Mapping of myeloperoxidase epitopes recognized by MPO-ANCA using human-mouse MPO chimers

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Myeloperoxidase (MPO) is one of the major target antigens of antineutrophil cytoplasmic autoantibodies (ANCA) found in patients with small-vessel vasculitis and pauci-immune necrotizing glomerulonephritis. To date, the target epitopes of MPO-ANCA remain poorly defined. Human MPO-ANCA do not typically bind mouse MPO. We utilized the differences between human and mouse MPO to identify the target regions of MPO-ANCA. We generated five chimeric MPO molecules in which we replaced different segments of the human or mouse molecules with their homologous counterpart from the other species. Of serum samples from 28 patients screened for this study, 43 samples from 14 patients with MPO-ANCA-associated vasculitis were tested against recombinant human and mouse MPO and the panel of chimeric molecules. Sera from 64 and 71% of patients bound to the carboxy-terminus of the heavy chain, in the regions of amino acids 517–667 or 668–745, respectively. No patient serum bound the MPO light chain or the amino-terminus of the heavy chain. All sera bound to only one or two regions of MPO. Although the pattern of MPO-ANCA binding changed over time (4–27 months) in 6 of 10 patients with several serum samples, such changes were infrequent. Other target regions of MPO-ANCA may not have been detected due to conformational differences between the native and recombinant forms of MPO. MPO-ANCA do not target a single epitope, but rather a small number of regions of MPO, primarily in the carboxy-terminus of the heavy chain.

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Knowledge about the target epitopes of autoantibodies can provide valuable insight into the mechanisms that initiate and regulate the autoimmune response. Epitope mapping can identify molecular mimics and elucidate the relationship between an alloantigen and autoimmune disease. The analysis of changes in target epitopes over time in an individual patient may also provide insight as to whether relapses are associated with reactivity to a new epitope or reactivation of an antibody response to the same epitope(s).

Antineutrophil cytoplasmic autoantibodies (ANCA) directed against myeloperoxidase (MPO) or proteinase 3 are associated with pauci-immune necrotizing and crescentic glomerulonephritis, microscopic polyangiitis, and Wegener's granulomatosis.[1] To date, the epitope specificity of MPO-ANCA remains poorly defined. Characterizations of the MPO-ANCA epitope specificity performed by analysis of competitive binding of various antibodies or antisera to MPO[2–4] or by analysis of binding to various fragments[5] or synthetic overlapping peptides[6] of MPO have led to inconclusive results. Furthermore, methods utilizing protein fragments or peptides fail to 'present' candidate target epitopes in a preserved three-dimensional conformation. Finally, very limited information is available as to whether the same epitopes are targeted during the onset of disease and relapse.[7]

Chimeric molecules have been used to determine the immunodominant epitopes of several autoantigens such as thyroid peroxidase[8] and the glomerular basement membrane's non-collagenous domain 1 (NC1) domain of the alpha 3 chain of type IV collagen.[9] Despite an 85% amino-acid identity (90% homology) between the human and mouse MPO sequences (Figure 1), the majority of human MPO-ANCA do not bind mouse MPO. We hypothesized that the areas of heterogeneity between human and mouse MPO are candidate target epitopes for MPO-ANCA. Furthermore, we hypothesized that the anti-MPO autoimmune response is directed against a limited number of immunodominant epitopes on MPO and that the same epitopes are targeted during disease onset and relapse.

We exploited the difference in ANCA reactivity to human and mouse MPO to identify immunodominant epitopes of

