1981) demonstrated that bovine asialoagalactothyroglobulin, derived from the parent molecule by cleavage of sialic acid and galactose residues, can bind to thyroid microsomal, lysosomal and Golgi membranes, and to bovine, as well as rat, thyroid cells in culture. In this report we demonstrate that human asialoagalactothyroglobulin is similarly able to bind to cultured human thyroid cells. Using blocking studies and two colour immunofluorescence we further show that the receptor for asialoagalactothyroglobulin shows a different distribution to the membrane associated thyroid microsomal antigen.

Correspondence: Professor I. M. Roitt, Department of Immunology, The Middlesex Hospital Medical School, Arthur Stanley House, 40–50 Tottenham Street, London W1P 9PG, UK.

MATERIALS AND METHODS

Human thyroglobulins. These were prepared as previously described for bovine thyroglobulin (Shifrin *et al.*, 1982). Briefly, 19S thyroglobulin was isolated by salt extraction of sliced thyroid glands followed by ammonium sulphate precipitation of the salt extract and gel filtration over a column of Sephacryl S-300 (Pharmacia). The material was eluted with 0·1 M potassium phosphate pH 7·5 and the 19S peak then rechromatographed on a further Sephacryl S-300 column.

Asialothyroglobulin was prepared by incubating 19S thyroglobulin (35mg/ml) in 0·1M sodium acetate pH 5·3 with 1,000 units/ml of *Vibrio cholerae* neuraminidase (Behring) at 37°C for 24 h. Several drops of toluene were added to the incubation mixture. The thyroglobulin was then rechromatographed on a column of Sephacryl S-300.

Asialoagalactothyroglobulin was prepared by incubating the asialothyroglobulin (15 mg/ml) obtained above with 2 units/ml of β-galactosidase from *Aspergillus niger* (Sigma) in 0·1M sodium acetate pH 5·2. A few drops of toluene were added to the mixture before incubation at 37°C for 24 h. The material was rechromatographed on a column of Sephacryl S-300.

Rabbit antisera. Rabbit antisera to either 19S thyroglobulin or asialoagalactothyroglobulin were raised by injecting 100 μg of the relevant thyroglobulin in Freund's complete adjuvant i.m. into New Zealand White rabbits, followed 2 weeks later by a similar injection in Freund's incomplete adjuvant and a further 2 weeks later by 100 μg of alum precipitated thyroglobulin. The rabbits were bled out 13 days later, antisera were precipitated with 40% saturated ammonium sulphate and dialysed into PBS.

Affinity purified antibody was prepared by adsorption to a column of 19S thyroglobulin coupled to Sepharose CL-6B (Pharmacia), elution of the bound antibody with 0·05M diethylamine HCl pH 11·5 and neutralization with 0·1M orthophosphoric acid, followed by dialysis into PBS.

Fluoresceination of antibodies was performed by incubating 0·05mg fluorescein isothiocyanate (BDH)/mg protein for 16 h at 4°C in 0·25M carbonate–bicarbonate buffer pH 9·0. The conjugated antiserum was separated from free fluorochrome by passage down a Sephadex G-25 column (Pharmacia) and dialysed into PBS.

Human antisera. Autoantibodies were measured using the Thymune-T and Thymune-M haemagglutination kits (Wellcome). Patient JM was positive for both anti-thyroglobulin (titre $>1:5{,}000$) and anti-microsomal (titre $1:160^2$) autoantibodies, patient FQ was positive only for microsomal antibodies (titre $>1:1280^2$), and patient HY was positive only for anti-thyroglobulin (titre 1:5,000). Serum from patient FQ reacted with thyroid membrane microsomal antigen in thyroid monolayers up to a dilution of 1:2,000. Binding of this antibody was visualized using FITC conjugated sheep anti-human $F(ab')_2$ (prepared in our laboratory) or TRITC conjugated goat anti-human IgG (Miles).

Ouchterlony. Reactivity of rabbit and human antibodies with the three thyroglobulin preparations was tested using double diffusion in 2% agar in 0·08 M barbitone buffer pH 8·2 on glass slides. After incubation overnight at room temperature the slides were washed extensively in PBS and then stained with 1% Coomassie brilliant blue R (Sigma).

