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# Anti-idiotypic activity against anti-myeloperoxidase antibodies in pooled human immunoglobulin

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## SUMMARY

We investigated the ability of six different pooled human immunoglobulin (PHIG) preparations to inhibit the binding of anti-myeloperoxidase (MPO) antibodies to MPO. All six PHIG preparations inhibited the binding of anti-MPO antibodies from six sera to MPO in a concentration-dependent manner in the concentration range 0·016–10 mg/ml. There was considerable variation in the ability of each PHIG preparation to inhibit the binding of anti-MPO antibody in a given serum. Further differences were seen in the ability of a given PHIG to inhibit anti-MPO binding in different sera. $F(ab')_2$ fragments from two PHIG preparations also inhibited in a concentration-dependent manner anti-MPO binding to MPO in all six sera in the concentration range 0·002–2·65 mg/ml, with a maximum inhibition of 42%. Little inhibition was seen with $F(ab')_2$ of normal human IgG from individual donors (1·8–12·2% at the maximum concentration of 2 mg/ml). $F(ab')_2$ fragments from three anti-MPO containing sera and two affinity-purified anti-MPO antibodies were eluted by affinity chromatography from Sepharose-bound PHIG $F(ab')_2$ and showed anti-MPO antibody activity. We have shown that PHIG and $F(ab')_2$ fragments of PHIG inhibit anti-MPO binding to MPO, and further that $F(ab')_2$ fragments of PHIG bind to $F(ab')_2$ fragments of anti-MPO antibodies. These observations indicate binding between the variable regions of PHIG and the antigen binding site of anti-MPO antibodies, and are consistent with an anti-idiotypic reaction. The variability seen in the inhibitory effect of the different PHIG preparations in anti-MPO-positive sera implies differences in their anti-idiotype content, while the variability of the inhibitory effect of a particular PHIG preparation between different sera suggests heterogeneity in the idiotypic repertoire of anti-MPO antibodies. Such variations in the inhibitory effect of different PHIG preparations on antibody binding may be an important determinant of their therapeutic effect.

**Keywords** anti-myeloperoxidase idiotype anti-idiotype immunoglobulin

## INTRODUCTION

The benefit of intravenous pooled normal human immunoglobulin (PHIG) in autoimmune disease was first reported in children with autoimmune thrombocytopenia in 1981 [1]. Subsequently PHIG was shown to be of benefit in a number of other autoimmune and immune-related diseases [2,3]. Several studies have shown that PHIG contains anti-idiotypic antibodies to several autoantibodies. These include antibodies to factor VIIIc [4], DNA, thyroglobulin, intrinsic factor [5,6], neuroblastoma [7], acetylcholine receptor [8], gp11b/111a [9] and neutrophil cytoplasmic antigens [10]. These studies raised the suggestion that the mechanism for the therapeutic effect of PHIG in autoimmune diseases was through idiotypic regulation of the immune response. In patients with a vasculitis and anti-neutrophil cytoplasmic antibodies (ANCA) who were treated with PHIG, a possible beneficial effect was seen with a reduction in disease activity, together with a fall in serum ANCA levels [11]. It is not known whether different PHIG preparations are equally effective in inhibiting the binding of a single autoantibody. It is also unknown whether a single PHIG preparation inhibits in a comparable manner the same autoantibody in different sera. Anti-myeloperoxidase (MPO) antibodies are found in the sera of patients with microscopic polyarteritis and idiopathic crescentic glomerulonephritis [12,13]. The aims of our study were to determine whether PHIG inhibited the binding of anti-MPO to MPO, and if so whether this inhibition was through idiotype–anti-idiotype interactions, and finally whether different PHIG preparations had comparable inhibitory activity.

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## MATERIALS AND METHODS

We studied sera from six patients with biopsy-proven microscopic polyarteritis and anti-MPO antibodies. Six different therapeutic PHIG preparations were studied: Sandoglobulin (SG) (Sandoz), Venoglobulin (VG) (Alpha), Gamimune N (GI) (Cutter), Anti-D (AD BPL) (Blood Products Laboratory), Anti-D (AD Ortho) and Intramuscular Immunoglobulin (IMIg) (BPL).

