An investigation of the clonality of human autoimmune thyroglobulin antibodies and their light chains

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SUMMARY

Thyroglobulin antibodies in the sera from 31 patients with a variety of disorders were studied by isoelectric focusing. Only one gave a spectrotype indicative of a monoclonal response, the other 30 giving spectrotypes characteristic of polyclonal responses. There was evidence of clonal dominance in some of the sera and each gave a different spectrotype. Light chains were prepared from five thyroglobulin antibodies purified by affinity chromatography. There was no restriction in the spectrotypes when compared with light chains prepared from normal immunoglobulin.

INTRODUCTION

Human autoantibodies to thyroglobulin (Tg) are commonly found in patients with Hashimoto’s thyroiditis and in a variety of other disorders. In contrast to TgAb raised in another species, the homologous antibodies are directed against a limited number (Roitt, Campbell & Doniach, 1958; Shulman & Witebsky, 1960) of shared determinants (Nye, Pontes de Carvalho & Roitt, 1980). These autoantibodies have been demonstrated in IgG, IgA and IgM classes (Fahey & Goodman, 1964) but IgM always contributes less than 1% of the total (Torrigiani & Roitt, 1963). The proportion of the activity in each of the IgG subclasses closely parallels the amounts of each subclass in normal serum (Hay & Torrigiani, 1973). However, antibodies secreted by a single clone of B cells will have the same idiotypic specificity independently of any change in the immunoglobulin class or subclass. Thus, similar idiotypes have been demonstrated for membrane-bound IgD and IgM on the same human B lymphocytes (Salsano et al., 1974) and for monoclonal IgG, IgA and IgM anti-IgG autoantibodies isolated from the serum of a patient with Felty’s syndrome (Abraham, Welch & Trieshmann, 1978). Furthermore, complete sharing of L chain spectrotypes has been demonstrated for murine IgG and IgM anti-streptococcal antibodies in the same animal (Perlmutter, Briles & Davie, 1977).

It is important for our understanding of the origin of autoimmune diseases and perhaps even for their treatment, to establish the number of immunoglobulin (Ig) variable region genes recruited in the autoantibody response. Similarities in Ig hypervariable region genes, as expressed in cross-idiotypic specificities for monoclonal cold agglutinins (Williams, Kunkel & Capra, 1968), monoclonal antigamma globulin antibodies (Kunkel et al., 1973) and rhesus antibodies (Ferre, Natvig & Michaelsen, 1977) have been detected in sera from different patients. In the murine system many L chain spectrotypes of anti-streptococcal antibodies were shared between different mice of the same strain (Perlmutter et al., 1977).

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In an isoelectric focusing (IEF) study Trieshmann, Abraham & Santucci (1975) demonstrated anti-IgG autoantibodies of restricted heterogeneity. Thirteen purified antibodies all contained a major peak with an acid isoelectric point (pI). Immunoelectrophoretic studies with human autoimmune TgAb, purified by affinity chromatography (Hearn et al., 1976), suggested some restriction in heterogeneity when compared with the heterologous guinea-pig anti-human Tg. The aim of this study was to investigate the degree of restriction in the expression of genes coding for human autoimmune TgAb by IEF and to compare the IEF patterns (spectotypes) for TgAb in the sera from different patients. Light chains prepared from affinity chromatography-purified TgAb were also investigated, in order to exclude the variability associated with isotypy and allotypy of the heavy chains.

**MATERIALS AND METHODS**

**Sera.** TgAb titres were measured by haemagglutination of Tg-coated sheep red blood cells (Roitt & Doniach, 1969) in serum samples sent to this laboratory for routine analysis. Thirty-one sera (29 female, two male) with titres greater than 1:5,000 were investigated; 14 were from patients with Hashimoto's thyroiditis and 17 from patients with a variety of other disorders including thyrotoxicosis, pernicious anaemia, rheumatoid arthritis, myasthenia gravis and diabetes mellitus with or without an associated thyroid disorder. Normal human serum with no detectable TgAb activity was obtained from healthy individuals.

