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Thyroid microsomal/thyroid peroxidase autoantibodies show discrete patterns of cross-reactivity to myeloperoxidase, lactoperoxidase and horseradish peroxidase

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SUMMARY

The recent cloning of the thyroid peroxidase (TPO) has shown that it is identical to the thyroid microsomal antigen (TMA), a potent antigen involved in autoimmune thyroid disease (ATD), which shares significant sequence homology with myeloperoxidase. The present study shows that autoantibodies (aAb) to the TMA/TPO antigen cross-react with human leucocyte myeloperoxidase, bovine lactoperoxidase and horseradish peroxidase. Cross-reactivity to myeloperoxidase was only apparent by ELISA using reduced and alkylated antigen preparations or by immunoblotting following denaturation with SDS. Sequential absorption of sera on SDS-denatured thyroid microsomes immobilized on Sepharose-4B followed by absorption on native microsomes removed all aAb specificities to TMA/TPO and the three peroxidase preparations, giving compelling evidence on the genuine cross-reactive nature of these aAbs. Sera from different patients contain different qualitative and quantitative specificities of aAb to the TMA/TPO antigen, confirming the polyclonal nature of this autoimmune response.

INTRODUCTION

Autoantibodies (aAb) to the thyroid microsomal antigen (TMA) are believed to play a pathogenic role in destructive autoimmune thyroid disease (ATD) by their ability to fix complement (Khoury *et al.*, 1981; Weetman & McGregor, 1984). Previous studies from our laboratory characterized the TMA as a 105,000 MW protein by immunoprecipitation and Western blotting analysis (Banga *et al.*, 1985, 1986) and subsequent studies have confirmed this (Hamada *et al.*, 1985, Kajita *et al.*, 1985) Biochemical and immunofluorescence studies on the distribution of TMA indicate that it is located in the exocytotic vesicles engaged in the secretion of thyroglobulin (Tg) into the colloid of the thyroid follicle (Roitt *et al.*, 1964), although it is clearly distinct from membrane bound Tg (Roitt *et al.*, 1964; Banga *et al.*, 1985). The close antigenic similarity between the TMA and thyroid peroxidase (TPO), an enzyme involved in the generation of thyroid hormones form Tg in these exocytotic vesicles, led to the postulation that TMA and TPO were comparable molecules (Czarnocka *et al.*, 1985; Portman *et al.*, 1985). The recent cloning of the human TPO gene gives further support to the close identity of the TMA and the TPO molecules (Kimura *et al.*, 1987; Seto *et al.*, 1987; Libert *et al.*, 1987). These studies have also shown that the extracellular sequence of TPO has significant homology with human myeloperoxidase (MPO) (Libert *et al.*, 1987; Kimura & Ikedo-Saito, 1988), an enzyme involved in the microbicidal activity of neutrophil leucocytes (Klebanoff & Hamon, 1972).

The pathogenicity of the aAb in ATD patients has led to studies on the nature and number of auto-antigenic epitopes on the TMA/TPO molecule (Kohno *et al.*, 1986; Hamada *et al.*, 1987; Doble *et al.*, 1988). Our studies indicate that the aAb to the TMA/TPO are polyclonal and far more heterogeneous than had been previously recognized, with at least six independent determinants discernible (Doble *et al.*, 1988). A recent report indicated that a monoclonal antibody to TPO cross-reacted with bovine lactoperoxidase (LPO) but not with horseradish peroxidase (HRP) (Ruf *et al.*, 1987). In view of the sequence homology of the TPO molecule to MPO, together with this cross-reactivity to LPO, we have examined the cross-reactivity of the aAb to the TMA/TPO to purified MPO, LPO and HRP by a combination of ELISA and immunoblotting techniques. Our results show for the first time that different ATD sera containing TMA/TPO aAb show selective cross-reactivity with

Abbreviations: aAb, autoantibodies, ATD, autoimmune thyroid disease; HRP, horseradish peroxidase; Ig, Immunoglobulin; LPO, lactoperoxidase; MW, molecular weight; MPO, myeloperoxidase, NHS, normal human serum; PBS, phosphate-buffed saline; Tg, thyroglobulin; TMA/TPO, thyroid microsomal antigen/thyroid peroxidase.

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these other peroxidases under different conditions. Furthermore, absorption experiments indicate unequivocally the cross-reactive nature of these aAb rather than a fortuitous presence of other peroxidase-specific aAb in ATD patients.

