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Autoantibodies against Forssman glycolipids in Graves' disease and Hashimoto's thyroiditis

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SUMMARY

Sera from patients with Graves' disease and Hashimoto's thyroiditis have been shown to react with the Forssman glycolipid antigen (Gb5) using the techniques of high performance thin-layer chromatography (HPTLC) immunostaining and ELISA. Human monoclonal antibodies (MoAbs) have been prepared by fusion of human myeloma with peripheral lymphocytes from patients with Graves' disease. A MoAb, TRMo-4, reacted strongly and specifically with Gb5. These results suggest that anti-Forssman antibody may be involved in the pathogenesis of these autoimmune diseases. The detection of anti-Forssman glycolipid antibody may provide a useful means for clinical diagnosis and therapy.

Keywords autoimmune disease Graves' disease Hashimoto's disease autoantibodies Forssman glycolipid

INTRODUCTION

Glycolipids are important constituents of the plasma membranes and they constitute part of the glycoconjugate network extending from the membrane surface. The carbohydrate constituents of glycolipids are localized prominently at the cell surface and often share antigenic determinants with other glycoconjugates. They are important in governing the properties and functions of cells (Hakomori, 1981; Marcus, 1984; Feizi, 1985). A specific carbohydrate structure on the cell surface can serve as both a potential receptor for an endogenous ligand and a determinant of the host's susceptibility to infectious agents (Hakomori, 1981). In spite of many speculations, little is known about the normal regulation of the immune response to glycolipids or the possible role of humoral or cell-mediated immunity to glycolipids in disease (Marcus, 1984). During the past decade considerable evidence has accumulated indicating that anti-glycolipid antibodies can be detected in sera of patients with several disorders of immune regulation including Waldenstrom macroglobulinaemia (Naiki & Marcus, 1977), systemic lupus erythematosus (SLE) (Hirano *et al.*, 1980; Endo *et al.*, 1984), multiple sclerosis (Fry, Weissbarth & Lehrer, 1974; Arnon *et al.*, 1980; Endo *et al.*, 1984; Kasai, Pachner & Yu, 1986), cold agglutinin disease and cold haemoglobinuria (Feizi, 1981; Schwarting, Kundu & Marcus, 1979), rheumatoid arthritis (Moore and Dorner, 1980; Nishimaki, Kano & Milgrom, 1978), AIDS (Witkin *et al.*, 1983), amyotropic lateral sclerosis (Pestronk *et al.*, 1988), and acute lymphoblastic leukaemias (Nakahara *et al.*, 1980). Recently, autoantibodies to gangliosides and sulphated glucuronic acid-containing glycolipids (SGGLs) have been detected in sera of patients with demyelinating neuropathy and motor neuron disease (Chou *et al.*, 1985; Ariga *et al.*, 1987; Ilyas *et al.*, 1988; Yu *et al.*, 1990). These studies indicate that anti-glycolipid antibodies may be involved in the pathogenesis of these autoimmune diseases.

Graves' disease and Hashimoto's thyroiditis are autoimmune diseases caused by disorders of the thyroid gland. McKenzie, Zakariji & Sato (1978) have reported that the thyrotropin (TSH) receptor in the thyroid plasma membrane is responsible for the production of thyroid-stimulating antibodies, which are present in the sera of patients with these disorders. The increased levels of immunoglobulins (IgG) in sera indicate that both humoral and cellular immune mechanisms might be involved in these diseases. In the present study, the occurrence of IgG antibodies against Forssman glycolipid in sera of patients with Graves' disease and Hashimoto's thyroiditis was investigated using a sensitive ELISA and a high performance thin-layer chromatography (HPTLC) immunostaining method.

MATERIALS AND METHODS

Symbols used for gangliosides and other glycolipids are based on the system of Svennerholm (1963) and the nomenclature recommended by IUPAC (1977).

