Clin Exp Immunol 1997; **107**:127–134

Anti-native and recombinant myeloperoxidase monoclonals and human autoantibodies

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(Accepted for publication 9 September 1996)

SUMMARY

Myeloperoxidase (MPO) is one of the main antigen targets of anti-neutrophil cytoplasmic antibodies (ANCA) in systemic vasculitides. It has been suggested that anti-MPO antibodies may recognize a single epitope on recombinant MPO. If confirmed on native MPO, this might allow specific therapeutic intervention with anti-idiotypic MoAbs to prevent antibody–antigen interaction which is thought to cause activation of neutrophils and vasculitis. We searched for restriction in the epitope recognition profile in 50 patients with anti-MPO autoantibodies, using both native and recombinant MPO. Mouse monoclonals were purified and tested in competition assays. At least four epitopes were identified on native MPO using these monoclonals and only two were conserved on recombinant MPO. We found that human MPO autoantibody response was not restricted to a single epitope on native MPO, as all sera tested did not show the same profile in competitive studies with monoclonals. Furthermure, 30% of human anti-native MPO sera failed to recognize rMPO.

Keywords myeloperoxidase recombinant epitope anti-neutrophil cytoplasmic antibodies vasculitis

INTRODUCTION

Myeloperoxidase (MPO) is one of the two major antigen targets for anti-neutrophil cytoplasmic antibodies (ANCA) [1–4]. ANCA are specific serological markers used for the diagnosis and monitoring of small-vessel vasculitis such as Wegener's granulomatosis (WG) or microscopic polyangiitis (MPA) [5–10]. Anti-MPO antibodies are found mainly in MPA, but also in several other autoimmune diseases. MPO, a haemoprotein predominantly present in azurophilic granules of polymorphonuclear leucocytes, plays a major role in the antimicrobicidal and cytotoxic activity of polymorphonuclear leucocytes by its ability to generate potent oxidant-chlorinated species [11]. It is a 120–150-kD dimer composed of one heavy (55–60 kD) and one light (14–15 kD) chain and carries two identical prosthetic haeme groups. The two heavy chains are joined by a disulphide link, and mild reduction leads to a 72–75-kD hemiperoxidase. MPO is synthesized as a 89-kD precursor which undergoes subsequent processing via intermediate forms (84 kD) to yield mature subunits [12]. The cDNA coding for human MPO has been cloned, and recombinant MPO (recMPO) (84 kD) is now available, purified from chinese hamster ovary (CHO) cell lines [12]. recMPO is largely similar to the mature enzyme purified from polymorphonuclear leucocytes, with the same cytotoxic activity and very similar glycosylations and physical properties.

Previous MPO mapping studies performed on recMPO but not on native MPO have suggested that human autoreactivity may be restricted to one immunodominant epitope [13,14]. The purpose of this study was to confirm these data on native MPO, since recMPO might not present all the epitopes recognized by human anti-MPO autoantibodies.

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PATIENTS AND METHODS

Patients

Serum samples obtained from venipuncture of patients tested for ANCA were aliquoted and stored at −20°C until use. Fifty consecutive patients proved ANCA-positive by indirect immunofluorescence (IIF) on ethanol-fixed neutrophils and anti-MPO-positive by antigen-specific ELISA. They were also tested on antigen-specific ELISA for proteinase 3 (PR3) (IBL, Hamburg, Germany), and lactoferrin (LF) as previously described [15,16]. Anti-nuclear autoreactivity was searched for by IIF on HEp2 cells (Immuno-Concept, Sacramento, CA).

ANCA assays

Polymorphonuclear neutrophils were separated on a density gradient (Polymorphoprep; Nycomed, Oslo, Norway), washed and