MATERIALS AND METHODS

Patients sera

Sera from 20 patients with ATD, with highest detectable levels of aAb to the TMA, as assessed by ELISA, were obtained from the thyroid clinic at King's College Hospital and used in this study. The sera were used irrespective of the presence or absence of aAb to Tg or the thyroid status of the patients or the type of ATD.

Thyroid microsomes

These were prepared as described previously (Banga *et al.*, 1985; Doble *et al.*, 1988). Human liver microsomes were a gift from Dr J.M.Tredger, Kings College School of Medicine.

Preparation of LPO, MPO and HRP

LPO from bovine milk and HRP from horseradish were obtained from Sigma Chemical Co. (Poole, Dorset) and used without further purification.

MPO was purified from the blood of patients with chronic myeloid leukaemia essentially as described by Mathieson, Wong & Travis (1981), except that a further purification step by isoelectric focusing in Ultrodex (Pharmacia-LKB, Milton Keynes) was incorporated to obtain fractions which were virtually pure. Two separate preparations of MPO of different purities were utilized in this study.

ELISA assay

This was performed as described elsewhere (Weetman *et al.* 1983), except that optimal protein concentration for thyroid microsomes and MPO was 10 μg/ml whilst for LPO and HRP a concentration of 5 μg/ml was utilized. For assays with reduced antigens, LPO, MPO and HRP were reduced with dithiothreitol and alkylated with iodoacetamide before coating the wells of ELISA plates at concentrations used for native proteins. All assays were performed in triplicate at a serum dilution of 1:100 for thyroid microsomes and at 1:20 for the other peroxidase preparations.

Immunoblotting

SDS–PAGE was performed exactly as described previously (Banga *et al.*, 1985) using a 5–15% polyacrylamide gel under non-reducing or reducing conditions. Routinely, 40–50 μg total thyroid microsome protein or approximately 4–5 μg Tg, LPO, MPO or HRP were electrophoresed. All sera containing aAb were used at a final 1:100 dilution, whilst absorbed sera were used at 1:50 dilution since some dilution of the serum occurred following batch absorptions.

Absorption of sera

This was performed as already described (Banga *et al.*, 1985). For absorption of sera, native proteins or SDS-denatured proteins immobilized on Sepharose-4B (Pharmacia-LKB) were used. Thyroid microsomes or LPO were solubilized in 1% SDS and denatured by placing in boiling water for 30 seconds before coupling to Sepharose-4B. This was achieved by using CNBr-activated Sepharose-4B (Pharmacia-LKB), according to the

Table 1. Cross-reactivity of aAb to TMA/TPO to other peroxidases

		ELISA							Immunoblotting							
		TMA	LPO		MPO		HRP		Non-reducing				Reducing			
Patient	Clinical diagnosis	N	N	R+A	N	R+A	N	R+A	TMA	LPO	MPO	HRP	TMA	LPO	MPO	HRP
1	H	+	+	−	−	+	−	−	+	+	+	+	+	+	+	+
2	H	+	+	−	−	−	−	−	+	+	+	+	−	−	−	−
3	PPT	+	+	−	−	−	−	−	+	+	−	+	+	+	−	+
4	H	+	+	+	−	+	−	−	+	+	+	−	+	+	+	−
5	H	+	+	+	−	+	−	−	+	+	−	+	+	+	−	+
6	H	+	−	−	−	−	−	−	−	−	−	+		ND		
7	M	+	+	−	−	+	−	−	−	−	−	−		ND		
8	G	+	−	−	−	+	−	−	+	−	−	+	+	+	+	+
9	H	+	−	+	−	+	−	−	+	+	−	+	+	+	+	+
10	G	+	−	−	−	−	−	−	+	−	−	+	+	+	+	+
11	G	+	−	−	−	+	−	−	+	+	−	−	−	+	+	+
12	G	+	−	+	−	+	−	−	+	+	−	−	+	+	−	−
13	G	+	−	−	−	+	−	−	+	−	−	+	−	+	+	+
14	H	+	+	−	−	+	−	−	+	−	−	+	−	−	+	+
15	G	+	−	−	−	−	−	−	+	−	−	−	−	−	+	−
16	H	+	−	−	−	+	−	−	+	+	−	−	+	+	−	+
17	H	+	−	−	−	−	−	−	+	−	−	−	−	−	−	+
18	H	+	−	−	−	+	+	+	+	−	−	+	+	+	+	+
19	G	+	−	+	−	+	−	−	−	−	−	−		ND		
20	G	+	+	−	−	+	−	−	−	−	−	−		ND		

H, Hashimoto thyroid disease; G, Graves' disease;, PPT, post-partum thyroid disease; M, primary myxoedema; N, native protein; R + A reduced and alkylated protein; ND, not determined since these sera do not react under non-reducing conditions with TMA.