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Neutral glycolipids and gangliosides used as references and standards were isolated from human and sheep erythrocyte membranes and human brain in our laboratories (Ariga *et al.*, 1980; Sekine *et al.*, 1984; Kasai, Sillerud & Yu, 1986). The isolation procedures for the total lipids, and neutral and acidic glycolipids from thyroid membranes and lymphocytes of Graves' disease were as described by Ariga *et al.* (1988). Briefly, total lipids were extracted with chloroform:methanol (2:1 and 1:1, v/v) and chloroform:methanol:water (30:60:8, v/v) and filtered. The combined extracts were then applied to a DEAE-Sephadex A-25 column (acetate form, 3 ml bed volume), followed by elution with 20 ml of chloroform:methanol:water (30:60:8, v/v). The neutral lipids were eluted in this fraction. Acidic lipids were then eluted with 20 ml of chloroform:methanol:0·8 M sodium acetate (30:60:8, v/v). Further purification of the neutral and acidic glycolipids was carried out by Iatrobeads column chromatography (Ariga *et al.*, 1988).

Sera were taken from 10 patients clinically diagnosed as having Graves' and Hashimoto's diseases. Control sera were obtained from 10 normal healthy individuals.

Preparation of MoAbs against thyrotropin (TSH) receptor

The MoAbs against TSH receptor were prepared according to the procedure of Edwards *et al.* (1982) with modifications (Yoshida *et al.*, 1988) as follows. Peripheral blood lymphocytes were separated from defibrinated blood of 28 patients with Graves' disease by centrifuging over Ficoll-Conray, washed in McCoy's tissue culture medium containing 50 U/ml penicillin, and then centrifuged at 400 *g* for 15 min at 4°C. The lymphocytes ($\sim 10^8$ cells) were mixed with LICR-LON-HMy2 cells (10^7 cells) and centrifuged. The pellet was resuspended in 1 ml of RPMI-1640 (GIBCO, New York, NY) containing 40% polyethylene glycol (PEG-1000, Sigma, St Louis, MO) and incubated for 5 min at 37°C. The cells were centrifuged and resuspended in HAT medium. The screening of antibodies was performed by ELISA using lymphocytes or thyroid membranes from Graves' disease. Positive hybrids were selected in HAT medium, expanded and cloned by limited dilution. The cloned cells were grown up in flasks, and the supernatants from these cells were tested for the presence of MoAbs to the TSH receptor. The IgG subfraction was purified by DEAE-Cellulose column chromatography.

Enzyme-linked immunosorbent assay

ELISA was carried out according to the method of Kohriyama *et al.*, (1987). Fifty microlitres of an ethanol solution of glycolipid (1 ng/ml) were added to each well of a flat-bottomed polystyrene microtitre plate. The solution was dried at room temperature overnight. Fifty microlitres of 1% BSA/PBS were added to each well and incubated at 37°C for 30 min. After washing each well with 200 μl of 0·05% Tween 20/PBS by decanting three times, 50 μl of the test antiserum or MoAbs, diluted 1:300 with 1% BSA/PBS, were added and incubated for 1·5 h. After washing, 50 μl of peroxidase-conjugated anti-human IgG antibodies, diluted 1:1000 with 1% BSA/PBS, were added and incubated for 1·5 h and washed again. The enzyme activities were measured by adding 200 μl of a mixture of 0·02% *o*-phenylenediamine and 0·012% of hydrogen peroxide in PBS, pH 5·0, followed by incubation for 15 min. The reaction was terminated by adding 20 μl of 4 M sulphuric acid and the absorbance was measured at 492 nm.

High performance thin-layer chromatography immunostaining

HPTLC immunostaining was carried out according to the method of Ariga *et al.* (1988). Glycolipids were chromatographed on HPTLC plate with the developing solvent system of chloroform:methanol:water (60:40:8, v/v). After chromatography, the plate was air-dried and dipped in a solution of 0·2% polyisobutylmetacrylate in *n*-hexane. After air-drying, the plate was overlaid with the antiserum or MoAbs at a dilution of 1:200 or more in 1% BSA/PBS for 1·5 h at room temperature. After washing with PBS, individual sample lanes were covered with anti-human IgG at a dilution of 1:500 in BSA/PBS for 1·5 h and washed as described above. After washing with PBS, the plate was incubated for a few minutes with a solution of 4-chloronaphthol (15 mg/25 ml) containing 0·02% hydrogen peroxide.