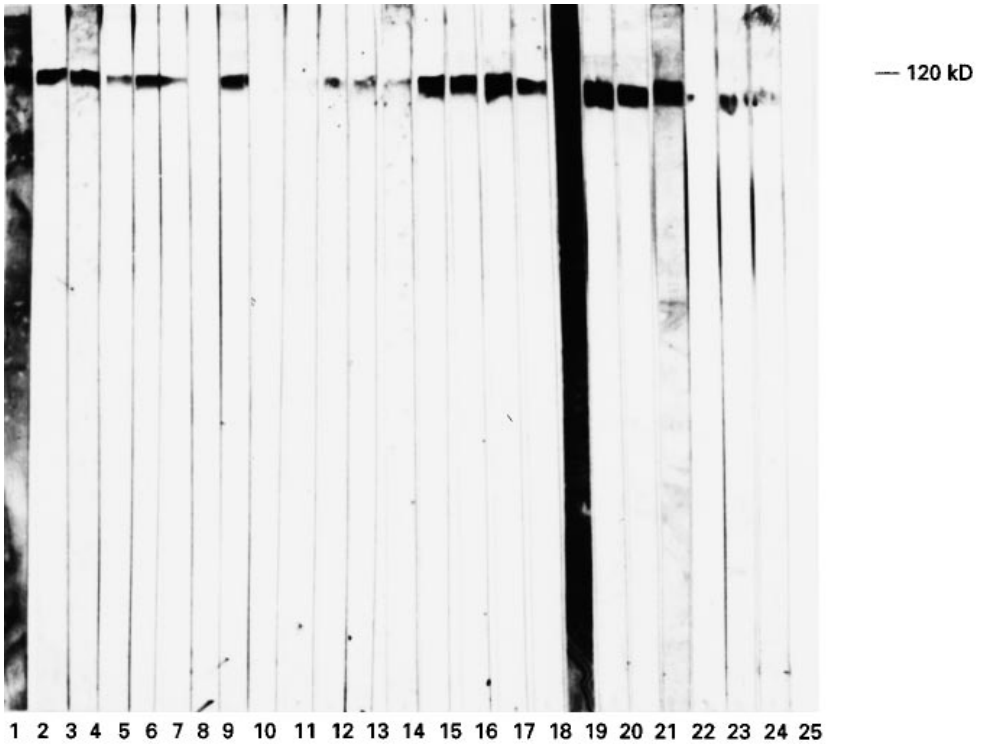

Fig. 1. Non-reduced, non-boiled native myeloperoxidase (MPO) Western blot analysis with 24 human anti-MPO sera. Lanes 1 and 18 show non-specific binding, lanes 2–6, 8, 11–17, 19–21, 23, 24 show binding at 120 kD, the molecular weight of the MPO tetramer, lanes 7, 9, 10, 22 show no binding.

cytocentrifuged. The slides were then fixed in ethanol, and immunofluorescence was performed with a 1:30 dilution serum. Fluorescence was revealed with a FITC-conjugated anti-human IgG, IgA, IgM reagent (Dako, Glostrup, Denmark).

Anti-native MPO ELISA was performed with purified MPO (Calbiochem, La Jolla, CA) as previously described [17]. Briefly, microplates were coated with 0·5 μg/ml MPO. After non-specific sites were blocked, supernatants, diluted purified monoclonals or human sera were incubated. Binding was detected using an alkaline phosphatase-conjugated goat polyclonal anti-mouse IgG reagent (Bioatlantic BA02402222; Nantes, France) or an anti-human IgG reagent (Sigma A3150; St Louis, MO) diluted 1:1000. pNPP was then added and absorbance read at 405 nm. All sera or monoclonals were tested in duplicate.

Anti-recMPO ELISA (1 μg/ml) was also performed with recombinant MPO purified from a CHO cell line kindly provided by N. Moguilevski (Université Libre de Bruxelles, Nivelles, Belgium) [12]. Positivity was defined as binding greater than the mean binding of 39 normal blood donors +2 s.d. for both native and recombinant anti-MPO ELISA.

Anti-MPO Western blot analysis was performed as previously described [18]. Briefly, 1 μg/lane Calbiochem MPO or 2 μg/lane recMPO diluted 1:1 in Laemmli sample buffer were loaded on a 12% acrylamide separating gel with 4% acrylamide stacking gel. After transfer, nitrocellulose strips were incubated either with monoclonals diluted 1 μg/ml or human sera diluted 1:20. Binding was detected with an alkaline phosphatase-conjugated goat polyclonal anti-mouse IgG reagent (Bioatlantic BA02402222) or anti-human IgG reagent (Sigma A3150) diluted 1:1000, before addition of the substrate (NBT Sigma N6876; BCIP Sigma B8503). Molecular weight-marker proteins were used as reference (Amersham Life Sciences, Aylesbury, UK).

Anti-LF ELISA and Western blot analysis were performed as previously described [15].

Monoclonal antibodies

Four mice were immunized once with MPO (Calbiochem) in acetate buffer with Freund's complete adjuvant (FCA) and twice with MPO in acetate buffer with Freund's incomplete adjuvant (FIA). They were boosted in FIA 4 days before the fusion. Spleens were removed for cell hybridization. The cell fusion partner was the X63 AG8-653 mouse myeloma cell line [19]. Fusions were performed with polyethylene glycol (PEG) 1500 (Boehringer, Mannheim, Germany) in RPMI 1640 medium (GIBCO, Paisley, UK) supplemented with 20% fetal calf serum (FCS; Institut Jacques Boy, Reims, France), hypoxanthine, aminopterin and thymidine (Sigma) and a lymphocyte culture supernatant (SLM; Diagast Laboratoires, Lille, France). Fourteen days later, hybridoma colony supernatants were screened for anti-MPO activity by an antigen-specific ELISA procedure. Positive hybridoma colonies were cloned by limiting dilution in HT (hypoxanthine, thymidine) medium, and after growth, rescreened as above. Hybridoma growth was then performed in RPMI 1640 medium supplemented with 10% FCS. MoAbs were then produced in ascitic fluid from BALB/c mice injected intraperitoneally with hybridomas. Ascitic fluids were screened as above, and monoclonals were isotyped (Sangstat Medical Co, Menlo Park, CA) and purified by affinity chromatography on a protein A Sepharose gel (Hytrap; Pharmacia, Uppsala, Sweden), biotinylated and used for further studies.