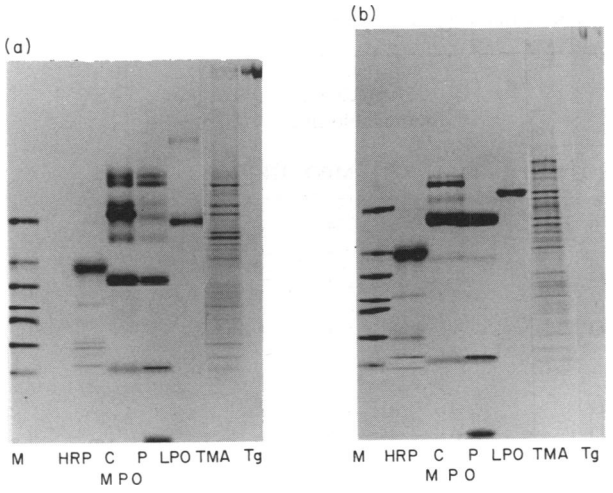

Figure 1. SDS–PAGE analysis of thyroid microsomes and purified LPO, MPO and HRP under non-reducing (a) and reducing conditions (b) and Coomassie blue staining. Two different preparations (lane P and lane C) of MPO were used in this study. Lane M denotes marker proteins for MW which are (from top to bottom) 69,000, 45,000, 36,000, 29,000, 24,000, 20,000 and 14,000.

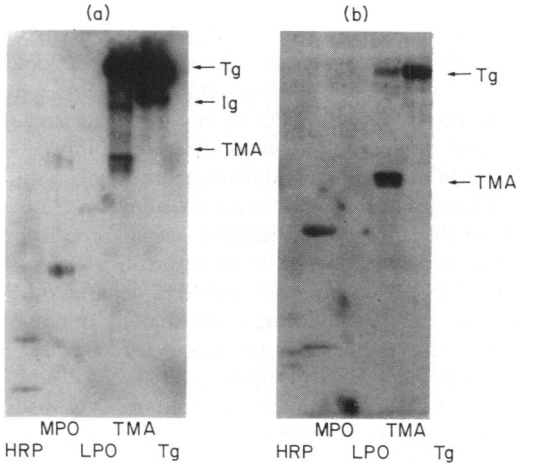

Figure 2. Autoradiograph following SDS-PAGE and immunoblotting with serum number 1 under non-reducing (a) and reducing conditions (b). The arrows indicate co-migratory positions of Tg, Ig and TMA. Binding of the TMA/TPO aAb to TMA, LPO, MPO and HRP components is clearly recognized under both electrophoretic conditions. The MPO preparation used was Prep P.

manufacturers instructions, except that phosphate-buffered saline (PBS) was substituted for the coupling buffer in all reactions.

For absorption on native thyroid microsomes or human liver microsomes, 3 mg microsomes were pelleted by ultracentrifugation at 100,000 *g* for 1 hr at 4°C. This was followed by the addition of 400 μl of either serum number 4 or serum number 18 (Table 1) to the pelleted microsomes, which were then dispersed gently by glass homogenization. The mixture was left on a recipro-shaker for 4 hr at room temperature and thence overnight in the cold room. The next day the microsomal membrane protein in the serum mixture was pelleted by ultracentrifugation and discarded. The absorption step with the serum supernatant was repeated twice to achieve complete absorption.

For absorption on SDS-denatured thyroid microsome–Sepharose-4B (10 mg protein/ml beads), 600 μl serum was added to 0·5 ml packed beads in a 1·5-ml Eppendorf tube and the tube placed on a recipro-shaker for 4 hr at room temperature and thence overnight in the cold room. The next day the Sepharose beads were pelleted by brief centrifugation in a microfuge and the supernatant removed. The Sepharose beads were regenerated by elution of the bound aAb with 50 mM diethylamine, pH 11·5, re-quilibrated in PBS and re-used. The absorption steps were repeated on two further occasions to achieve complete absorption. Sera were absorbed on LPO–Sepharose-4B, SDS-denatured LPO-Sepharose-4B or HRP-Sepharose-4B (all at 10 mg protein/ml beads) as described above. For double absorption, sera were initially absorbed three times with the first immobilized ligand followed by a further three absorptions with the second immobilized ligand.