RESULTS

Figure 1 shows the ability of sera of patients with Graves' and Hashimoto's disease to recognize Forssman glycolipid (Gb5) in the HPTLC-immunostaining assay. In this figure, the patients' sera did not react with glucosyl ceramide (GlcCer), galactosyl ceramide (GalCer), lactosyl ceramide (LacCer), globotriaosyl ceramide (Gb3), globotetraosyl ceramide (Gb4), asialo-gangliotriosyl ceramide (GA2), asialo-gangliotetraosyl ceramide (GA1) and gangliotetraosyl ceramide (GM1). Nor did the patients' sera bind to neolactosyl ceramide (nLc4), sialylparagloboside

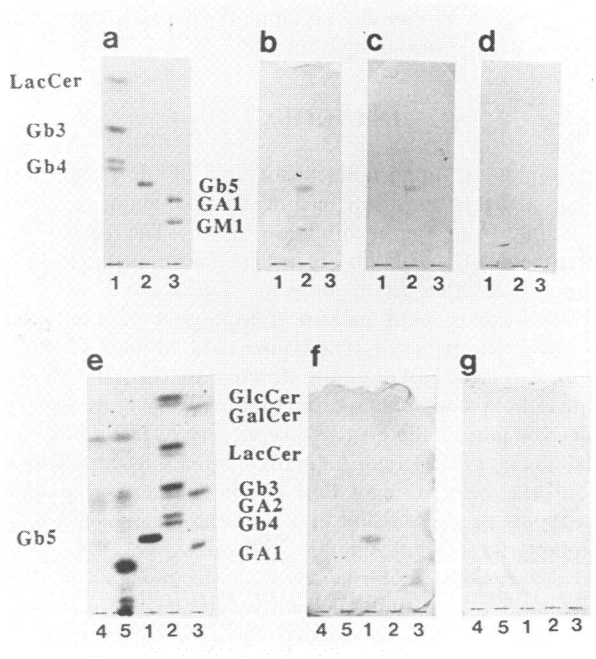

Fig. 1. HPTLC immunostaining of neutral glycolipids and gangliosides. Plates a and e were visualized by spraying with the orcinol-sulphuric acid reagent. Plates b and c were immunostained with sera of patients with Graves' disease and Hashimoto's thyroiditis, respectively. Plates d and g were immunostained with the sera of normal controls. Plate f was immunostained with sera of a patient with Graves' disease. The plates were developed with chloroform:methanol:water (60:40:8, v/v). Lanes 1, 2 and 3 contained the glycolipid standards (0·3 μg) as indicated in plates a and e. Lanes 4 and 5 were the neutral glycolipid fractions from thyroid membranes and lymphocytes of patients with Graves' disease, respectively.

(LM1), hematoside (GM3) or other ganglio-series gangliosides (data not shown), or other neutral glycolipids (Fig. 1e, lanes 4 and 5) or acidic lipids (data not shown) isolated from Graves' thyroid membranes and lymphocytes. The control sera did not bind to any of the glycolipids tested. Figure 2 and Table 1 show the binding activities of patients' sera to various glycolipids as estimated by ELISA. Sera from patients with Graves' disease and Hashimoto's thyroiditis had significantly higher titres for Gb5 as compared to normal controls. There were no significant differences in titres for binding activities between Graves' disease and Hashimoto's thyroiditis; both binding activities were specific to Gb5 (Fig. 2). The patient's sera did not react with LacCer, Gb3, Gb4, GA1, GM1, and GD1a (Table 1).