*Anti-MPO ELISA*

Microtitre plates were coated with MPO (Calbiochem, Nottingham, UK) at a concentration of 10 μg/ml in 0·05 M bicarbonate buffer pH 9·6 for 16 h at 4°C. The plates were blocked with 2% bovine serum albumin (BSA). Sequential incubations were performed at 37°C for 1 h as follows: (i) sera or anti-MPO $F(ab')_2$ in 0·1 M PBS/0·05% Tween 20 (v/v); (ii) alkaline phosphatase-conjugated sheep anti-human IgG (Fc) (The Binding Site, Birmingham, UK) at a dilution of 1:1600 or alkaline phosphatase-conjugated goat polyclonal anti-$F(ab')_2$ (Sigma, Poole, UK) at a dilution of 1:2000 respectively; (iii) *p*-nitrophenyl phosphate substrate (1 mg/ml) in 10% diethanolamine buffer pH 9·8 and the optical density (OD) read at 405 nm. The plates were washed between incubations with 0·15 M saline/0·05% Tween 20 (v/v).

*Preparation of IgG $F(ab')_2$ fragments from patients' sera, normal sera and PHIG*

IgG was purified separately from three sera containing anti-MPO antibodies and two normal human sera by affinity chromatography on protein-A Sepharose. A Sepharose-protein A column was equilibrated with 0·1 M PBS pH 7·4. Serum (5 ml) was applied to the column in 0·1 M PBS pH 7·4, washed with the same buffer until no protein was found in the flow through fraction, and bound IgG eluted with 0·1 M glycine HCl pH 2·6. The pH of the eluate was adjusted rapidly to pH 7·4 with 1 M Tris and the protein concentration determined by the OD. $F(ab')_2$ fragments were prepared from patient IgG, normal human IgG and PHIG by pepsin digestion followed by chromatography on protein-A Sepharose. IgG preparations isolated as above were dialysed against 0·2 M acetate buffer pH 4 for 2 h and then incubated with pepsin (Sigma) 1 mg/50 mg IgG (2% w/w). The reaction was stopped after 16 h by dialysis against 0·1 M PBS pH 7·4 for 16 h at 4°C. $F(ab')_2$ fragments were purified by affinity chromatography of the pepsin digest on Sepharose-bound protein A. The protein-A Sepharose column was equilibrated with 0·1 M PBS pH 7·4. The pepsin digest was applied to the column in 0·1 M PBS pH 7·4 and the flow through fraction containing $F(ab')_2$ collected. The concentration of $F(ab')_2$ was determined by OD.

*ELISA to check purity of IgG $F(ab')_2$ fragments*

Microtitre plates were coated with mouse monoclonal anti-human IgG (Fc) (The Binding Site) at 10 μg/ml in 0·05 M bicarbonate buffer pH 9·6 at 4°C. The plates were blocked with 2% BSA and sequential incubations performed at 37°C for 1 h as follows: (i) purified IgG $F(ab')_2$ fragments; (ii) horseradish peroxidase (HRP)-conjugated sheep anti-human IgG (Fc) (The Binding Site) at a dilution of 1:25 000 (previously incubated for 1 h with 5% normal mouse serum in 2% BSA/PBS Tween); (iii) *o*-phenylenediamine substrate in 0·05 M citrate phosphate buffer pH 5. The reaction was stopped with 20% sulphuric acid after 5 min and the OD read at 492 nm. The plates were washed between incubations with 0·15 M saline/0·05% Tween.