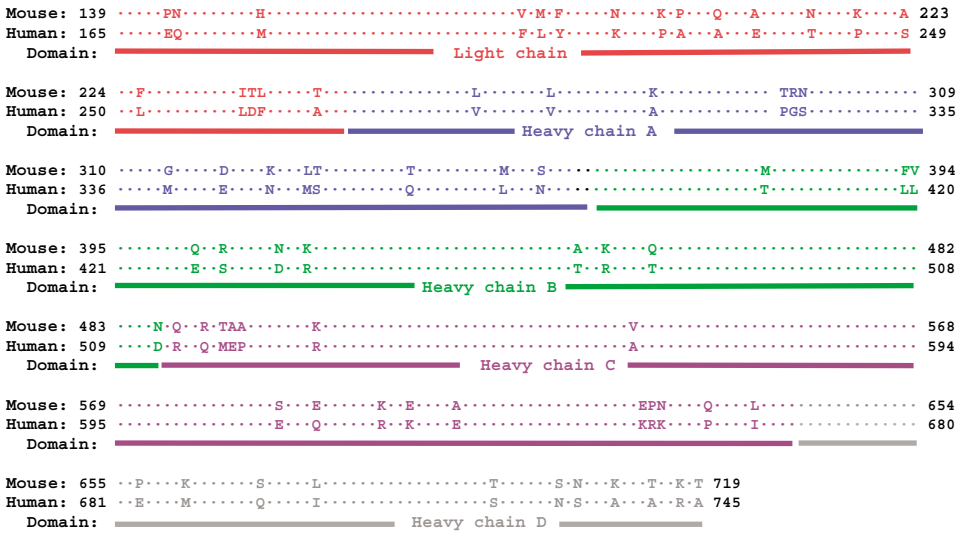

Figure 1 | Alignment of human and mouse MPO amino-acid sequences. Identical amino acids are represented by dots. Human–mouse chimeric molecules were generated by replacing segments of the human or mouse MPO cDNA with homologous segments from the other species. The five regions used to generate the human-mouse chimerics are delineated by different colors. The seven amino acids at the L–A junction that are cleaved in the maturation of MPO are not included in this figure.

human MPO using a panel of recombinant human MPO, recombinant mouse MPO, and five human–mouse chimeric molecules. This approach allows presentation of selected regions of MPO in a preserved three-dimensional configuration. Chimeric molecules were designed to present a region of human MPO on the framework of the mouse MPO molecule. If this human region contains the target epitope of an MPO-ANCA, the antibody binding to the chimeric would be greater than that binding to recombinant mouse MPO. Alternatively, chimeric molecules were generated by replacing a region of the human MPO molecule by its homologous region from mouse MPO. If that region contains the target epitope, the antibody binding to the chimeric would be less than that binding to recombinant human MPO.

RESULTS

Generation of chimeric molecules

The heavy chain of MPO was 'divided' into four segments, namely A, B, C, and D (Figure 2). We used capital letters to denote a human segment and lower-case letters to denote a mouse segment. 'L' or 'l' denotes the MPO light chain. Human and mouse recombinant MPO and five of the six chimeric MPO molecules (full description in Materials and Methods) were successfully expressed in human embryonic kidney 293 cells and the purified recombinant proteins verified by Western blot analysis (Figure 3). We were unable to express the 'labcD' despite three transfections of human embryonic kidney 293 cells. For each construct, we detected bands of 70 and 90 kDa by Western blot analysis, corresponding to the pro-MPO and mature MPO forms (Figure 3).

MPO-ANCA binding to the chimeric MPO molecules

To assess whether the MPO light chain harbors target epitopes, we compared the binding pattern of each serum

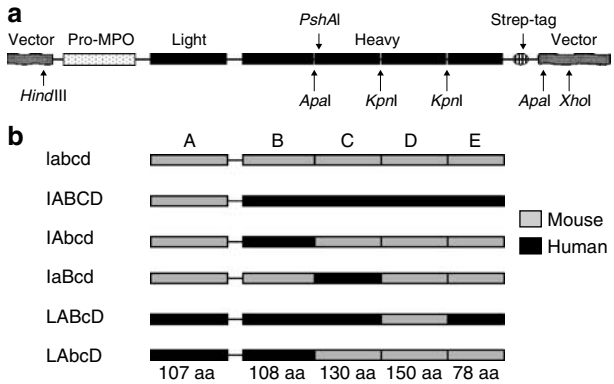

Figure 2 | Generation of chimeric molecules. Graphic representation of the MPO cDNA expression construct showing the Strep-tag® (IBA, Germany) and the restriction enzyme sites used in the generation of the human–mouse chimeric molecules. Five chimeric molecules were generated. L/l denotes the MPO light chain. The MPO heavy chain is 'divided' into four regions, denoted as A, B, C, and D. Capital letters and black bars denote human segments, small letters and gray bars denote mouse segments.

sample to the chimerics expressing the murine light chain ('lABCD', 'lAbcd', and 'laBcd') and the molecules expressing the human light chain ('LABCD', 'LABcD', and 'LAbcd'). No patient serum demonstrated a consistent binding to the light chain.