Table 1. Clinical features of the patients

No.	Diagnosis	nMPO	rMPO	nWB	rWB	aK
1	W					
2	W					
3	W					
4	SLE					
5	WG	1440	86	P	P	$2{\cdot}8 \times 10^9$
6	W	1440	31	P	P	$1{\cdot}4 \times 10^9$
7	PAN	3200	31	P	P	$1{\cdot}4 \times 10^9$
8	MPA	76	17	P	N	
9	UV	190	<5	P	P	4×10^7
10	IgAN	10	<5	P	N	2×10^7
11	WG	34	<5	P	P	$1{\cdot}1 \times 10^9$
12	WG	160	<5	P	P	8×10^7
13	MPA	30	<5	P	N	
14	WG	21	<5	P	P	4×10^7
15	MPA	45	6	P		
16	?	8	<5	NS		
17	?			N		
18	GNEC	160	7	P	P	$1{\cdot}4 \times 10^8$
19	MPA	13	<5	N		
20	WG	7				
21	?	++	161	P	P	
22	WG	11	7	N		
23	GNEC	11	<5	N		
24	APC	48	<5	N	N	$1{\cdot}4 \times 10^7$
25	?	27	<5	P	N	
26	W	26	26	P	N	6×10^9
27	MPA	120	57	P	P	$1{\cdot}4 \times 10^9$
28	GNEC	10				
29	PAN	520	9	P	P	6×10^9
30	MPA	6500	80	P	P	7×10^8
31	GNEC	86	18	P	N	7×10^8
32	MPA	640	60	P	P	4×10^8
33	WG	<5	<5	N		
34	ARD		<5	N		
35	MPA	25	11	NS		2×10^8
36	GNEC	>7000	130	P	P	6×10^8
37	GNEC	30	5	N		2×10^8
38	CSS	19	18	P		7×10^8
39	MPA	1200	39	P	P	$1{\cdot}1 \times 10^8$
40	GNEC		9	P	N	
41	CGN	800	128	P	P	6×10^8
42	MPA	36	5	N		2×10^7
43		96	17	P	P	2×10^8
44		300	67	P	P	$1{\cdot}4 \times 10^9$
45	RA	6500	107	P		$3{\cdot}5 \times 10^8$
46	MPA	320	44	P	P	4×10^9
47	MPA	1920	128	P	N	6×10^9
48	MPA	8	<5	P		
49	MPA	15	5	N		
50	MCD	400	8	P	P	$1{\cdot}2 \times 10^9$

Diagnosis: WG, biopsy-proven Wegener's granulomatosis; W, clinically suspected Wegener's granulomatosis; SLE, systemic lupus erythematosus; PAN, periarteritis nodosa; MPA, microscopic polyangiitis; ACP, atrophic polychondritis; GNEC, extracapillary glomerulonephritis; CGN, chronic glomerulonephritis; ARD, acute renal deficiency; CSS, Churg–Strauss syndrome; MCD, mixed connective disease; IgAN, IgA nephropathy; RA, rheumatoid arthritis; UV, undefined vasculitis.

ANCA tests: nMPO, Titre on native MPO ELISA; rMPO, titre on recMPO (1 μg/ml); rWB, recMPO Western blot; nWb, native non-reduced MPO Western blot; NS, non-specific binding; N, negative; P, positive. The cut-off value for positivity in nMPO and rMPO ELISA is 5.

aK, Affinity constant.