*Inhibition of anti-MPO activity*

The dilution of each patient sera giving 50% of maximum anti-MPO activity was determined in a preliminary anti-MPO ELISA. Sera at these dilutions were then incubated for 1 h at 37°C, in a series of inhibition assays, with PHIG, normal human IgG, myeloma IgG (IgG1 and IgG2 κ (Immunodiagnostic Research Laboratory, University of Birmingham, Birmingham, UK)) or albumin in the concentration range 0·016–10 mg/ml, or $F(ab')_2$ fragments of PHIG in the concentration range 0·002–2·65 mg/ml, or of normal human IgG in the concentration range 0·003–2 mg/ml. The anti-MPO ELISA was then performed as previously described. Per cent inhibition was calculated as follows:

$$\text{Per cent inhibition} = \frac{\text{OD without inhibitor} - \text{OD with inhibitor}}{\text{OD without inhibitor}} \times 100$$

*Preparation of cyanogen bromide-activated Sepharose 4B MPO and PHIG $(Fab')_2$ affinity chromatography columns*

PHIG $F(ab')_2$ (14 mg) or MPO (8 mg) was coupled to cyanogen bromide-activated Sepharose 4B (1 g/10 mg protein) (Sigma). The PHIG $F(ab')_2$ and MPO were dialysed against 0·1 M $NaHCO_3$/0·5 M NaCl pH 8·3 for 16 h at 4°C. The Sepharose was washed (×3) with 1 mM HCl and the PHIG $F(ab')_2$ or MPO added to the gel and rotated for 16 h at 4°C. The gel was washed (×2) with 0·1 M $NaHCO_3$/0·5 M NaCl pH 8·3. Tris (0·1 M) HCl pH 8 was added and the gel rotated for 16 h at 4°C. Finally the product was washed with three cycles of alternating 0·1 M acetate/0·5 M NaCl pH 4 and 0·1 M Tris/0·5 M NaCl buffer pH 8.

*Purification of anti-MPO antibody from anti-MPO sera*

Anti-MPO antibodies were purified from two anti-MPO-containing patient sera. Serum (5 ml) was applied to the Sepharose-bound MPO column in 0·1 M PBS pH 7·4, washed with the same buffer until no protein was found in the flow-through fraction, and bound anti-MPO eluted with 0·1 M glycine HCl pH 2·6. The pH of the eluate was adjusted rapidly to pH 7·4 with 1 M Tris and the protein concentration determined by the OD. $F(ab')_2$ fragments of the affinity-purified anti-MPO antibodies were prepared by pepsin digestion and affinity chromatography on protein A Sepharose as previously described.

*Affinity chromatography of patient IgG $F(ab')_2$ or purified anti-MPO $F(ab')_2$ on Sepharose-bound PHIG $F(ab')_2$*

The PHIG $F(ab')_2$ column was equilibrated with 0·1 M PBS pH 7·4. Patient IgG $F(ab')_2$ or purified anti-MPO $F(ab')_2$ in 0·1 M PBS pH 7·4 was applied to the column and the flow-through fraction recirculated for 20 h at 4°C. The column was washed with PBS until no protein was found in the flow-through fraction, and bound $F(ab')_2$ was eluted with 0·1 M glycine HCl buffer pH 2·6 and immediately dialysed against 0·1 M PBS pH 7·4 for 2 h at room temperature. This was then concentrated using Minicon-B15 (Amicon) and the protein concentration determined by OD. The anti-MPO activity of the eluted $F(ab')_2$ was measured by the anti-MPO ELISA as previously described, and activity described as OD divided by mg of protein.

*Assay for MPO*

PHIG preparations were tested for the presence of MPO. Hydrogen peroxide (50 μl/0·4 mM) diluted in dianiside dihydrochloride was added to 50 μl of each of the PHIG preparations and to serial dilutions of standard MPO (range 0·625-12 μg/ml) in a microtitre plate. After 4 min at 37°C the OD was read at 450 nm and the amount of MPO in the test samples determined from the standard curve. The limits of detection of MPO in this assay were 1·25 μg/ml.

## RESULTS

*MPO and anti-MPO activity in PHIG*

Neither MPO nor anti-MPO activity was detectable in the PHIG preparations.

*Inhibition of anti-MPO activity by PHIG*

All six PHIG preparations inhibited the binding of anti-MPO antibodies in patient sera to MPO in a concentration-dependent manner through the range 0·016–10 mg/ml, with a maximum of 100% inhibition (Fig. 1). There was considerable variation in the inhibitory effect of the six PHIG preparations in any one patient serum and of any one PHIG preparation between the six sera. To analyse this further the concentration of each PHIG that inhibited by 50% the binding of anti-MPO antibodies in the six sera to MPO is shown in Fig. 2. Further, the ability of a given PHIG preparation to inhibit anti-MPO binding in the six sera is shown in Fig. 3.