The reactivity of MPO-ANCA sera to the remaining four chimerics (lAbcd, laBcd, LABcD, and LAbcd) was analyzed. As we did not express the 'labcD' chimeric, we were not always able to distinguish whether certain samples bound either or both of the B or D regions or the C and D regions. Based on the binding pattern of sera from our 14 patients, sera from 0 patients bound to region A (amino acids (a.a.) 1–108 of heavy chain), sera from six patients bound to region

B (a.a. 109–237), sera from nine patients bound to region C (a.a. 238–388), and sera from 10 patients bound to region D (a.a. 389–469) at some point during the course of disease (Figure 4). Sera from six patients bound to one region, sera from six patients reacted to two regions, and sera from two patients to possibly three regions over time (Table 1). No individual serum sample bound to more than two regions of MPO.

In 4 of 10 patients with more than one sample, the binding pattern never changed over a period of 3–20 months. In six patients (patient nos. 1, 2, 5, 6, 10, and 11) the binding pattern changed over a period of 7–27 months. For three patients (nos. 1, 2, and 11) the target immunodominant region changed from D to C. For patient no. 6 the target immunodominant region changed from B to D and for patient no. 10 it changed from B to C (Table 1). Due to the small number of patients, we could not correlate the antibody-binding pattern with changes in clinical levels of disease activity.

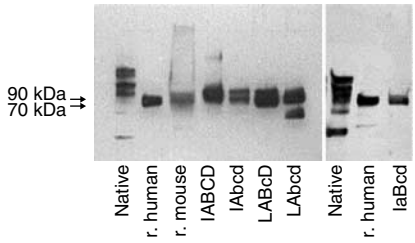

Figure 3 | Immunoblot of recombinant mouse and human MPO and human–mouse chimeric molecules. This immunoblot shows the recombinant proteins and chimeric molecules detected by polyclonal rabbit anti-human MPO antibodies. Proteins analyzed are native = native human MPO, r. human = recombinant human MPO; r. mouse = recombinant mouse MPO.

DISCUSSION

Knowledge about the immunodominant target regions of MPO can provide insight into the MPO-ANCA immune response. Several studies suggested that the immunodominant epitopes of MPO are restricted in number and are conformational in nature,[2–4,10] but failed to identify them.

Chimeric molecules have been used to determine the immunodominant epitopes of several autoantigens in a preserved three-dimensional configuration.[8] This approach pinpointed seven amino acids in the NC1 domain of the alpha 3 chain of type IV collagen that are essential to the structure of the target epitope of anti-glomerular basement membrane antibodies.[9,11] Epitope mapping of proteinase 3 using human–mouse and human proteinase 3–elastase chimeric antibodies determined a restricted number of target epitopes, which varied from patient to patient.[12] The goal of this study was to identify the target immunodominant epitope(s) of MPO. In this respect, our use of recombinant human MPO limits identification of some target epitopes, especially those present only on the dimerized holoenzyme. This limitation is reflected in our ability to analyze the target

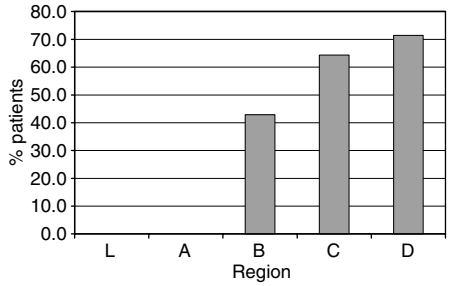

Figure 4 | Percentage of patients with antibodies to each region of MPO (some patients bound more than one region, as detailed in Table 1).