Four MoAbs, TRMo-1, TRMo-2, TRMo-3 and TRMo-4, were prepared from clones obtained by fusion of lymphocytes from Graves' disease with human myeloid cells (LICR-LON-HMY2 cells) (see Materials and Methods). All these MoAbs contained only the IgG class of immunoglobulin and show heterogeneous bioactivities as described by Yoshida *et al.* (1988). Figures 3 and 4 show the reactivities of the four MoAbs with various authentic glycolipids. The MoAb TRMo-4 reacted strongly with Gb5, but not with other neutral glycolipids such as LacCer, Gb3, Gb4, nLc4, GA2 and GA1 (Figs 3e and 3g). This MoAb also did not react with brain gangliosides and gangliosides from thyroid membranes (Fig. 3g, lanes 6 to 9). The reactivities of other MoAb toward Gb5 were considerably lower (Fig. 4). Figure 5 shows the titration curves of various glycolipid antigens (50 ng) using different dilutions of MoAb TRMo-4, which is highly specific to Gb5, but not to other glycolipids. Using different amounts of Gb5 (between 10 and 100 ng), a titration curve for the MoAb TRMo-4 was obtained by employing the ELISA technique. As shown in Fig. 6, the integrated curve increased almost linearly up to 100 ng of the pure antigen.

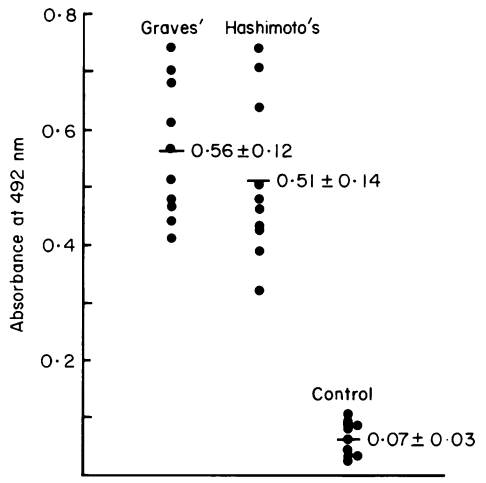

Fig. 2. Activities for anti-Gb5 antibodies in the sera using ELISA. Sera were diluted 1:300 or greater. The absorbance of the developed colour of the peroxidase reaction was determined at 492 nm.

DISCUSSION

Recent studies have focused on the immunological properties of glycolipids and this field has undergone a remarkable development and expansion (Marcus, 1984; Rapport & Huang, 1985). There are several reports implicating glycolipids as autoantigens responsible for the development of several autoimmune diseases (Hakomori, 1981; Marcus, 1990; Yu *et al.*, 1990). Graves' disease and Hashimoto's thyroiditis are autoimmune thyroid diseases and are characterized by increased levels of immunoglobulins and autoantibodies reactive with thyroid tissues in patient's sera; therefore, both humoral and cellular immune mechanisms are thought to be involved in the pathogenesis of these disorders (Volpe, 1984).

It is well known that human sera contain naturally occurring antibodies against a variety of carbohydrate determinants (Yasuda *et al.*, 1982; Kaise *et al.*, 1985; Marcus, 1990). Therefore, it is important to define carefully the normal range of antibodies against a particular carbohydrate determinant (Marcus, 1990). In the present study, we have demonstrated positive binding of antibody to Forssman antigen (Gb5) with a dilution of the patients' sera at 1:300 or greater. This finding suggests that anti-Forssman glycolipid antibodies in the sera are of relatively high titres in Graves' disease. No significant differences in binding of the antibody to Forssman glycolipid were found between Graves' disease and Hashimoto's thyroiditis. Although humans have generally been considered to be a Forssman-negative species, this glycolipid has nevertheless been reported to be present in the gastric and colonic epithelia of about 20% of the Chinese people and has also been detected in gastric and colonic carcinomas in most individuals who lack Forssman glycolipid in their normal mucosa (Hakomori, Wang & Young, 1977; Kitamura *et al.*, 1979; Kijimoto-Ochiai, Takahashi & Makita, 1981; Mori *et al.*, 1982). Yasuda *et al.* (1982) reported that human sera contained anti-Forssman, GA2, GA1 and GM1 antibodies, but no correlation between ABO blood group type and anti-glycolipid antibody titre was found. Anti-Forssman glycolipid antibodies were also found in sera of patients with juvenile rheumatoid arthritis (Moore & Dorner, 1980), Kato, Kubo & Naiki (1978) reported the presence of anti-Gb4 and/or Gb3 antibody activities in sera of

Table 1. Activities for anti-glycolipid antibodies in the sera using ELISA (antibody titres (absorbance) at 492 nm; mean ± s.d.)