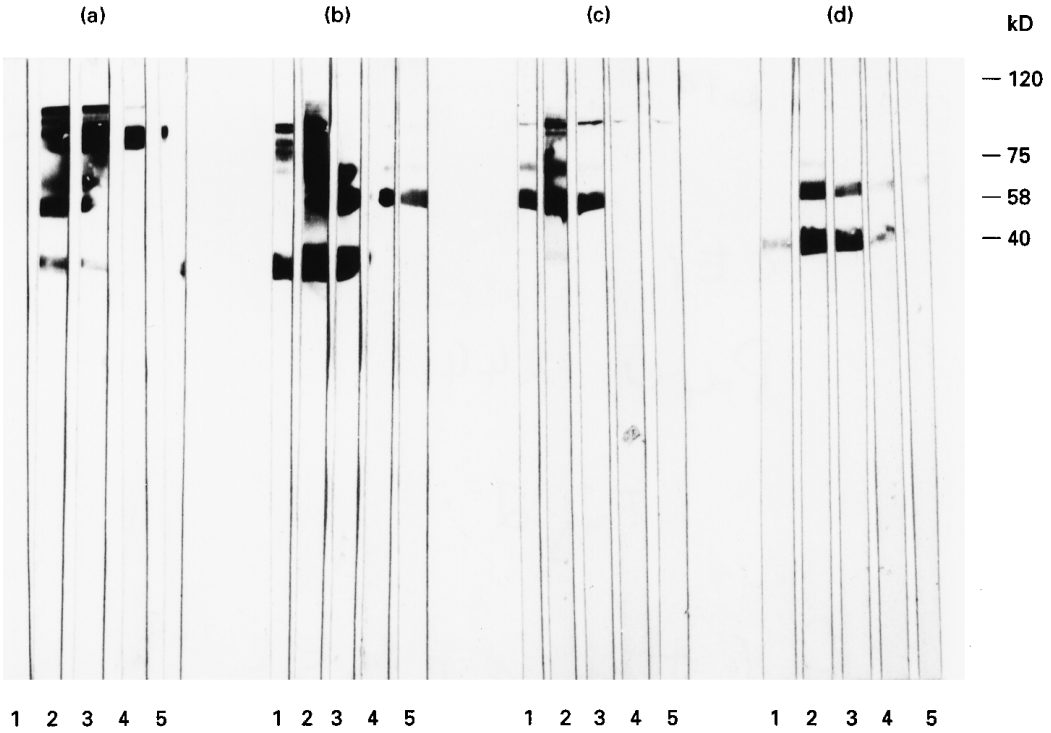

Fig. 2. Myeloperoxidase (MPO) Western blot analysis with anti-MPO monoclonals. (a) Non-reduced, non-boiled MPO. (b) Non-reduced, boiled MPO. (c) Reduced, non-boiled MPO. (d) Reduced, boiled MPO. 1, 2F2; 2, 1H2; 3, 1B10; 4, 5B8; 5, 2C7.

Affinity constant determination

Monoclonal or serum affinity was determined as the reciprocal value of MPO molar concentration in the liquid phase resulting in 50% inhibition of anti-MPO binding to MPO in ELISA, as described [20–23]. Briefly, monoclonals or serum at limiting concentration were incubated with increasing amounts of MPO (from 0·017 μg/ml to 20 μg/ml) for 2 h at 37°C. The mixture was then transferred on MPO-coated plate, and standard ELISA was performed using conjugated antibodies, as described above. The affinity constant was the reciprocal value of MPO giving 50% inhibition of maximum anti-MPO binding.

Competitive ELISA

Monoclonal/monoclonal competition assays. Serial dilutions of the biotinylated monoclonals were first incubated in duplicate in native MPO ELISA, and the concentration giving 50% of maximum binding was chosen for further inhibition studies (limiting concentration). Serial dilutions of non-biotinylated antibodies were incubated in anti-native MPO ELISA, followed by incubation of biotinylated antibodies at limiting concentration. Binding was detected by streptavidin alkaline phosphatase (Bioatlantic BA310-00022) and pNPP. Absorbance was read at 405 nm.

Monoclonal/human serum competition assays. After incubation of a serial dilution of anti-MPO monoclonals in anti-native MPO ELISA, human sera were added at limiting concentration (showing 50% of maximum binding in MPO ELISA). Binding was revealed using an alkaline phosphatase-conjugated goat anti-human IgG reagent (Sigma A3150) exhibiting no cross-reactivity with mouse antibody. Results are expressed as the percentage of inhibition of serum binding calculated according to the formula: % inhibition $= (S - (S/M))/S \times 100$, where $S =$ binding of serum and $S/M =$ binding of test serum after preincubation of monoclonals

Human serum/monoclonal competition assays. A serial dilution of human anti-MPO-positive sera was incubated before addition of monoclonals at limiting concentration. Binding was then revealed using an alkaline phosphatase-conjugated anti-mouse IgG, followed by addition of pNPP.

RESULTS

Patients

At time of diagnosis, the 50 selected patients (46% female, mean age 64 years) were all P-ANCA-positive on IIF, positive in native MPO ELISA and negative in LF ELISA. One was double-positive for MPO and LF in ELISA and Western blot analysis. Fourty-four were tested in MPO Western blot analysis. In non-reduced and non-boiled MPO Western blot analysis, 32 showed 120-kD band binding, two were non-specific (background binding), 10 showed no binding. Examples are shown in Fig. 1. If MPO was reduced or boiled, there was no recognition on Western blot analysis.