Table 1 | Patient characteristics and pattern of MPO-ANCA binding for each sample tested

					Target region: sequential samples[b]							
Patient	Age	Gender	Diagnosis	Time interval (months)[a]	I	II	III	IV	V	VI	VII	VIII
1	57	F	MPA	7	D	C/D[c]	C					
2	59	F	WG	17	D	C	C	C				
3	36	F	WG	16	C	C						
4	63	M	MPA	3	D	D	D	D				
5	55	M	MPA	16	B&D	D/B[c]						
6	45	F	WG	18	B	B	B	D	D	D	D	D
7	76	F	WG	0	D/B[c]							
8	75	F	MPA	20	C	C						
9	50	M	RENAL	0	C							
10	21	F	RENAL	24	B	B/D[c]	C/B[c]	C				
11	46	F	MPA	27	D	D/B[c]	D/B[c]	D/B[c]	D/B[c]	C	C	C
12	71	F	RENAL	0	D							
13	75	F	MPA	11	C	C						
14	78	M	MPA	0	B&D							

Abbreviations: F, female; M, male; MPA, microscopic polyangiitis; MPO-ANCA, myeloperoxidase-antineutrophil cytoplasmic autoantibodies; renal, pauci-immune necrotizing glomerulonephritis without extra-renal vasculitis; WG, Wegener's granulomatosis.
[a]Time interval in months between the first and last serum sample.
[b]Antibody target region for each serum sample available from each patient.
[c]Unable to ascertain if reactivity is to one, the other or both regions of MPO.

regions of sera from 50% of the patients originally screened for this study. The recombinant human MPO molecule may also differ from the native molecule as a result of changes in folding and post-translational modifications such as glycosylation or the expression of the 'pro-form' as opposed to the 'mature' MPO molecule.[13] Nevertheless, using the observation that most human MPO-ANCA patient sera bind human but not mouse recombinant MPO, we were able to define a limited number of antigenic regions of interest.

In our study, sera from 71% of patients did not bind recombinant mouse MPO. Similarly, serum from only 1 of 36 (2.7%) patients with MPO-ANCA vasculitis bound rat MPO.[14] These results confirm that MPO-ANCA recognize epitopes on human MPO that are absent on rat or mouse MPO.

Several investigators have concluded that the target epitopes of MPO are conformational in nature by demonstrating that MPO-ANCA bound only the MPO holoenzyme, but not the denatured protein.[2,10] Using expressed protein fragments of MPO, researchers have similarly deduced that the epitopes are conformational in nature and demonstrated that the immunodominant epitopes are likely on the heavy chain of MPO.[6,15,16] We confirm this observation as all the patient sera bound to the heavy chain, and none bound to the light chain. Based on the crystal structure of MPO,[17,18] the three-dimensional model of MPO reveals that, in the dimer form, the light chain is largely 'hidden' in the groove between the two MPO monomers and is poorly 'accessible' to antibody binding (Figure 5).

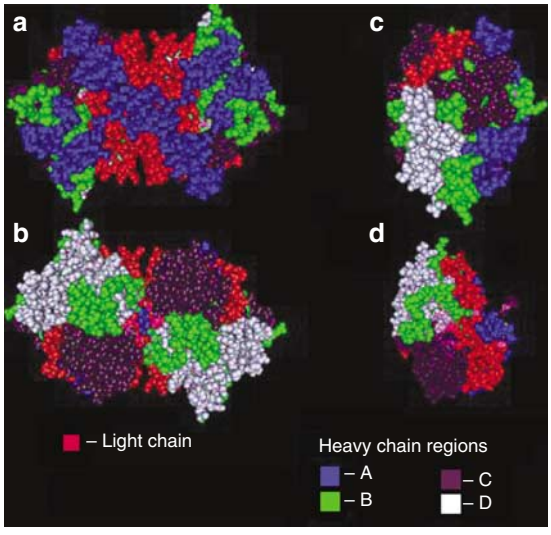

Figure 5 | Three-dimensional modeling of MPO. MPO is a 140 kDa dimer, with each monomer composed of a heavy (59 kDa) and a light chain (13.5 kDa). This figure shows four views of MPO. (**a**, **b**) show two views of the MPO dimer. (**c**) shows a 'profile' view of the MPO monomer, and (**d**) shows the surface of the MPO monomer that faces the other monomer. The latter view reveals that, in the dimeric form, the light chain is 'sandwiched' in the groove between the two monomers, and is partially hidden from the surface of the molecule (based on the MPO structure from National Center for Biotechnology Information structure 1 DNW).

In contradistinction with anti-glomerular basement membrane autoantibodies where the majority of patients react to a single, well-defined epitope,[9] the MPO-ANCA sera tested in this study most frequently bound epitopes restricted to the C or D region of the heavy chain. Based on the three-dimensional model of MPO, these two regions are 'intertwined' on the same 'hemisphere' of the molecule (Figure 4).