Thyroid tissue. Tissue from three different glands obtained at surgery from two patients with thyrotoxicosis and one patient undergoing radical neck surgery for carcinoma of the larynx were digested with collagenase type IV (Worthington) 5mg/ml for 3 h at 37°C and filtered through a 200 μm mesh. The cells were plated out at a concentration of 10^5/ml on 13mm glass coverslips in a Linbro 24 well plate and cultured in RPMI 1640 (Flow) supplemented with 2 mM glutamine, 8 μg/ml insulin, 5μg/ml transferrin, 10^{-8}M hydrocortisone and 20% fetal calf serum (GIBCO). After 24 h monolayer cultures consist mainly of viable thyroid cells (Khoury *et al.*, 1981).

In separate experiments, digested tissue from a normal gland and a thyrotoxic patient's gland were plated out onto 35mm diameter Petri dishes pre-coated with agarose (type VI, Sigma) to prevent cells from attaching to the bottom. Intact follicles with normal polarity (basal/vascular surface of thyroid cells exposed to outside of follicle) become reconstituted after 2 days of culture in medium containing 0·5% FCS, whilst follicles with reversed polarity (apical surface of thyroid cells exposed to outside of follicle) are obtained after 5 days of culture with 10% FCS (Hanafusa *et al.*, 1982).

Immunofluorescence. To detect the binding of the different thyroglobulin preparations to cultured thyroid cells, the viable monolayers were washed with BSS containing 5% bovine serum albumin and incubated with 19S thyroglobulin, asialothyroglobulin or asialoagalactothyroglobulin at concentrations ranging from 10 μg to 1mg/ml for 1 h at room temperature. The cultures were then extensively washed with BSS-BSA, and affinity purified rabbit anti-thyroglobulin antiserum added at a dilution of 1:50 for 30 min at room temperature. After further washings FITC goat anti-rabbit Ig was added for 30 min at room temperature.

To assess the ability of the different thyroglobulin preparations to block the access of the microsomal autoantibodies to the microsomal membrane antigen, cultures were first incubated for 1 h at room temperature with one of the different thyroglobulin preparations followed by patient FQ anti-microsomal serum at 1:1,000 dilution (one dilution below end point). This was followed by FITC sheep anti-human $F(ab')_2$. Possible inhibition of the binding of thyroglobulin by microsomal autoantibody was assessed by reversing the order of the first two layers, using the human antibody at 1:10 dilution and the binding of thyroglobulin revealed using rabbit anti-human thyroglobulin followed by FITC goat anti-rabbit Ig.

Simultaneous visualization of the presence on the thyroid cell membranes of thyroglobulin and microsomal antigen was achieved by sequential incubation of the monolayers with the following; patient anti-microsomal serum (1:10), TRITC goat anti-human IgG, asialoagalactothyroglobulin (1mg/ml), rabbit anti-human thyroglobulin (1:50), FITC goat anti-rabbit Ig. The cultures were then fixed with 5% acetic acid in ethanol at $-20°C$ for 10 min, mounted with glycerol on a microscope slide and examined under a Zeiss fluorescence microscope.

In an attempt to assess the binding of the different thyroglobulin preparations to either the basal or apical surfaces of the thyroid cells, intact follicles with either normal or reversed polarity were stained as described above for thyroid monolayers.

RESULTS

Antigenic relationships between the different thyroglobulin preparations

Using the Ouchterlony double diffusion system in agar, both rabbit antisera against 19S thyroglobulin or asialoagalactothyroglobulin and human thyroglobulin autoantibodies gave precipitin lines against all the thyroglobulins tested (19S, asialo- and asialoagalactothyroglobulin). The results indicated complete antigenic identity between the different thyroglobulins and shows that neither sialic acid nor galactose residues contribute to any of the epitopes. A patient's serum giving positive results in haemagglutination only with microsomal antigen failed to give a precipitin line with any of the thyroglobulins.