**Rabbit anti-human Tg.** Rabbits were immunized with multisite intramuscular injections of a total of 500 µg Tg in Freund's complete adjuvant, followed by five booster injections over a period of 10 weeks. The first booster injection consisted of 500 µg Tg in Freund's complete adjuvant, the other four of 300 µg alum-precipitated Tg. Booster injections 2 and 3 were given subcutaneously and 4 and 5 intravenously. The rabbits were bled at 2-weekly intervals after each booster injection prior to the next injection.

**Human thyroglobulin.** This was prepared by ammonium sulphate precipitation (Derrien, Michel & Roche, 1948) using four thyroid glands from patients undergoing thyroidectomy; one patient had a cyst, two non-toxic goitre and one a toxic nodular goitre. Further purification was achieved by Sepharose 6B gel filtration to remove any remaining immunoglobulin, using 0·15 M phosphate-buffered saline, pH 7·2 (PBS), as the eluant. Purified Tg was aliquoted and stored at −20°C.

**Iodinations.** Ig fractions were partially purified by ammonium sulphate precipitation prior to iodination: 20 µl serum was incubated with 1 ml 40%, ammonium sulphate for 1 hr at 4°C, mixing by vertical rotation. After centrifugation at 1,000 g for 10 min, the supernatant fluid was aspirated and the precipitate dissolved in 20 µl PBS and iodinated in the same tube. Ig and Tg were iodinated by the chloramine T technique of Hunter & Greenwood (1962) to specific activities of 1 and 10 µCi/µg (0·1 and 3 atoms I/molecule) respectively. Table 1 which compares TgAb titres before and after iodination indicates that a substantial amount of activity remained. Small losses could easily be caused by non-specific adsorption onto columns and glassware during the purification procedure.

<table>
<thead>
<tr>
<th>Table 1. TgAb haemagglutination titres of immunoglobulin fractions before and after iodination</th>
</tr>
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<tbody>
<tr>
<td><strong>Patient</strong></td>
</tr>
<tr>
<td>Co</td>
</tr>
<tr>
<td>Jo</td>
</tr>
<tr>
<td>Mo</td>
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<tr>
<td>Nu</td>
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<tr>
<td>Le</td>
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Preparation of immunosorbents. Sepharose 4B (Pharmacia AB, Uppsala, Sweden) was activated by the method of Axén, Porath & Ernback (1967). Human Tg, Cohn fraction II (Koch–Light Laboratories, Colnbrook, Buckinghamshire, England) or human Ig prepared by ammonium sulphate precipitation were linked to the activated material by incubation at 4°C for 48 hr with vertical rotation. The immunosorbents were washed on a glass sinter with 0.15 M PBS until there was no protein in the eluate.

Purification of anti-thyroglobulin antibodies by affinity chromatography. Anti-thyroglobulin antibodies were purified from five different 125I-labelled human immunoglobulins. The iodinated Ig (approximately 500 μCi) in a volume of 2.5 ml 0.05 M phosphate buffer containing 0.1% bovine serum albumin (BSA) was passed down a 1 ml Sepharose 4B column to remove protein which bound non-specifically to the adsorbent and then recycled five times through 1 ml Sepharose 4B–Tg (2.7 mg Tg) to adsorb the specific TgAb. The supernatant, which contained no TgAb activity, was discarded and the Sepharose was washed with three 10-ml aliquots of modified PBS I (containing 0.1% Tween 20 and 0.1% BSA). The third aliquot of wash solution contained less than 1% of the total radioactivity. Specific TgAb was eluted with 4 ml 6 M guanidine HCl: 0.3 M tris HCl, pH 8.0. The approximate amounts of specific TgAb were between 10 and 41% of the total radioactive Ig. Small amounts of radioactivity (0.01–0.1% of the total) remained on the immunosorbent after elution. Thus over 95% of the specific TgAb was always eluted. When normal 125I-Ig was subjected to this procedure approximately 0.15% was eluted with guanidine HCl.