Our results corroborate some of the results of Fujii *et al.*,[16] who studied the pattern of reactivity of 20 sera from 20 MPO-ANCA patients against a panel of 10 partially overlapping recombinant fragments of MPO. In their study, 53% of patients bound to the terminal 147 amino acids of the heavy chain (corresponding to a region encompassing part of our C region and the entire D region). However, our results diverge from theirs, in that 84% of their sera bound the first 62 amino acids and 63% the next 68 amino acids near the amino-terminus of the heavy chain (corresponding to our A region and a junction of our regions A and B, respectively). These results most likely derive from the fact that Fujii *et al.*[16] expressed fragments of human MPO which were not presented in a preserved three-dimensional molecular structure.

The finding of a restricted number of target epitopes was previously suggested based on studies using the method of competition among various antibodies.[2–4] Although studies of competitive binding of antibodies to their target antigen are helpful in determining the relative number of epitopes, they generally fail to identify the 'location' (target amino acids) of these epitopes. This general approach is further hindered by influences of protein–protein interaction, since the binding of one antibody to MPO may alter the molecule or block the binding of other antibodies to adjacent epitopes, for example.[4]

It is important to note that although we report the restriction of ANCA reactivity to relatively large regions of the MPO molecule (C and D regions corresponding to a total of 230 amino acids), the actual number of amino acids that differ between the human and mouse molecules within these regions is much smaller (28 amino acids) (Figure 1). Within the C and D regions, there are four small regions of marked heterogeneity between the mouse and human MPO molecules that are separated by long stretches of amino-acid identity. We will assess whether these non-contiguous divergent amino acids are important to the structure of the target epitopes creating MPO chimerics with smaller areas of differences between the human and mouse molecules.

We tested serial serum samples in 10 of our 14 patients. Although we observed changes in the antibody-binding pattern with time in six patients, these changes were infrequent, occurring once over 3–20 months in all but one patient. Owing to the relatively small number of patients in our study, and the inherently variable nature of ANCA vasculitis,[19] we could not determine a clear correlation between the antibody-binding profile and specific disease manifestations or levels of activity or changes thereof. To do so with confidence requires the prospective

analysis of multiple serum samples from a large group of patients.

In summary, we employed an approach to the epitope mapping of MPO that preserves its three-dimensional structure. Our data reveal that MPO-ANCA react to one or more epitopes on the recombinant human, but not mouse, MPO molecule. Using our panel of chimeric mouse–human MPO molecules, we demonstrated a restriction of antibody reactivity to two intertwined target regions corresponding to the carboxy-terminus of the heavy chain. We plan to further pinpoint the target epitopes of MPO-ANCA by replacing smaller fragments of the mouse or human MPO with homologous regions from the other species, focusing on the discrete foci of marked heterogeneity in the amino-acid sequences between human and mouse at the carboxy-end of the MPO molecule.

MATERIALS AND METHODS

Patients and sera

Ninety-five archival serum samples from 28 patients with MPO-ANCA vasculitis were screened for use in this study. Of these, 52 samples from 14 patients were excluded from further analysis due to lack of binding to the recombinant human MPO (29 samples), binding to the 'no-antigen' control (10 samples) or similar reactivity to both mouse and human recombinant MPO (13 samples from eight patients).

Fourteen MPO-ANCA positive patients with microscopic polyangiitis, Wegener's granulomatosis or pauci-immune crescentic glomerulonephritis (age range 21–75 years old; 10 females) provided 43 serum samples (1–8 samples per patient) for testing against our panel of chimeric molecules (Table 1) collected at various times during their disease course and stored at −20°C. These serum samples were derived from patients with different and changing degrees of disease activity, ranging from active vasculitis to remission on and off immunosuppressive therapy. Thirteen of 14 patients provided at least one serum sample, while having signs of active vasculitis. Serum samples from 11 healthy volunteers (age range 21–60 years old; seven females) served as negative controls. This study was conducted in accordance with the Declaration of Helsinki Principles with an Institutional Review Board approved protocol. Informed consent was obtained prior to the blood collections.