*Inhibition of anti-MPO activity by F(ab')$_2$ PHIG*

F(ab')$_2$ fragments were prepared from two PHIG preparations (Anti-D BPL and Gamimune), and were also found to inhibit anti-MPO binding through concentration range 0·002–2·65 mg/ml, with a maximum inhibition of 42% (Fig. 4).

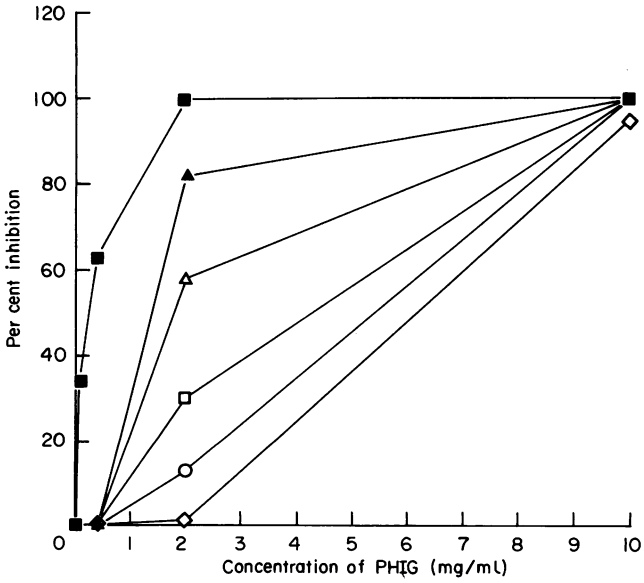


**Fig. 1.** Inhibition of the binding of anti-myeloperoxidase (MPO) antibodies to MPO in a typical serum by six pooled human immunoglobulin (PHIG) preparations (SG (□), VG (△), GI (▲), AD BPL (■), AD Ortho (○), IMIg (◇))—see text for explanation of abbreviations.

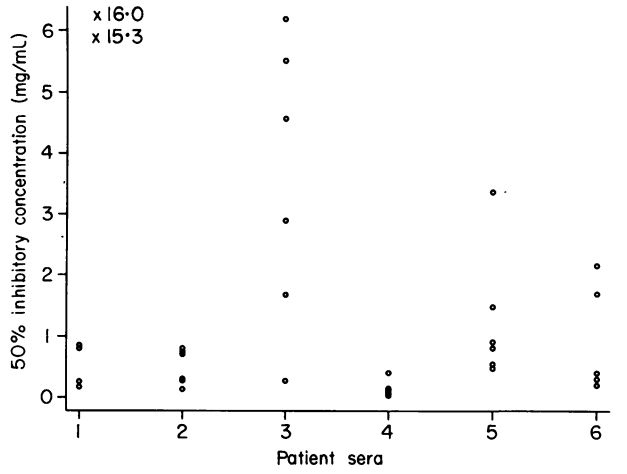


**Fig. 2.** Graph showing the concentration of each pooled human immunoglobulin (PHIG) that inhibited by 50% the binding of anti-myeloperoxidase (MPO) antibodies from six different sera to MPO.