RESULTS

Binding of TMA/TPO aAb to other peroxidases by ELISA

By ELISA, all 20 ATD sera were positive for thyroid microsome antibodies and since the conditions for ELISA are such that Tg antibodies do not interfere and are not measured in this assay (Weetman *et al.*, 1983), these aAb are likely to be directed at the TMA/TPO molecule (Table 1). The binding of these 20 ATD sera to native or reduced and alkylated preparations of LPO, MPO and HRP was ascertained by ELISA (Table 1). A proportion of the sera were positive [taken as the levels greater than the mean +2 SD of 50 normal human sera (NHS)] for native LPO [8 out of 20 (40%) sera] whilst a fewer number of sera reacted with reduced LPO [5 out of 20 (25%) sera]. Only one of the sera persistently showed reactivity with native or reduced HRP [serum number 18 (5%)]. No cross-reactivity of sera was apparent to native MPO, whilst a large proportion of the sera react with reduced MPO [14 out of 20 (70%) sera] (Table 1). This limited cross-reactivity of anti-TMA/TPO aAb with other peroxidases by ELISA was studied further using immunoblotting analysis.

Cross-reactivity of TMA/TPO aAb to other peroxidases by SDS–PAGE and immunoblotting

Thyroid microsomes from Graves' glands and purified preparations of LPO, MPO and HRP were analysed by SDS–PAGE by electrophoresing under non-reducing or reducing conditions and immunoblotting with ATD sera. For MPO, two different preparations of MPO were utilized. Purified human Tg was also electrophoresed, in order that anti-Tg aAb could also be recognized. The results for protein staining of SDS–PAGE gels are shown in Fig. 1. The Tg and thyroid microsomes show polypeptide bands already described (Banga *et al.*, 1985; Doble *et al.*, 1988). Under non-reducing conditions purified LPO showed two protein bands co-migrating at approximately 67,000 MW and a variable intensity, weaker band at 130,000 MW (Fig. 1a), whilst under reducing conditions a single protein-staining band co-migrating at approximately 80,000 MW was apparent (Fig. 1b). The two preparations of MPO used in this

Table 2. Cross-reactive aAb remaining in serum number 4 following various absorptions

	ELISA*				Reducing immunoblotting			
Absorption step	TMA	LPO	MPO	HRP	TMA	LPO	MPO	HRP
Unabsorbed	+	+	−	−	+	+	+	−
Absorb liver microsomes	+	+	−	−	+	+	+	−
Absorb thyroid microsomes	−	−	ND	ND	−	+	+	−
Absorb SDS-denatured thyroid microsomes	−	−	ND	−	−	+	+	−
Absorb SDS-denatured thyroid microsomes then absorb thyroid microsomes	−	−	ND	−	−	−	−	−
Absorb LPO	+	+	ND	−	+	+	+	−
Absorb SDS-denatured LPO	+	+	ND	−	+	−	−	−
Absorb LPO then SDS-denatured LPO	+	+	−	−	+	−	−	−

ND, not determined.
*Native proteins only.

study show considerable heterogeneity under non-reducing conditions (Fig. 1a, lanes C and P), whilst under reducing conditions pure MPO (lane P) showed two bands at approximately 60,000 MW and 14,000 MW together with a weaker band at 43,000 MW (Fig. 1b), comprising the heavy, light and intermediate polypeptide chains of the MPO enzyme complex (Miyasaki *et al.*, 1986). The crude preparation of MPO (lane C) lacked significant amounts of light chain (Fig. 1a, b); the weaker staining band at this position appears to be distinct from the MPO light chain. The migration of the HRP polypeptides did not change significantly under different conditions of electrophoresis, where a major band at 42,000–45,000 MW was apparent together with four, weakly staining low molecular weight components also recognizable (Fig. 1).