Thyroglobulin binding to cultured thyroid cells

Using rabbit anti-thyroglobulin antiserum we were unable to detect thyroglobulin bound to the thyroid cell monolayers without first incubating the cultures with exogenous thyroglobulin. Binding was only observed with the partially glycosylated asialoagalactothyroglobulin when used at a concentration of 1·0mg/ml (Fig 1a). No binding to monolayers could be detected using asialothyroglobulin or fully glycosylated 19S thyroglobulin. Furthermore, no binding of any of the thyroglobulin preparations could be visualized on either the basal or apical surface of intact reconstituted thyroid follicles with either normal polarity (basal surface exposed to outside after reconstitution in 0·5% FCS) or reversed polarity (apical surface exposed to outside after reconstitution in 10% FCS). The exterior surface of cells with reversed polarity did, however, stain with anti-microsomal autoantibodies.

Blocking studies

Neither 19S nor asialoagalactothyroglobulin used at concentrations as high as 1·0 mg/ml were able to block the access of human anti-microsomal autoantibody to its membrane expressed antigen. Conversely, high titre microsomal autoantibody was unable to inhibit the binding of asialoagalac-

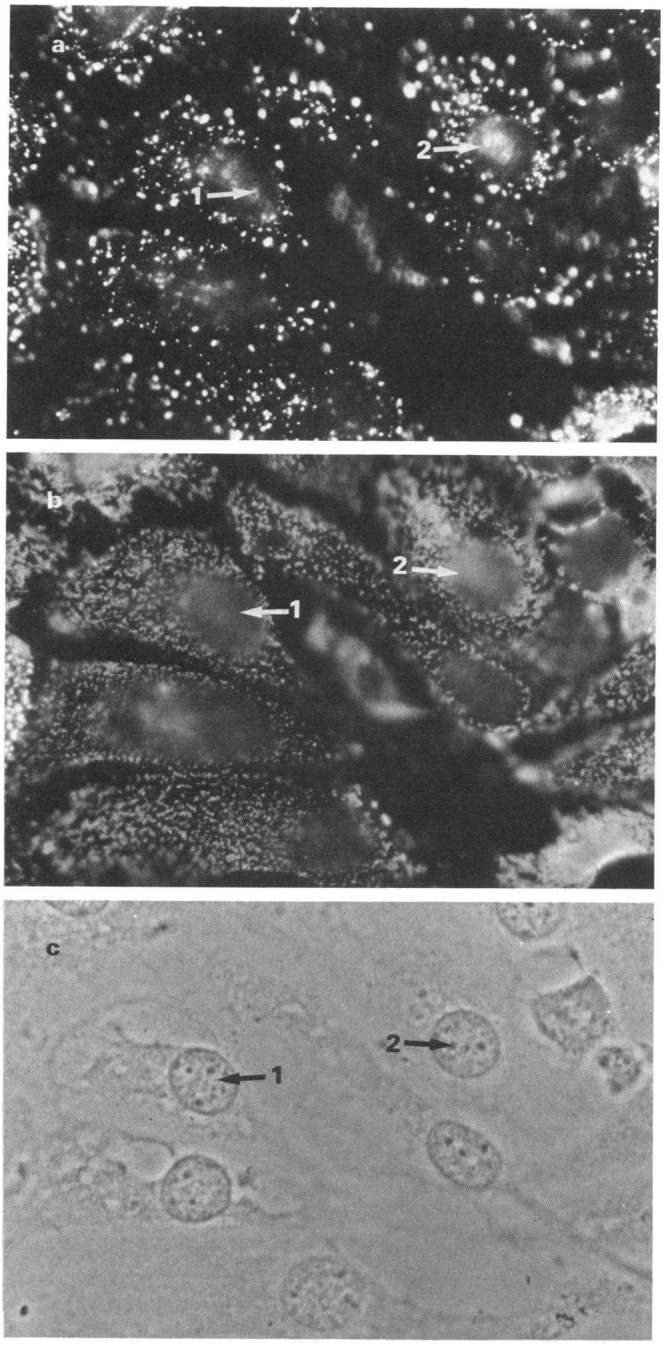

Fig. 1. Double immunofluorescence staining of cultured thyroid cell monolayers showing (a) Binding of agalactothyroglobulin. (b) Microsomal antigen. (c) Phase contrast of the same field. Arrows indicate the nuclei of individual cells. Magnification ×450.

tothyroglobulin to cultured human thyroid cells, suggesting that the binding site for asialoagalactothyroglobulin is distinct from the membrane associated microsomal antigen.