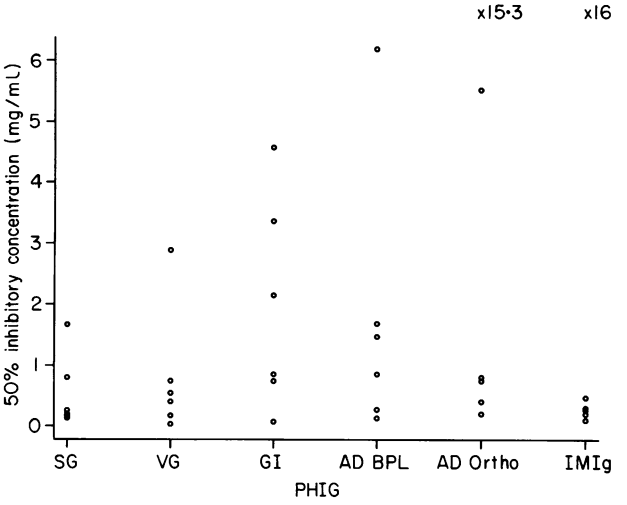


**Fig. 3.** Graph showing the concentration of pooled human immunoglobulin (PHIG) inhibiting by 50% the binding of anti-myeloperoxidase (MPO) antibodies to MPO in each of six different sera.

*Inhibition of anti-MPO activity by albumin, myeloma IgG and single-donor normal human IgG*

No inhibition of anti-MPO binding was seen with albumin. The maximum inhibition of anti-MPO binding with myeloma IgG1 and IgG2 was 18·4% and 10·1% respectively, at a maximum concentration of 10 mg/ml. Two normal human IgG preparations inhibited all three patient sera by 16·2–36% at a maximum concentration of 10 mg/ml. F(ab')$_2$ fragments of normal IgG inhibited anti-MPO binding by 1·8–12·2% at a maximum concentration of 2 mg/ml.

*Affinity chromatography of patient IgG F(ab')$_2$ and purified anti-MPO F(ab')$_2$ on Sepharose-bound PHIG F(ab')$_2$*

Anti-MPO antibody activity was detected in F(ab')$_2$ from three anti-MPO antibody-containing sera eluted by affinity chromatography on a PHIG (Gamimune) F(ab')$_2$ column.

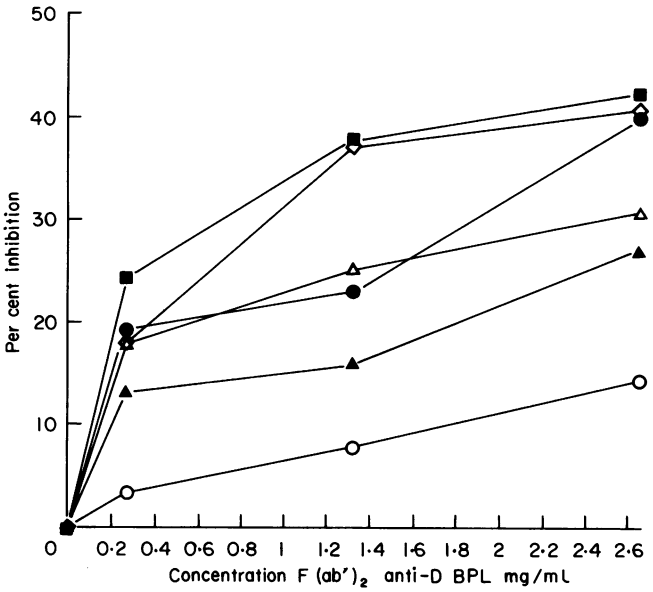


**Fig. 4.** Inhibition by $F(ab')_2$ anti-D BPL (mg/ml) of the binding of anti-myeloperoxidase (MPO) antibodies in six different sera to MPO. ○, Serum 1; ●, serum 2; ▲, serum 3; ■, serum 4; △, serum 5; ◇, serum 6.

**Table 1.** Affinity chromatography of patient IgG $F(ab')_2$ and affinity-purified anti-myeloperoxidase (MPO) $F(ab')_2$ on Sepharose-bound pooled human immunoglobulin (PHIG) $F(ab')_2$. Specific anti-MPO activity as measured by OD per mg of loaded $F(ab')_2$ and eluted $F(ab')_2$ is shown

| Loaded IgG $F(ab')_2$ | Amount loaded (mg) | Specific anti-MPO activity (OD/mg) | Amount eluted (mg) | Specific anti-MPO activity (OD/mg) |
|---|---|---|---|---|
| Patient 1 | 11·2 | 3·62 | 0·14 | 3·25 |
| Patient 2 | 15·0 | 10·41 | 0·38 | 3·57 |
| Patient 3 | 15·0 | 9·22 | 0·44 | 9·18 |
| Anti-MPO 1 | 2·3 | 52·0 | 0·05 | 30·6 |
| Anti-MPO 2 | 2·4 | 1·98 | 0·04 | 1·95 |

There was no enrichment of anti-MPO antibody activity in the $F(ab')_2$ eluates compared with the loaded patient $F(ab')_2$. There was specific binding between PHIG $F(ab')_2$ and the $F(ab')_2$ of affinity-purified anti-MPO (Table 1).