The clinical presentation consisted of: 12 WG, 14 MPA, two periarteritis nodosa (PAN), seven extracapillary glomerulonephritis (GNEC) and one rheumatoid arthritis (RA). The two patients with PAN actually showed small-vessel vasculitides and should have been classified as MPA, as previously described [24]. These data are summarized in Table 1.

Monoclonal antibodies

Five monoclonals were raised and showed positive binding in native MPO ELISA and Western blot analysis. They were all negative in LF ELISA. 1H2 and 1B10 were IgG2b, 2C7 and 5B8 were IgG1, 2F2 was IgG2a. In non-reduced and non-boiled native

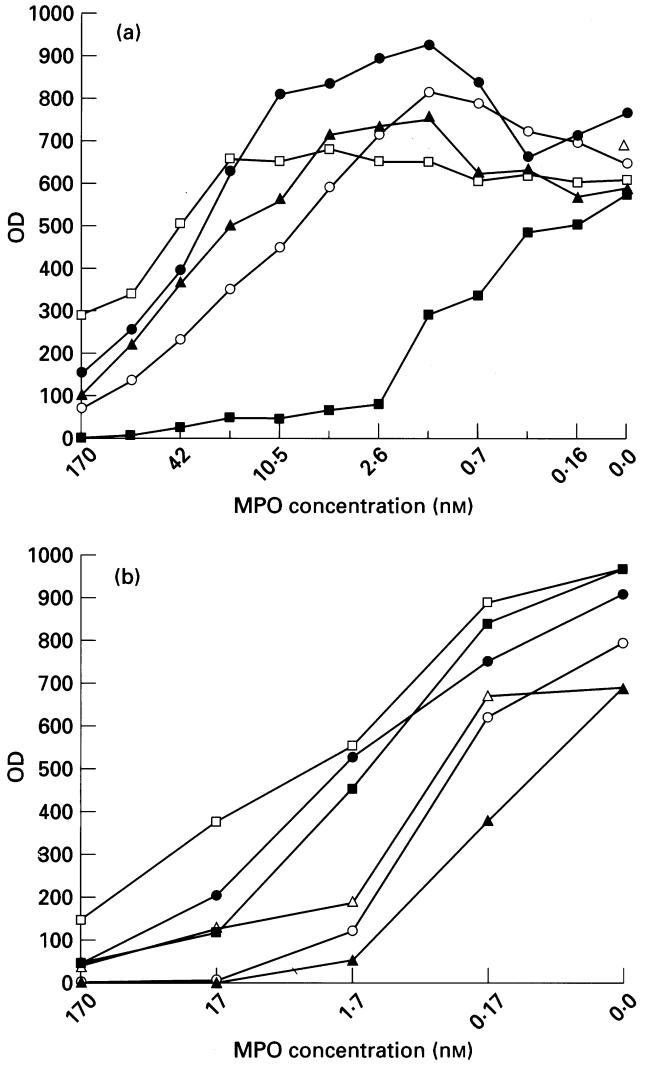

Fig. 3. (a) Determination of affinity constants of monoclonals. The affinity constant was calculated as the reciprocal value of the myeloperoxidase (MPO) molar concentration giving 50% inhibition of anti-MPO monoclonal or human anti-MPO-positive sera. ■, 1H2; □, 2F2; ●, 5B8; ○, 2C7; ▲, 1B10. (b) Determination of affinity constants of human anti-MPO sera. ■, No. 44; □, no. 41; ●, no. 45; ○, no. 46; ▲, no. 47; △, no. 50.

MPO Western blot analysis, all monoclonals recognized a 120-kD band. In reduced and non-boiled MPO Western blot analysis, 1H2, 1B10 and 2F2 recognized two bands: one at 72 kD as the molecular weight of hemiperoxidase and one at 58 kD as the molecular weight of MPO heavy chain. When MPO was boiled, 1H2 and 1B10 recognized a 58-kD band and a 40-kD degradation product of MPO. 2F2 recognized only the 40-kD band. 2C7 and 5B8 exhibited faint binding when MPO was boiled and reduced, but recognized a 58-kD band when MPO was only boiled (Fig. 2).

Affinity constant determination

Affinity constants were calculated, as described, as the reciprocal value of the MPO concentration inhibiting 50% of MPO binding. Figure 3a presents the binding of monoclonals after preincubation with decreasing concentrations of MPO. The calculated affinity constants are expressed in mol^{-1} as follows:

2F2	$2{\cdot}5 \times 10^7$ mol^{-1}
5B8 and 1B10	$2{\cdot}5–5 \times 10^7$ mol^{-1}
2C7	$5–10 \times 10^7$ mol^{-1}
1H2	7×10^8 mol^{-1}

Polyclonal affinity constants showed large differences from one serum to another (ranging from 10^7 to 10^{10} mol^{-1} (Table 1)). Figure 3b shows some examples of human anti-MPO binding curves after preincubation with decreasing concentrations of MPO.