	GD1a	GM1	GA1	LacCer	Gb3	Gb4
Graves' disease (10)	0·06±0·02	0·07±0·02	0·10±0·03	0·06±0·03	0·06±0·01	0·07±0·00
Hashimoto's thyroiditis (10)	0·05±0·02	0·06±0·01	0·10±0·02	0·07±0·03	0·07±0·00	0·08±0·01
Normal control (10)	0·05±0·03	0·05±0·01	0·10±0·01	0·08±0·02	0·07±0·00	0·07±0·01

The number in parentheses indicates the number of experiments.

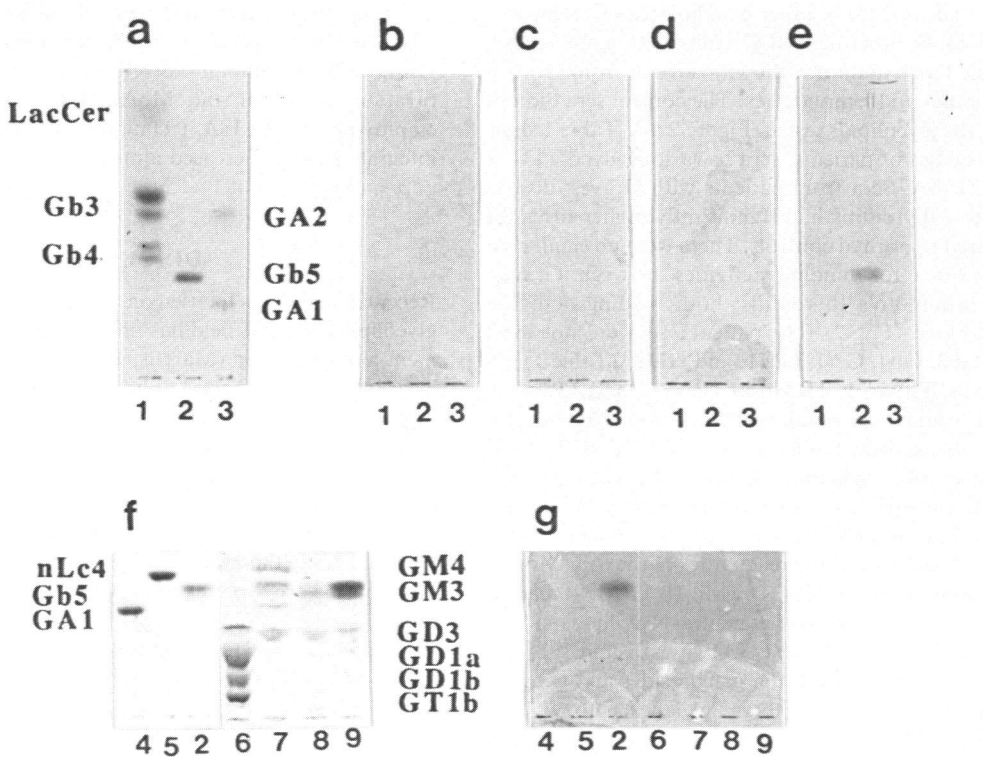

Fig. 3. HPTLC immunostaining of neutral glycolipids and gangliosides using human monoclonal antibodies against thyrotropin (TSH) receptor. Plates a and f were visualized by staining with the orcinol-sulphuric acid reagent. Plates b–e were immunostained with the MoAbs, TRMo-1, TRMo-2, TRMo-3 and TRMo-4, respectively. The plate g was immunostained with MoAb TRMo-4. Plates a–e were developed with chloroform:methanol:water (60:40:8, v/v). The plates f and g were developed with chloroform:methanol:0·2% $CaCl_2 \cdot 2H_2O$ (55:45:10, v/v). Lanes 1, 2, 3 and 7 contained the glycolipid standards (0·3 μg) as indicated in plates a and f. Lane 6 was ganglioside mixtures from human grey matter. Lanes 8 and 9 were acidic lipid fractions of thyroid membranes from Graves' disease.