Preparation of light (L) chains from specific TgAb. Fractions eluted from the immunosorbents were pooled and 40 mg normal human Ig Cohn fraction II (Sigma) in a volume of 4 ml was added to each as a non-radioactive carrier. L chains were prepared by the method of Merz et al. (1975). The solution was reduced with 40 mg dithiothreitol (Miles Laboratories, Kankakee, Illinois, USA) for 2 hr at room temperature, alkylated with 80 mg iodoacetamide (BDH Chemicals Ltd, Poole, England) and incubated at room temperature, in the dark, for 18 hr. L chains were purified by gel filtration on a 90 × 1 cm column of Sephadex G-100 (Pharmacia, Uppsala, Sweden) using 5 M guanidine HCl, pH 5–5, as the eluant.

Fig. 1 shows a typical elution pattern: the first peak contained undegraded Ig and heavy (H) chains; the second peak contained L chains. Fractions 14 and 15 were pooled, concentrated approximately five times and dialysed by equilibrium dialysis against 8 M urea. The purified L chains were stored at −20°C prior to IEF.

L chains were also prepared from the whole Ig fraction of a serum sample containing TgAb, and a normal serum. As an additional control 125I-Co immunoglobulin was adsorbed on a column of Sepharose 4B–Cohn fraction II Ig containing 12 mg of protein prior to affinity chromatography in order to remove any anti-immunoglobulin activity before purification of the TgAb, so ensuring that any anti-IgG activity was largely removed.

Isoelectric focusing. IEF was performed using a flat-bed gel system (Nye & Roitt, 1980).

IEF of whole serum. The cooling system was set at −4°C and samples applied at the anode. A

Fig. 1. The separation of 125I-labelled H and L chains by Sephadex G-100 gel filtration, showing optical density at 240 nm (o—o) and counts per 10 sec × 10−3 (●—●).
constant power supply was set at 15 W and a maximum of 20 mA, while the voltage increased to 1,200. Approximately 45 min after sample application the voltage had reached 1,200 and the gel was focused for a further 60 min. The pH was then measured with a micro pH electrode (Pye Unicam/Ingold) and the gel incubated in 18% sodium sulphate for 10 min at room temperature. This solution was aspirated as completely as possible and the immunoglobulins ‘fixed’ by incubating the gel overnight at room temperature in 5–10 ml sheep anti-human Ig or sheep anti-rabbit Ig according to the species being focused. The antiserum was aspirated and the gel washed with three changes of PBS over a 24-hr period. Protein bands with anti-Tg activity were located by reaction with 125I-Tg. The gel was incubated with approximately 10 μg 125I-Tg (100 μCi) dissolved in 20 ml of modified PBS II (containing 0.05%, Tween 20, 0.02% sodium azide with an additional 6% sodium chloride) for 6 hr at room temperature. Unbound 125I-Tg was removed by washing with three changes of modified PBS II over a 24-hr period. Exposure to X-ray film for 1–3 days in an X-ray cassette revealed protein bands with anti-Tg activity.

**IEF of light chains.** The cooling system was set at 8°C and samples applied at the anode. The constant power supply was set at 1 W and a maximum of 10 mA while the voltage increased to 200. The gel was focused overnight, protein was fixed in 5% trichloroacetic acid, 5% sulphosalycilic acid and 30% methanol for 2 hr and allowed to swell in 5% glacial acetic acid prior to exposure to X-ray film for 3 days.

**Tests for anti-immunoglobulin (rheumatoid factor).** The Rheumaco-Wellcotest (Wellcome Reagents Ltd, Wellcome Research Laboratories, Beckenham, Kent) and the RAHA test (Fujizoki Pharmaceutical Company Ltd, Tokyo, Japan) were used for the classical haemagglutination reactions.

The ability of aggregated human Cohn fraction II Ig to inhibit the binding of 125I-Mo to Sepharose 4B–Tg was tested. The affinity chromatography procedure was carried out as described previously except that 500 μg unlabelled Mo was employed plus trace amounts of 125I-Mo (giving approximately 250,000 counts per 10 sec) for each sample, in 1·0 ml 0·05 M phosphate buffer containing 0·1% BSA. These samples contained either no additional protein, 20 mg/ml Cohn fraction II human Ig (aggregated by heating at 60°C for 10 min), 20 mg/ml horse ferritin (an unrelated protein) or 40 mg/ml Tg. A normal 125I-Ig was subjected to the same procedure to give an estimate of the non-specific binding.