Competitive ELISA

Monoclonal/monoclonal competitive ELISA. 1H2 and 1B10 competed for binding on the same epitope and partially inhibited 2F2 binding. 2C7 and 5B8 partially competed for MPO binding (Figs 4 and 5a). 2F2, 5B8 and 1B10 did not fully inhibit their own binding. This could be explained by their low affinities and partial release during the washing procedure before the addition of the biotinylated antibody.

Monoclonal/human serum competitive ELISA. The percentages of inhibition of human anti-MPO-positive sera after preincubation of anti-MPO monoclonals are presented in Table 2. Inhibition ranged from 0% to 65% (mean 12·5%, s.d. 16%).

Human serum/monoclonal competitive ELISA. Since monoclonal affinity might have been lower than polyclonal affinity, increasing dilutions of polyclonals were first incubated and then a limiting concentration of monoclonal, in order to search for inhibition of monoclonal binding. Results were very similar (data not shown). In all these competition assays, inhibition was considered as significant when above 30%.

On the whole, the binding of 14 out of 30 (46%) sera on native MPO was inhibited by at least one monoclonal. Seven were inhibited by one monoclonal (23%), four by two monoclonals (13%), two by three monoclonals (6%) and one by the five monoclonals (3%). 1H2 inhibited the binding of 11 out of 30 sera (37%), 2C7 and 5B8 inhibited the binding of five out of 30 sera (17%), and 2F2 and 1B10 inhibited the binding of two out of 30 sera, as shown in Fig. 5b.

Recombinant MPO

On recMPO ELISA, 2C7 and 2F2 showed little binding on MPO, and 1H2, 1B10 and 5B8 exhibited no binding (Fig. 5c). On recMPO Western blot in non-reducing and non-boiling conditions, only 2C7 recognized a band at 84 kD.

Only 28 out of 40 anti-native MPO-positive sera were positive on recMPO ELISA (70%). When a higher concentration of recMPO was used for coating microplates (2 μg/ml), four other sera exhibited binding. When 29 sera with binding in native MPO Western blot analysis were tested in recMPO Western blot analysis, 20/29 bound recMPO in Western blot studies, including 16 positive in recMPO ELISA (1 μg/ml), two positive in recMPO ELISA (2 μg/ml) and two negative in recMPO ELISA (1 and 2 μg/ml). Out of the nine sera which did not bind recMPO in Western blot analysis, six were positive and three negative in recMPO ELISA (1 or 2 μg/ml). In native MPO ELISA, titres of anti-recMPO-positive sera ranged from 8 to >7000 (mean 1219, s.d. 2060), and titres of anti-recMPO-negative sera from 8 to 190 (mean 70, s.d. 59). The correlation coefficient between titres in