Double staining

We were able to visualize the separate binding sites for asialoagalactothyroglobulin and thyroid microsomal autoantibodies by the double immunofluorescence technique. The fluorescein (staining agalactothyroglobulin) and rhodamine (staining microsomal antigen) dots were clearly distinct and exhibited different distribution patterns (Fig. 1a & b).

DISCUSSION

The autoantibodies in Hashimoto's thyroiditis are directed against thyroglobulin and/or thyroid microsomal antigen (Roitt *et al.*, 1956; Belyavin & Trotter, 1959). In trying to assess the role these antigens play in the pathogenesis of the disease it is important to know whether they are associated with the surface membrane of the thyroid cells. Recently we demonstrated, using both immunofluorescence and complement-mediated antibody-dependent cytotoxicity techniques, that thyroid microsomal antigen is expressed on the surface of human thyroid cells but we were unable to detect thyroglobulin bound to these cells even after pre-incubation with 19S thyroglobulin (Khoury *et al.*, 1981). Although binding of radiolabelled anti-thyroglobulin antibody to cultured thyroid cells has been reported in one study (Fenzi *et al.*, 1982), it has not previously been demonstrated that exogenous human thyroglobulin is able to bind to these cells. Recently Consiglio *et al.* (1981) observed that treatment of bovine thyroglobulin with neuraminidase partially increased binding to thyroid membranes but this was markedly enhanced after additional digestion with β-galactosidase. We have now demonstrated that human thyroglobulin will bind to the surface membrane of human thyroid cells in monolayer culture after removal of terminal sugars from the iodoprotein. In our studies it was necessary to remove both sialic acid and galactose residues before binding could be visualized. We failed to detect binding of asialoagalactothyroglobulin to intact reconstituted thyroid follicles. This could be due to either the lower sensitivity of staining cells in suspension rather than as monolayers or perhaps an inability of the intact follicle to express as high a concentration of surface asialoagalactothyroglobulin receptors as the monolayer cells particularly under the conditions required for reconstituting the follicles with reversed polarity, although in these follicles the microsomal antigen could still be visualized. This disparity in staining behaviour indicated that the microsomal/microvilli autoantigen and the asialoagalactothyroglobulin receptors were distinct entities; this was amply confirmed by concomittant staining with two fluorescent probes and by the failure of asialoagalactothyroglobulin and anti-microsomal antibodies to influence each other's binding to the thyroid cells. These findings also exclude the possibility that removal of carbohydrate from the fully glycosylated iodoprotein exposes normally hidden determinants which are then recognized by microsomal antibody.

The cells infiltrating the Hashimoto thyroid have an increased proportion of B lymphocytes compared to peripheral blood and are able to synthesize relatively large amounts of thyroglobulin and/or microsomal antibody *in vitro* without further stimulation (Weetman *et al.*, 1982; McLachlan *et al.*, 1983). Around forty per cent of the lymphocytes isolated from these glands are of the $OKT4^+$ subset (McLachlan *et al.*, 1983; Wall *et al.*, 1983) which recognize antigen in combination with class II MHC antigens, provide T cell help for antibody production (Reinherz *et al.*, 1980) and release lymphokines producing delayed type hypersensitivity reactions. Since asialoagalactothyroglobulin possesses the same autoantigenic determinants as the parent molecule, its ability to bind to the surface of thyroid cells has important implications, especially in the light of our recent observations of HLA-DR expression in sections of thyrotoxic glands and by normal thyroid cells after culture *in vitro* with lectins (Hanafusa *et al.*, 1983; Pujol-Borrell *et al.*, 1983). We have postulated that other agents (virus, lymphokine, interferon) could induce HLA-DR expression *in vivo* on thyroid cells (Bottazzo *et al.*, 1983). The presence of thyroglobulin and/or thyroid microsomal antigen together with HLA-DR on the surface of thyroid cells may allow the presentation of normally non-immunogenic autoantigens to T lymphocytes, thereby acting as a powerful stimulus for the

production of chronic inflammatory reactions and of autoantibody which might then either be directly cytotoxic or 'arm' K cells for ADCC (Podleski, 1972; Calder *et al.*, 1973).

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