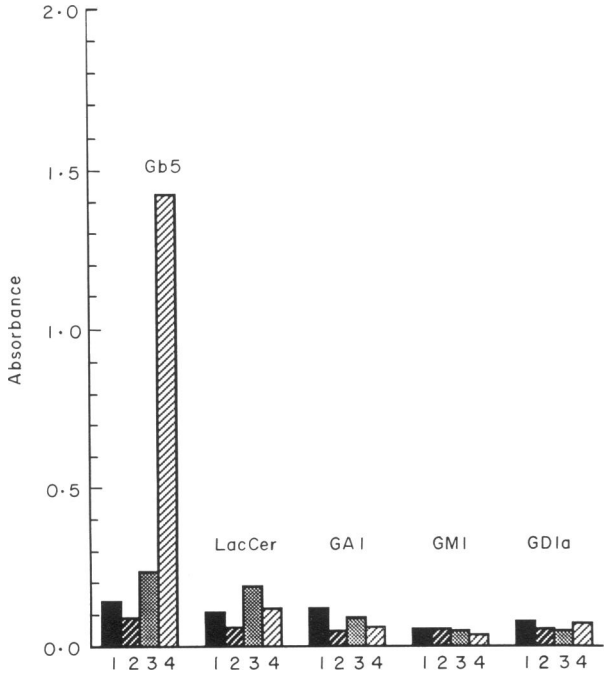

Fig. 4. Specificities for human MoAbs and activities for anti-glycolipid antibodies as measured by ELISA. Lanes 1 to 4 are activities for the MoAbs TRMo-1, TRMo-2, TRMo-3 and TRMo-4, respectively. The absorbance of the developed colour was determined at 492 nm.

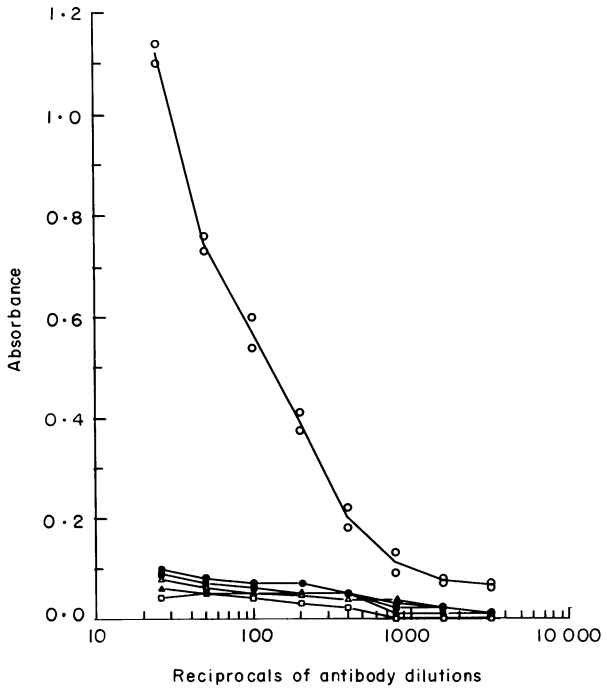

Fig. 5. Affinity of the MoAb TRMo-4 for neutral glycolipids and gangliosides. ●, LacCer; ▲, Gb4; ○, Gb5; ■, GA1; □, GM1; △, GD1a. The absorbance of the developed colour was determined at 492 nm.

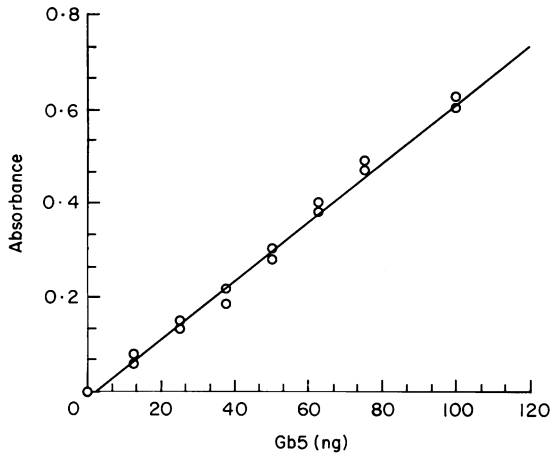

Fig. 6. Calibration curve for quantitative determination of Forssman glycolipid (Gb5) using the monoclonal antibody TRMo-4. The absorbance of the developed colour was determined at 492 nm.