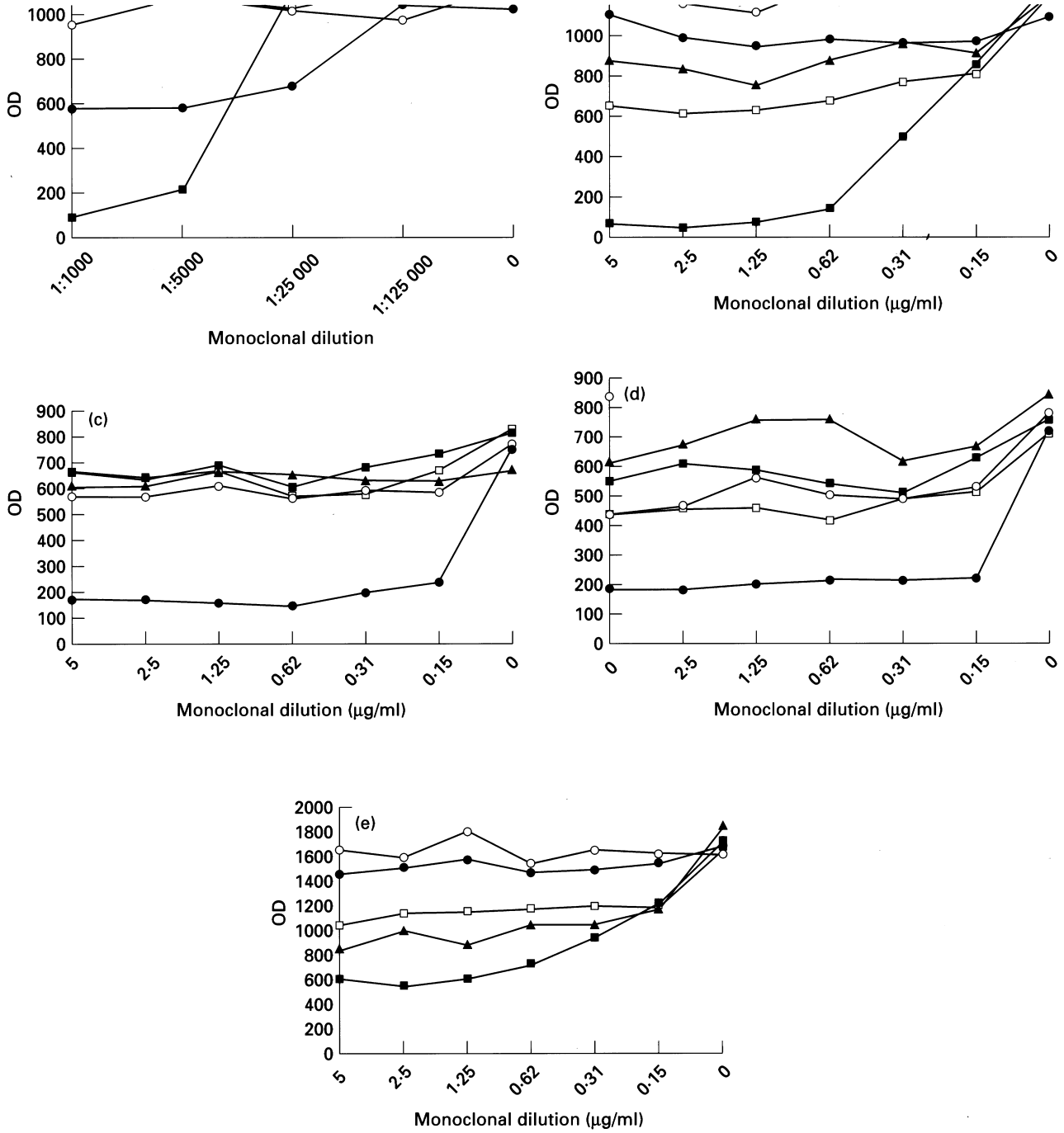

Fig. 4. Competitive studies between monoclonals. Increasing dilutions of the purified monoclonals (1H2 (a), 1B10 (b), 2C7 (c), 5B8 (d), and 2F2 (e)) were first incubated and washed before limiting concentration of a single biotinylated antibody was added. (a) ■, 1H2; □, 5B8; ●, 1B10, ○, 2C7. (b,c,d,e) ■, 1H2; □, 1B10; ●, 2C7; ○, 5B8; ▲, 2F2.

anti-native MPO ELISA and anti-recMPO ELISA was $r = 0{\cdot}6$ ($P < 0{\cdot}05$).

DISCUSSION

Five monoclonals were raised after BALB/c immunization with commercial native MPO. Since we previously showed that commercial native MPO was contaminated by LF, anti-MPO monoclonal specificity was confirmed by MPO and LF ELISA and Western blot analysis. Three groups of mouse anti-MPO monoclonals were defined: group 1 (1H2 and 1B10) recognized an epitope resistant to heat and reduction on MPO heavy chain present in a heat-degradation fraction of 40 kD. This epitope was not present on recMPO. Group 2 (2F2) recognized an epitope resistant to reduction on MPO heavy chain. This epitope was slightly altered on recMPO, since it bound 2F2 in recMPO ELISA

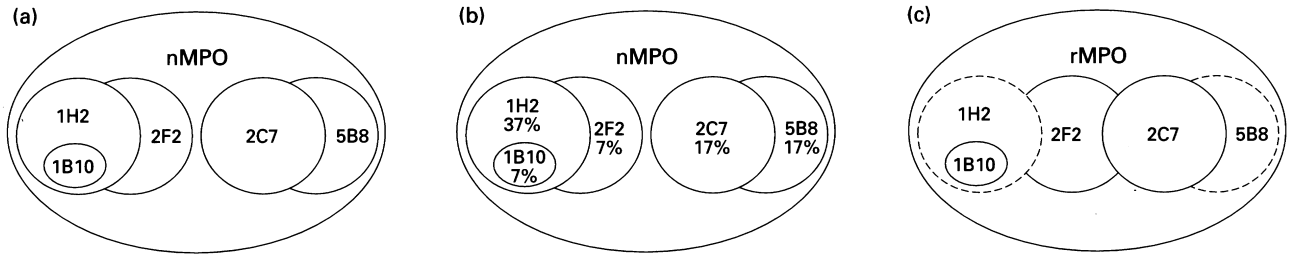

Fig. 5. Myeloperoxidase **(**MPO) epitope mapping. (a) Monoclonal competitive studies on nMPO. (b) Competitive studies between monoclonals and human sera on nMPO: percentages of human sera which were inhibited by the different monoclonals. (c) Monoclonal binding on rMPO.