Generation of chimeric molecules

Full-length cDNA for human MPO was provided by Dr William Nauseef (University of Iowa, Iowa City, IA, USA); cDNA for mouse MPO was obtained by polymerase chain reaction (PCR) cloning from RNA isolated from WEHI 3BD cells[20] (American Type Culture Collection, Manassas, VA, USA). Using PCR, a *Hin*dIII restriction site and a Kozac consensus translation initiation sequence were introduced 5′ to the coding sequence (Figure 2). At the 3′ end of the cDNA, we introduced 18 nucleotides encoding an in-frame StrepTag® peptide (IBA, Göttingen, Germany) followed by a stop codon and an *Xho*I site. Both the human and murine cDNA for MPO were cleaved by *Hin*dIII/*Xho*I and subcloned into a pcDNA3 vector (Invitrogen, Leek, The Netherlands).

Six human–mouse chimeric molecules were generated (Figure 2). In describing the chimeric molecules, we used capital letters to denote a human segment and lower-case letters to denote a mouse segment. 'L' or 'l' denote the MPO light chain. The heavy chain was 'divided' into four segments, namely A, B, C, and D (Figures 3, 4a and b) based on the regions of heterogeneity between the mouse and human molecules and the presence of convenient restriction enzyme sites (*Apa*I and *Kpn*I) usable on both the human and mouse MPO cDNA. For example, in 'lAbcd' only the 'A' region of the heavy chain is of human origin and the other parts originate from the mouse sequence. The recombinant mouse and human MPO constructs are referred to as 'labcd' and 'LABCD', respectively. The L, A, B, C, and D regions correspond to human a.a. numbers, 165–272, 279–386, 387–516, 517–667, and 668–745, and murine a.a. numbers 139–246, 253–360, 361–490, 491–641, and 642–719, respectively.

The 'lABCD' chimeric was created by sequential PCR technique,[21] where the 'l' was amplified from the 'labcd' and the 'ABCD' part from the 'LABCD'. The PCR products were purified, combined, and used as template to allow fusion of the two products in a second PCR reaction using the forward murine 'l' primer and the reverse human 'D' primer. The final PCR product was then cleaved using *Hin*dIII/*Xho*I and inserted into the pcDNA3 vector.

'lAbcd' and 'LAbcd' were generated from 'lABCD' and 'LABCD', respectively. The 'bcd' segment was generated by standard PCR using 'labcd' as template, and subcloned into the previously digested 'lABCD' and 'LABCD' cDNA using restriction enzymes *Psh*AI (New England Biolabs, Ipswitch, MA, USA) at a.a. 386 and *Xho*I (New England Biolabs, Ipswitch, MA, USA) 3′ to the MPO coding sequence (Figure 2).

The 'laBcd' chimeric was generated from 'labcd' and a newly generated 'Bcd' fragment. Sequential PCR technique was applied to generate the segment 'Bcd'. The murine sequence contains an *Apa*I site at the 'a–b' join that the human sequence lacks. Thus, a new *Apa*I site was introduced at the 5′ end of 'B' during the PCR generation of the 'B' fragment. In a second PCR, the segment 'cd' with an *Apa*I site in the 3′ end was generated using the 'labcd' as template. A third PCR used the segments 'B' and 'cd' as templates to create 'Bcd'. The 'bcd' fragment was excised from 'labcd' using the *Apa*I sites and the new 'Bcd' was subcloned into the 'pcDNA3-la' DNA. This approach was also used to generate the 'labcD' chimeric molecule, using 'labcd' as the initial template.

The 'LABcD' chimeric was generated using 'LABCD' as template. The restriction enzyme *Kpn*I (New England Biolabs) was utilized to cut out 'C' at a.a. 516 and 667 and replace it by the corresponding mouse 'c' segment, which was isolated after similar *Kpn*1 digestion.

For each chimera, the final cDNA construct cloned into the expression vector was sequenced using external and internal sequencing primers to verify that it was complete and corresponded to the desired sequence.

Human embryonic kidney 293 cells were cultured in Dulbecco's modified Eagle's medium/F12 medium (Gibco: Invitrogen Corp, Carlsbad, CA, USA) plus 10% fetal bovine serum (Gibco). For each construct, 8×10^5 cells were transfected with 20 μg of plasmid by electroporation (Bio-Rad, Hercules, CA, USA) at 200 V and 960 μF. Geneticin® (400 μg/ml) (Gibco)-resistant cell clones producing recombinant protein, as determined by enzyme-linked immunosorbent assay, were expanded and transferred to serum-free Dulbecco's modified Eagle's medium/F12 1 week prior to collection of cell culture supernatant.