All 20 ATD sera were examined by immunoblotting under non-reducing or reducing conditions to ascertain the cross-reactivity to LPO, MPO and HRP following denaturation with SDS. An example is shown in Fig. 2 of the immunoblotting pattern obtained following staining with an ATD patients' serum (serum number 1, Table 1) that reacts by ELISA with TMA/TPO. The aAb in serum number 1 reacts with a TMA/TPO polypeptide band co-migrating at approximately 117,000 MW under non-reducing conditions (Fig. 2a) and continue to maintain reactivity with the TMA/TPO component co-migrating at 105,000 MW under reducing conditions (Fig. 2b). Although, by ELISA, serum number 1 cross-reacts with native LPO and reduced MPO (Table 1), by immunoblotting analysis reactivity with LPO persists under both non-reducing and reducing conditions (Fig. 2a, b); furthermore, reactivity with MPO and HRP components is also clearly apparent under both electrophoretic conditions (Fig. 2a, b).

Analysis of cross-reactive aAb by ELISA and immunoblotting

By complementing ELISA and non-reducing/reducing SDS–PAGE immunoblotting analysis to ascertain binding of anti-TMA/TPO aAb from 20 ATD patients to LPO, MPO and HRP, our results can be summarized as follows (Table 1).

(i) Cross-reactivity of anti-TMA/TPO aAb to native or reduced LPO can be measured by ELISA with some sera; a number of these LPO-positive sera by ELISA continue to maintain reactivity with LPO by non-reducing and reducing SDS-PAGE immunoblot analysis (serum numbers 4 and 5; Table 1) whilst others show differential reactivity with LPO by non-reducing/reducing analysis (serum numbers 1, 2, 3, 7, 9, 12, 14, 19 and 20; Table 1). Furthermore, a small proportion of sera do not show any significant reactivity with native or reduced LPO by ELISA, but only react with SDS-denatured LPO upon non-reducing and/or reducing conditions by immunoblotting analysis (serum numbers 11, 13, 16 and 18; Table 1).

(ii) Cross reactivity with native MPO could not be demonstrated by ELISA in our panel of 20 ATD sera, although reactivity with reduced MPO or with SDS-denatured MPO is clearly apparent (Table 1).

(iii) Cross reactivity with native or reduced HRP by ELISA could only be consistently demonstrated with one ATD serum (serum number 18). A greater proportion of sera cross-react with HRP following denaturation with SDS, either under non-reducing or reducing conditions (Table 1). Some sera do not show any presence of cross-reacting aAb to HRP by any of the three techniques (serum numbers 4, 12 and 15; Table 1). Furthermore, by immunoblotting analysis, different sera react with different components of HRP. Some ATD sera show cross-reactivity solely with the major 42,000–45,000 MW polypeptide component of HRP, for example (serum number 3, 6 and 14; not shown), whilst some sera react with the lower MW components of HRP only (serum number 1; fig. 2, and serum 13, not shown), whilst some sera show reactivity with all the subunit components of HRP (serum 18, Fig. 4, sera 2 and 5, not shown). Since HRP is composed of a single polypeptide chain (Welinder, 1986), the lower MW components are likely to be degraded products of HRP in the commercial preparation since aAb show reactivity to these polypeptides.