individuals with blood group p and P^K phenotypes. Yasuda *et al.* (1982) found higher titre antibodies against Forssman glycolipid, GM3 (NeuGc) and LM1 (NeuGc) in patients with leukaemia and lymphomas. However, it is not clear whether or not the anti-Forssman glycolipid antibodies are pathogenic, although the possibility exists that the presence of autoantibodies could be responsible for the autoimmune phenomena occurring in Graves' disease and similar disorders. McGinnis *et al.* (1988) reported that antibodies to SGGLs in SLE and rheumatoid arthritis appeared to occur in low titres. Although the titres for anti-SGGL antibodies were increased in peripheral neuropathies associated with SLE, we could not detect anti-Forssman antibodies in sera of patients with SLE and similar disorders (Ariga *et al.*, unpublished data).

Abnormal ganglioside patterns of thyroid membranes have been reported in Graves' disease (Lee *et al.*, 1977; Sawada *et al.*, 1982). Moreover, recent studies have suggested that immunoglobulins in sera of patients with Graves' disease can serve as the effectors for the thyroid overactivity and that this action is mediated by the thyrotropin (TSH) receptor (McKenzie *et al.*, 1978; Valente *et al.*, 1982; Volpe, 1984). Several studies by Kohn and co-investigators (Kohn *et al.*, 1980; Yavin *et al.*, 1981; Valente *et al.*, 1982; Lacetti *et al.*, 1985) have demonstrated that TSH receptor is composed of a glycoprotein with a ganglioside component and that the MoAbs against TSH receptor interact more potently with human thyroid gangliosides. They further suggest that gangliosides may be important in the normal function of the TSH receptor, perhaps in a modulatory role. Such a modulatory role for gangliosides has been shown for a number of growth hormone receptors (Hakomori, 1990). In the present study, we prepared four human MoAbs which reacted with TSH receptor (Yoshida *et al.*, 1988), and interestingly, one of the MoAbs, TRMo-4, interacted specifically with Forssman glycolipid. This finding is consistent with our observation that sera from patients with Graves' and Hashimoto's diseases contain autoantibodies against Forssman glycolipid. The MoAb, TRMo-4, may therefore be useful for the detection of small amounts of Forssman glycolipid antigen in various tissues or cells (Fig. 6).

The origin of the anti-Forssman antibody in Graves' disease is unknown. However, it is known that Forssman glycolipid antigen is highly immunogenic (Uemura, Yuzawa & Taketomi, 1979). Antibodies against this glycolipid have been found in sera of patients with Guillain–Barré syndrome (Koski, Chou & Jungalwala, 1988), lymphomas or leukaemias (Milgrom *et al.*, 1975), and rheumatoid arthritis (Moore & Dorner, 1980). Lacetti *et al.* (1985) reported that one of the human MoAbs against TSH receptor reacted with a minor unknown ganglioside from the disialoganglioside fraction from the thyroid membranes of patients with Graves' disease. Unfortunately, we could not detect Forssman glycolipid antigen in thyroid membranes and lymphocytes in patients with Graves' disease using the HPTLC immunostaining technique (Figs 1 and 3). It is possible that the Forssman antigen may be present in such a low amount that it is even undetectable using the highly sensitive HPTLC immunostaining method. Another plausible explanation is that the immunogenic carbohydrate determinant of Forssman antigen may be masked or present on other glycoconjugates in patients' lymphocytes and/or thyroid membranes (Yoshida *et al.*, 1988). Further studies are needed to clarify the role of this antigenic determinant in various autoimmune disorders. Nevertheless, the detection of anti-Forssman antibody should be useful in developing new avenues of research in clinical diagnosis and therapy.

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