**RESULTS**

**IEF of whole serum**

Only one serum (Mn) out of 31 gave a spectrotype characteristic of a monoclonal response. Serum Mn was from a patient with rheumatoid arthritis and no clinical or biochemical evidence of thyroid disease. The other 30 sera gave spectrotypes characteristic of a typical polyclonal antibody response (Fig. 2), the numbers of bands being similar to that observed with rabbit anti-human Tg (Fig. 3). There is evidence of clonal dominance in some of the sera, for example in Ru, which was from a patient with a TgAb-secreting thyroid tumour (kindly sent to us by Professor Robert Goudie); one or both of the dominant groups of bands could be due to antibody of restricted clonality secreted by the thyroid tumour. There were some similarities between individual groups of bands in different samples, for example at pH 6·8 in the higher titre sera, but the overall spectrotypes were always very different.

**IEF of purified light chains**

The spectrotypes of L chains from five different TgAb populations prepared by affinity chromatography from five different serum samples revealed considerable heterogeneity (Fig. 4). The band patterns were similar but differed in the relative abundance of the different spectrotypes. Purified TgAb L chains included all the spectrotypes found in a normal iodinated L chain population although there were quantitative differences in the intensity of some of the bands; TgAb contained more alkaline and less acid species than the L chain from total Ig. It is of interest that L chains from patient Jo contain a dominant band at pH 6·7 (Fig. 4) and the spectrotype of the whole
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serum sample shows a dominant clone between pH 7.7 and 8.1 (Fig. 2). The other four sera did not show particularly dominant clones of either whole Ig or L chain. When $^{125}$I-Ig from a normal serum sample was subjected to the affinity chromatography procedure used to purify TgAb, approximately 0-15% was eluted from the column, an amount which was too low to be detected by the X-ray film (C2, Fig. 4).

Despite the selection of sera with very high TgAb titre, it was possible that some of the L chain spectrotypes could be contaminated with anti-Ig which had bound to TgAb on the immunosorbent, since antiglobulins are present in low amounts even in normal sera (Torrigiani & Roitt, 1967). The observed spectrotypes would then represent a mixture of anti-Ig and anti-Tg L chains. All of the sera were subsequently tested for rheumatoid factor and gave negative results by the Rheumaco-
Fig. 3. IEF spectrotypes of rabbit anti-human TgAb in five serum samples taken at 2-weekly intervals from the same rabbit. Normal rabbit serum (NRS) did not bind $^{125}$I-Tg.

Fig. 4. IEF spectrotypes of $^{125}$I-labelled L chains from five different purified TgAb L chain preparations (Co, Jo, Mo, Nu and Le) and four control preparations (C1–C4). C1 and C3 represent L chain preparations from the whole Ig fraction of a normal serum and a TgAb-containing serum respectively. C4 represents L chains from Co after adsorption with Sepharose 4B-Cohn fraction II Ig prior to affinity chromatography. C2 shows that small amounts of radioactivity obtained when a normal serum sample was subjected to the affinity chromatography procedure were too low to be detected by the X-ray film.
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Table 2. The effect of aggregated Cohn fraction II human immunoglobulin on the binding of $^{125}$I-Mo to Sepharose 4B-Tg