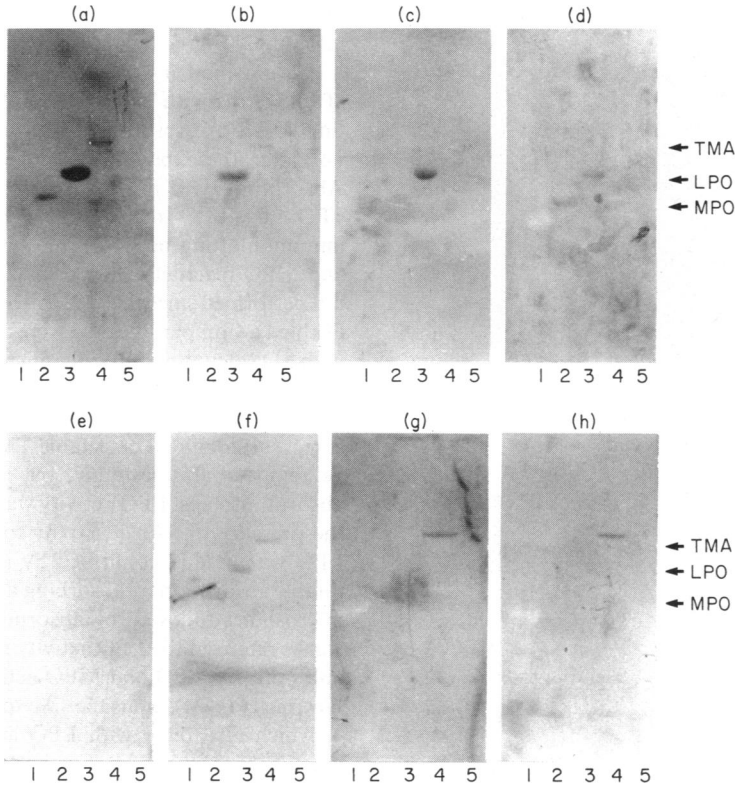

Figure 3. Autoradiographs of immunoblotting following SDS–PAGE under reducing conditions with serum number 4 after various absorption steps outlined in the text. Lanes 1, 2, 3, 4 and 5 refer to HRP, MPO, LPO, thyroid microsomes and Tg, respectively. Unabsorbed serum shows binding to MPO, LPO and TMA (a) which does alter following absorption on liver microsomes (b). Absorption on thyroid microsomes (c) or SDS-denatured thyroid microsomes (d) removes the TMA band, although LPO and MPO specificities are still present. Successive absorptions on SDS-denatured thyroid microsomes followed by absorption on native thyroid microsomes–Sepharose-4B removes the TMA band and all cross-reacting specificities (e). The TMA, LPO and MPO specificities are clearly present following absorption on LPO–Sepharaose-4B (f), whilst absorption on SDS-denatured LPO–Sepharose-4B (g) or a double absorption on LPO–Sepharose-4B followed by SDS-denatured LPO–Sepharose-4B (h) removes the LPO and MPO aAb specificities, but not all of the TMA specificities.

Table 3. Cross-reactive aAb remaining in serum number 18 following various absorptions

	ELISA*				Reduced immunoblotting			
Absorption step	TMA	LPO	MPO	HRP	TMA	LPO	MPO	HRP
Unabsorbed	+	−	−	+	+	+	+	+
Absorb thyroid microsomes	−	ND	ND	−	+	+	+	+
Absorb SDS-denatured thyroid microsomes	+	ND	ND	+	+	−	−	+
Absorb SDS-denatured thyroid microsomes then thyroid microsomes	−	−	ND	−	−	−	−	−
Absorb HRP	+	ND	ND	−	+	+	+	−
Absorb LPO	+	−	ND	−	+	−	−	+

ND not determined.
*Native proteins only.

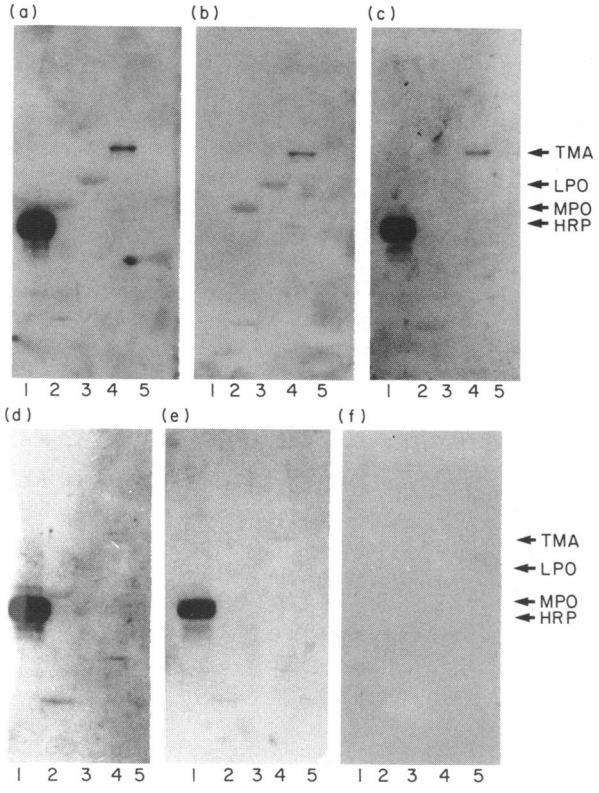

Figure 4. Autoradiographs following SDS–PAGE under reducing conditions and immunoblotting with serum number 18 after various absorption steps outlined in the text. Lanes 1, 2, 3, 4, and 5 refer to HRP, MPO, LPO, thyroid microsomes and Tg, respectively. Unabsorbed serum shows binding to HRP, MPO, LPO and TMA (a) whilst absorption on HRP–Sepharose-4B completely removes the HRP binding specificities, leaving the MPO, LPO and TMA specificities present (b). Absorption on LPO–Sepharose-4B removes the LPO and some of the MPO specificities, although the TMA and HRP bands are visible together with the light chain band of MPO (c). Following absorption on thyroid microsomes, the HRP, MPO, LPO and TMA are visible, although the latter three bands are reproducibly always considerably reduced in intensity (d). Absorption on SDS-denatured thyroid microsomes–Sepharose-4B removes LPO and some of the MPO specificities (e), whilst a double absorption on SDS-denatured thyroid microsomes followed by native thyroid microsomes–Sepharose removes the TMA band and all other cross-reacting specificities (f).