Purification of recombinant proteins

Proteins from cell culture supernatant were precipitated using ammonium sulfate at 4°C in the presence of protease inhibitors. The

suspension was centrifuged at 18 600 *g* for 30 min. The pellet was dissolved in and dialyzed against the sample buffer (50 mM Tris buffer, 0.02% azide, pH 7.5). The samples were run through an anion exchange column (UnoQ, Bio-Rad) and the flow-throughs containing the MPO protein were collected and concentrated using ultrafiltration devices (Vivaspin, VivaScience). The purity of the recombinant proteins was determined by analysis of silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and by Western blot analysis. Protein concentration was determined by measuring the absorbance at 280 nm and confirmed by comparing intensities of recombinant protein bands with a known amount of purified human MPO (Calbiochem) on silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting

A 4–15% Tris-HCl gel (Bio-Rad) was run in Laemmli buffer system at 100 V for 90 min and stained using a Silver Stain Plus kit (Bio-Rad). For immunoblotting, proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH, USA) (100 V for 60 min). The membranes were blocked with 10% non-fat milk in Tris-buffered saline + 0.05% Tween and then incubated overnight at 4°C with a rabbit anti-human MPO antibody diluted 1:5000. The membranes were washed and incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (Chemicon, Temecula, CA, USA) for 1 h; proteins were detected with Super-Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

Capture enzyme-linked immunosorbent assay of recombinant MPO proteins

StrepTactin®-coated plates (IBA, Germany) were coated overnight at 4°C with recombinant human and mouse MPO and chimeric molecules at 0.1 μg/well, the saturation concentration for the StrepTactin® plates. The plates were washed and blocked with fish gelatin buffer (2 g/l in Tris-buffered saline + 0.05% Tween) for 1 h at room temperature. MPO-ANCA sera, diluted 1:100 in fish gelatin buffer, were incubated for 3 h at room temperature. Polyclonal rabbit anti-human MPO antibody (1:5000 dilution) served as a positive control. Sera from healthy donors (diluted 1:100) served as negative controls. Secondary antibodies, alkaline phosphatase-conjugated goat anti-rabbit IgG (Pierce) and goat anti-human IgG (Pierce) (diluted 1:5000) were added for 1 h at room temperature. Bound antibodies were detected with an Alkaline Phosphatase Substrate Kit (Bio-Rad). Absorption was measured spectrophotometrically at 405 nm after 1 h. All assays were carried out in duplicate.

Data analysis

The reactivity of normal sera to the various recombinant proteins varied substantially. For this reason, we first normalized the reactivity of each serum sample against the 'background' by testing each serum sample against the blocking buffer as a no-antigen control ($OD_{background}$) according to the following equation:

$$X = \frac{OD_{chimeric} - OD_{background}}{OD_{chimeric}} \quad (1)$$

Equation (1) was used to calculate the reactivity of each sample to each recombinant molecule. The reactivity of 11 normal sera against each chimeric molecule was also determined using Equation (1). For the reactivity of a serum sample to a recombinant molecule to be considered positive, it had to be greater than the average + 2 s.d. of the normal sera for that molecule using Equation (1).

In order to compare the reactivity of a serum sample to the various chimeric molecules, we normalized its binding to each chimeric molecule relative to its binding to the recombinant human MPO according to Equation (2):

$$X = \frac{\frac{OD_{chimeric} - OD_{background}}{OD_{chimeric}}}{\frac{OD_{rec.human-MPO} - OD_{background}}{OD_{rec.human-MPO}}} \quad (2)$$

We assumed that the maximal binding of a serum sample would be to the recombinant human MPO. Thus, if the reactivities of a serum sample to the chimeric molecule and recombinant human MPO are the same, the ratio is 1. A ratio of 0.5 would indicate a 50% loss of reactivity to the chimeric molecule compared to the recombinant human MPO. We used a ratio >0.5 as the threshold for determining the substantial preservation of binding to the chimeric molecule when compared to that of the recombinant human MPO.

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