<table>
<thead>
<tr>
<th>Sample applied to columns</th>
<th>$^{125}$I-Mo</th>
<th>$^{125}$I-Mo</th>
<th>$^{125}$I-Mo</th>
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</thead>
<tbody>
<tr>
<td>+ 10 mg aggregated human Ig</td>
<td>$^{125}$I-Mo*</td>
<td>$^{125}$I-Mo*</td>
<td>$^{125}$I-Mo*</td>
</tr>
<tr>
<td>+ 10 mg horse ferritin</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>+ 20 mg human Tg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ $^{125}$I-LN†</td>
<td></td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>1st eluate (pH 7.2)</th>
<th>68‡</th>
<th>65</th>
<th>68</th>
<th>81</th>
<th>68</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 ml wash (PBS II)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st</td>
<td>9.4</td>
<td>9.1</td>
<td>8.6</td>
<td>8.7</td>
<td>13.8</td>
</tr>
<tr>
<td>2nd</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.05</td>
<td>0.24</td>
</tr>
<tr>
<td>3rd</td>
<td>0.17</td>
<td>0.14</td>
<td>0.18</td>
<td>0.06</td>
<td>0.08</td>
</tr>
<tr>
<td>Elution with</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>6 M guanidine HCl (pH 5.5)</td>
<td>7.9</td>
<td>7.5</td>
<td>7.6</td>
<td>0.13</td>
<td>0.19</td>
</tr>
</tbody>
</table>

* 500 µg in 0.5 ml.
† 500 µg normal immunoglobulin in 0.5 ml.
‡ Percentage of total radioactivity applied to each column.

Wellcotest and the RAHA test. Adsorption of one of the serum Ig preparations, $^{125}$I-Co, with Sepharose 4B–Cohn fraction II Ig prior to affinity chromatography did not change the observed spectrotypes (C4 and Co, Fig. 4). Furthermore, aggregated Cohn fraction II Ig did not inhibit the binding of $^{125}$I-Mo to Sepharose 4B–Tg. Table 2 shows that in the presence of modified PBS I, 7.9% of $^{125}$I-Mo bound specifically to the immunosorbent and was eluted with guanidine HCl. In the presence of aggregated human Ig this figure was reduced by only 0.4–7.5% and an unrelated protein, horse ferritin, had a similar effect. The binding of $^{125}$I-TgAb was specifically inhibited by unlabelled Tg such that only 0.13% was eluted from the column, a value similar to that obtained for the normal $^{125}$I-Ig (LN).

DISCUSSION

In 30 out of 31 human sera, the autoimmune TgAb response was polyclonal, and the antibody spectrotypes appeared to be as complex as those seen in rabbit anti-human Tg sera. Each of the serial rabbit samples gave a similar spectrotype showing that, once established, the same B cell clones produced TgAb throughout the immunization period. However, the obvious differences in the overall spectrotypes of TgAb from different human subjects indicate that many different B cell clones are involved in TgAb production in different patients.

Theoretically, antibody of monoclonal origin consists of a single molecular species with a particular pI value. However, deamidation and decarboxylation take place in biological fluids to produce degradation products and the IEF spectrototype of a monoclonal antibody will consist of one or two major bands, and three or four fainter bands of degradation products (Williamson, Salaman & Kreth, 1973). Such a spectrotype was observed in only one patient out of the 31 investigated. This patient had rheumatoid arthritis but no overt thyroid disease and it is possible that, in this unusual case, the TgAb is the product of a benign monoclonal gammopathy.

The presence of idiotypic restrictions could have been obscured by isotypic variations (due to class or subclass). In order to limit this effect we investigated the spectrotypes of L chains prepared from affinity chromatography-purified TgAb where just $\kappa$ and $\lambda$ isotypes need be considered; of these, only the $\lambda$ constant region has variants and these can be ignored for our purposes since they
do not involve charge differences. The spectrotypes of human L chains prepared from whole immunoglobulin fractions of normal serum contained surprisingly few bands. Similar patterns were observed by two other groups (Merz et al., 1975; Gibson, 1977). Assuming that the L chains had focused in the pH gradient according to their individual pI values, the only explanation for this result is that a large proportion of the amino acid changes, or combinations of amino acid changes, do not affect the overall charge of the L chains. There was no restriction in the TgAb L chain spectrotypes when compared with normal immunoglobulin L chains, although the former contained more of the alkaline and less of the acid species. In addition, preliminary investigations (Nye & Roitt, unpublished observations) showed that TgAb idiotypes were not shared between different individuals, since there was no cross-reaction between anti-idiotype antibodies raised against TgAb in the sera from different patients.