Absorption of ATD sera on native and SDS-denatured peroxidases

To show unequivocally that aAb to TMA/TPO cross-react with other peroxidases, necessitates absorption studies in order to prove that these aAb are not totally different species of aAb to LPO and MPO, or other peroxidases related to HRP in man that are fortuitously present in sera from ATD patients. Serum numbers 4 and 18 were selected for these studies and absorbed separately with native thyroid microsomes, SDS-denatured thyroid microsomes, native LPO, SDS-denatured LPO and HRP immobilized on Sepharose-4B. Absorptions on immobilized MPO could not be performed due to a lack of the large amounts of MPO that would have been required. Following absorption, the presence of remaining aAb in the sera reacting with TMA/TPO or other peroxidases was ascertained by ELISA and immunoblotting following SDS–PAGE to measure aAb to native or SDS-denatured, non-labile epitopes, respectively. The results for serum number 4 and serum number 18 are presented in Table 2, Fig. 3 and Table 3, Fig. 4, respectively.

Serum number 4 reacts with TMA/TPO and LPO by ELISA (Table 2) and with TMA/TPO, LPO and MPO by immunoblotting (Fig. 3a). Absorption with human liver microsomes does not alter these binding specificities (Table 2 and Fig. 3b). Absorption with native thyroid microsomes removes TMA/TPO and LPO reactivity by ELISA (Table 2) whilst by immunoblotting no TMA/TPO band was apparent, but LPO and MPO reactivity still persisted (Fig. 3c). MPO ELISA was not performed since the aAb epitopes on MPO are not accessible (Table 1). Comparable data were obtained following absorption with SDS-denatured thyroid microsomes (Table 2 and Fig. 3d). This shows that aAb to LPO or MPO can not be absorbed on native thyroid microsomes or SDS-denatured thyroid microsomes, although all detectable TMA/TPO activity appeared to be removed. Interestingly, by either of the aforementioned absorption steps, LPO activity was not apparent by ELISA, but the presence of aAb reactivity to SDS non-labile epitopes on LPO was readily confirmed by immunoblotting (Table 2). A double absorption by absorbing first on SDS-denatured thyroid microsomes followed by absorption on native thyroid microsomes removed the LPO activity measured by ELISA (Table 2) and both the LPO and MPO activity ascertained by immunoblotting (Fig. 3e). Thus the aAb to TMA/TPO in serum number 4, which also react with LPO and MPO, are genuine cross-reacting species of aAb. Further confirmation was obtained by absorption on native or SDS-denatured LPO, which showed that TMA/TPO activity could not be removed (Fig. 3f–h). Thus aAb to TMA/TPO can only be removed by successive absorptions on SDS-denatured and native thyroid microsomes, which removes all other cross-reacting aAb to LPO and MPO, whilst absorptions on native and/or SDS-denatured LPO removes only the LPO and MPO cross-reacting aAb without removing the bulk of TMA/TPO aAb. In addition, aAb cross-reacting with LPO and MPO appear to parallel each other on reduced immunoblotting (Table 2 and Fig. 3).

Absorption studies with TMA/TPO aAb from a different patient's serum also confirm the genuine cross-reactive nature of these aAb to other peroxidases, including those aAb cross-reacting with HRP. Serum number 18 is the only ATD patient that contains aAb to HRP that are measurable by ELISA (Table 1). Following immunoblotting, under reducing conditions, aAb reactivity with TMA/TPO, LPO, MPO and HRP is also readily apparent (Table 1 and Fig. 4a). This serum also contains aAbs that react weakly with two other components of the TMA/TPO of approximately 45,000 MW and 28,000 MW (Fig. 4a). Absorption with immobilized HRP removed aAb reactivity with HRP, both by ELISA (Table 3) and immunoblotting (Fig. 4b) whilst absorption with native LPO removes the LPO reactivity by ELISA (Table 3) and both LPO and MPO reactivity by immunoblotting (Fig. 4c). A comparable result was obtained following absorption with SDS-denatured LPO (not shown). Absorption with native thyroid microsomes removed TMA activity by ELISA (Table 3), whilst by immunoblotting weak activity with TMA was still visible and reactivity with LPO and MPO apparent, albeit weakly (Fig. 4d). Absorption on SDS-denatured thyroid microsomes removed LPO and MPO reactivity by immunoblotting (Fig. 4e), whilst TMA and HRP activity persisted both by ELISA and immunoblotting. In this