but not in recMPO Western blot. Group 3 (2C7 and 5B8) recognized an epitope resistant to heat but destroyed after reduction on MPO heavy chain. These monoclonals partly competed for binding on native MPO. However, the 2C7 but not the 5B8 epitope was present on recMPO. Therefore, at least four epitopes were identified on native MPO using mouse monoclonals (1H2/1H10, 2F2, 2C7 and 5B8), and only two were conserved on recombinant MPO (2C7 and partially 2F2) (Fig. 5). All four epitopes were resistant to heat, and two out of four to reduction.

Polyclonal human anti-MPO response also appeared to be multi-epitopic, as all sera tested did not show the same profile in competitive studies with monoclonals, or in native or recMPO ELISA. Furthermore, no single monoclonals or pooled monoclonals could completely inhibit human anti-MPO binding to MPO in ELISA. We supposed that these poor inhibitions were due to the lower affinity constants of the monoclonals, but preincubation of increasing dilutions of human sera gave no better inhibition of anti-MPO monoclonal binding. Most MPO epitopes recognized by human sera were destroyed by heat and reduction. In contrast, three MPO epitopes recognized by mouse monoclonals were resistant to heat. This suggests that the MPO epitopes recognized by human sera were conformational and only poorly related to MPO epitopes recognized by mouse monoclonals.

We found that almost 30% of anti-native MPO-positive sera failed to recognize recMPO, contrary to results in a previous study [25]. This poor sensitivity of recMPO ELISA became only slightly higher after the recMPO coating concentration was increased. Some sera were positive in recMPO Western blot analysis. All the anti-recMPO-positive sera bound native MPO in Western blot studies. Furthermore, we found that the binding of 37% of human sera was partially inhibited by 1H2 and that the epitope recognized by this monoclonal was not present on recMPO (Fig. 5)

On the whole, one-third of human anti-MPO antibodies were not detected on recMPO ELISA or in recMPO Western blot analysis, suggesting that all the epitopes recognized by human anti-MPO were not present on recMPO. We suggest that recMPO is not folded as is native MPO, thus leading to significant impairment of native MPO conformational epitopes. A link between two hemiperoxidases might also be needed for recMPO in order to obtain adequate MPO epitope presentation. All previously reported, MPO epitope mapping studies were performed on recMPO and suggested the existence of an immunodominant epitope [13,14]. Our competitive studies on native MPO do not point to an immunodominant epitope.

The clinical presentation of anti-MPO-positive patients was similar to that in previously published series [26,27]. The results of competition studies differed from one serum to another, and after preincubation of monoclonals, no specific inhibition profile could be correlated with one or another type of vasculitis. In addition, the affinity of human anti-MPO antibodies appeared variable.

It may be concluded that this recMPO should not be used for diagnosis or MPO epitope mapping since it lacks several relevant conformational MPO epitopes. Further studies are needed to obtain a recombinant MPO tetramer. MPO autoantibody response appeared to be multi-epitopic, and no correlation was found between the epitope profile and clinical presentation.

Table 2. Competitive studies between monoclonals and polyclonal human anti-myeloperoxidase (MPO) sera

No.	1H2	1H2	1B10	2C7	2F2	5B8
5	4	26	0	22	0	0
6	6	0	3	8	7	3
7	1	0	0	14	14	5
8		43	9	17	2	0
9		0	0	5	19	0
10		0	0	0	13	0
11		0	0	65	3	0
12	48	0	9	35	11	0
14		0	0	0	15	3
18	29	12	30	14	37	24
24		0	9	0	9	0
25		0	0	33	38	30
26	9	0	0	0	0	6
27	41	11	15	25	0	37
29	9	0	0	0	0	16
30	33	0	0	0	0	10
31	53	36	29	42	6	38
32	15	0	8	0	0	0
35		35	26	15	13	0
36	4	6	6	7	3	0
37	50	4	10	12	1	43
38	26					
39	4	0	4	6	0	0
41	21	11	7	0	4	0
42	38	24	44	44	18	37
43	0	24	0	0	0	0
44	34	44	0	19	0	0
45	46	44	0	0	0	0
46	39	38	0	0	0	0
47		1	3	0	6	0
50		0	9	5	12	23

Results are expressed as percentage of inhibition of human anti-MPO binding after incubation of monoclonals.

ACKNOWLEDGMENTS

This work was supported by the 'Délégation à la Recherche Clinique' of Nantes Hospital.

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