In contrast to the polyclonal nature of the TgAb response, the results of Trievesmann et al. (1975) showed a restriction in the expression of genes coding for autoimmune anti-IgG. Furthermore, in animal models it has been shown that the clonality of an immune response depends to a certain extent on the structure of the antigen. Limitation in the complexity of the antigen (Eichmann et al., 1970) and the carrier in an anti-hapten response (Richards et al., 1969) reduces antibody heterogeneity although this restriction is usually transient and heterogeneity tends to increase with prolonged stimulation (Montgomery & Pincus, 1973). An antibody response of restricted heterogeneity, which persisted for up to 2½ years, was achieved with the immunogen (DNP)2-gramicidin S, a compound which possesses two DNP groupings in an identical conformational environment, on a rigid stable decapeptide (Montgomery et al., 1975).

One could postulate that the autoimmune determinants on IgG are less complex or perhaps more rigid than those of Tg, even though human autoimmune TgAb are directed against not more than four (Roitt et al., 1958) or six (Shulman & Witebsky, 1960) epitopes, the majority of which are shared by different patients (Nye et al., 1980). Alternatively, IgG which circulates in far higher concentrations than Tg, may induce tolerance more effectively. Thus only a limited number of clones may have escaped clonal deletion in early life, or suppression by T cells or anti-idiotypic antibodies in later life.

In his V gene theory of autoimmunity Adams (1978) suggested that the development of autoimmunity was due to the inheritance of a limited number of variable region genes coding for Ig with specificities for autologous proteins. Our results provide no support for this view although they do not exclude the possibility of restriction in the H chain component, since even a limited number of H chain hypervariable regions can give rise to a very large number of antibody species with different pl values, both through coupling with various D and J regions (Sakano et al., 1979) and with class and subclass isotypes, and also through combination with a multiplicity of L chains. However, we have so far failed to demonstrate cross-reacting idiotypes on the TgAb in different patients, while genetic studies have shown that the inheritance of Ig genes and the development of spontaneous autoimmune disease are not linked in NZB mice (Bocchieri et al., 1981). Furthermore, B lymphocytes from normal individuals bind radioactive Tg to their surface receptors (Bankhurst, Torrigiani & Allison, 1973) and experimental animals generally have a lymphocyte repertoire which enables them to respond to autologous Tg in various adjuvants (Rose & Witebsky, 1956; Weigle, 1961; Rose et al., 1981). These observations together with our finding of typically polyclonal autoantibody responses, suggest that autoimmunity is not attributable to the inheritance of restricted V region genes but rather to a malfunction of the control mechanisms which normally suppress the production of autoantibodies by B cells.

It is widely accepted that two such mechanisms, helper and suppressor T cells (Cooke & Lydyard, 1981), and anti-idiotype antibodies (Jerne, 1974; Eichmann, 1978) can regulate immune responses. Since the autoimmune epitopes on the Tg molecule are both limited and shared by different patients, we had originally expected to find some restriction in the antibody clonality and some similarities in the antibody response in different patients, involving a limited number of idiotypes. Neither of these expectations have so far been fulfilled. Under these circumstances, the possibility of controlling the autoimmune response by treatment with anti-idiotypic sera to suppress individual idiotypes would appear to be difficult to achieve. An alternative strategy which aims to generate antigen-specific T-suppressors may provide a more feasible approach (Roitt et al., 1981).
since it is known that suppressor T cells directed against one epitope can inhibit the immune response to other epitopes on the same molecule (Adorini et al., 1979; Taniguchi, Saito & Tada, 1979). Monoclonal anti-idiotypes, preferably site-specific and therefore resembling the antigen itself, could act as ‘surrogate antigens’ to induce antigen-specific T suppressors provided we establish the appropriate form in which to present them to the immune system. Other advantages of monoclonal anti-idiotypes would be that the same preparations should be applicable to all patients with a given autoimmune response and that abundant supplies would be available even in cases where the autoantigen itself would be difficult to isolate in sufficient quantity for immunotherapeutic purposes.

We are grateful to Professor D. Doniach and Dr G. F. Bottazzo for making available to us sera from selected patients with Hashimoto’s thyroiditis. L. C. P. de Carvalho was the recipient of a grant from the Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil.

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