instance the HRP activity was considerably reduced (Fig. 4e). Double absorption on SDS-denatured thyroid microsomes followed by absorption on native thyroid microsomes removed the TMA/TPO activity and all cross-reacting activities of LPO, MPO and HRP (Fig. 4f and Table 3). Thus, in addition to LPO and MPO cross-reacting specificities, reactivity to HRP is also due to genuine cross-reacting aAb. Furthermore, with both the sera with which absorption studies were performed, LPO and MPO cross-reacting aAb specificities paralleled each other.

DISCUSSION

The autoimmune response to the TMA/TPO in ATD patients is polyclonal in nature, with at least six independent determinants recognized by a combination of immunochemical and enzymatic techniques (Doble *et al.*, 1988). The cross-reactivity of the aAb to TPO with other peroxidases demonstrates the heterogeneous nature of the autoimmune response and confirms the polyclonal nature of these aAb indicated by our earlier studies (Doble *et al.*, 1988). This cross-reactivity to other peroxidases not only manifests itself against another human peroxidase, (neutrophil MPO) but also against a peroxidase derived from bovine tissue (LPO) and plant tissue (HRP).

Absorption of aAb from two ATD patients' sera on native and SDS-denatured thyroid microsomes removes all reactivity with other peroxidases, whilst absorption of these sera on individual peroxidases only removes a fraction of the aAb species, showing unequivocally that the aAb are indeed cross-reacting with other peroxidases. Different ATD patients contain discrete populations of aAb that are directed to various auto-antigenic epitopes of TMA/TPO which show different patterns of cross-reactivity with the other three peroxidases examined in this study. A small number of ATD patients contain aAb to TMA/TPO alone, whilst others contain varying proportions of cross-reacting aAb to LPO, MPO and HRP, revealed solely by ELISA or by immunoblotting following SDS-denaturation (Table 1). Thus different ATD patients sera contain different qualitative and quantitative levels of aAb to TMA/TPO. The precise correlation of the clinical status of the ATD patients with any of these cross-reacting aAb species remains to be determined.

The structural basis of the aAb-recognizing epitopes on the TMA/TPO and those that cross-react with LPO, MPO and HRP are at present not known. The complete gene sequence of the TPO, MPO and HRP are known from the recent cloning of the molecules (Seto *et al.*, 1987; Libert *et al.*, 1987; Kimura *et al.*, 1987; Weil *et al.*, 1987; Fujiyama *et al.*, 1988). To date, LPO has not been cloned or sequenced. The fact that LPO and MPO activities are removed together in our absorption studies on two different ATD sera suggests a common structural basis of epitope(s) on these two peroxidases which cross-react with TMA/TPO. The cross-reactivity of the anti-TMA/TPO aAb to MPO correlates with the 42% homology in amino acid sequence reported between TPO and MPO (Libert *et al.*, 1987; Kimura & Ikeda-Saito 1988). However, the anti-TMA/TPO cross-reacting epitope on MPO was not accessible on the native MPO molecules by ELISA techniques and appears to be available for aAb binding only after reduction and/or SDS-denaturation of the MPO molecule. Autoantibodies in Graves' disease that react with neutrophil cell surface have been described. These aAb are readily displaced by thyrotropin (TSH) and their relationship to the MPO cross-reacting aAb described here is uncertain (Weitzman *et al.*, 1985).

It is now becoming clear that aAb to the TMA/TPO in ATD patients consist of a large number of aAb of different specificities directed to various auto-antigenic epitopes of the TMA/TPO molecule. Different ATD patients appear to contain quantitatively and qualitatively different aAb specificities. The presence and combinations of these specificities may well determine the pathogenicity of the microsomal aAb. Further characterization of the individual specificities of these aAbs to TMA/TPO molecule and the auto-antigenic epitopes to which they are directed will contribute towards a greater understanding of the role of the aAb in ATD.

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