## DISCUSSION

PHIG are therapeutic preparations of human polyspecific IgG derived from large but varying sized pools of healthy blood donors. The presence in PHIG of anti-idiotypic antibodies against disease-associated autoantibodies was first suggested by a study of two patients with anti-factor V111c autoantibodies [4]. Since then PHIG from several sources has been shown to express a wide spectrum of anti-idiotypic antibody activity to both naturally occurring [14,15] and disease-associated autoantibodies [4–10]. Anti-MPO antibodies are found in systemic vasculitis and idiopathic necrotising and crescentic glomerulonephritis [12,13]. Since the levels of P-ANCA, the immmunofluorescent pattern of anti-MPO antibodies, correlate with disease activity [16,17], they may be of pathogenic importance. MPO is cationic and binds to the anionic surface of endothelial cells, where it reacts with anti-MPO antibodies *in situ* [18]. Moreover, these antibodies have been shown to activate tumour necrosis factor (TNF)-primed neutrophils which degranulate and release reactive oxygen products, and may thus cause bystander damage to endothelium [19–21].

We have shown that PHIG and $F(ab')_2$ fragments of PHIG inhibit the binding of anti-MPO antibodies to MPO. As with previous studies showing the inhibitory effect of PHIG on various autoantibodies [5,10], we found that the inhibition was concentration-dependent. No inhibition of anti-MPO activity was seen with albumin, and the PHIG studied lacked both MPO and antibody activity to MPO. Normal human IgG inhibited the binding of anti-MPO to MPO, but this inhibition was greatly reduced when $F(ab')_2$ fragments of normal human IgG were tested. This suggests that the inhibition by whole IgG was in part due to steric hindrance. There was little inhibition by myeloma IgG, which only inhibited by a maximum of 18·4% at 10 mg/ml, whilst PHIG inhibited by 100% at the same concentration.

$F(ab')_2$ fragments of anti-MPO antibodies in patient sera bound to Sepharose-bound PHIG $F(ab')_2$, and after elution contained anti-MPO antibody activity. No enrichment of anti-MPO antibody activity was seen in the eluates, unlike in other studies [5,10], and this needs to be explained. The patient sera from which these $F(ab')_2$ fragments are derived contain antibodies against a range of antigens. The PHIG preparations are likely to contain anti-idiotypic antibodies against a wide range of antibodies [22], including those found in the patient $F(ab')_2$ preparations, and would therefore bind such antibodies. There is no particular reason why $F(ab')_2$ in PHIG should bind to a greater extent to anti-MPO binding $F(ab')_2$ than to $F(ab')_2$ in the patient sera directed against other antigens. Specific binding between affinity-purified anti-MPO $F(ab')_2$ and PHIG $F(ab')_2$ was demonstrated. With one of these affinity-purified anti-MPO $F(ab')_2$ there was a reduction in specific activity, suggesting that some idiotypes in this preparation were not recognized by PHIG.

We have shown that PHIG $F(ab')_2$ has antibody activity directed against the antigen binding site of anti-MPO antibodies, and these can be regarded as anti-idiotypic antibodies. In keeping with the findings of previous studies [5,10], there was considerable variation in the inhibitory effect of a particular PHIG preparation in different anti-MPO-containing sera. This suggests heterogeneity in the idiotypic repertoire of anti-MPO antibodies in different individuals. Studies using specific heterologous anti-idiotype antibodies will help to define these idiotypes on anti-MPO antibodies. In addition we were able to demonstrate a variation in the inhibitory capacity of different PHIG preparations in the same anti-MPO-containing serum. This implies variation in the spectrum of anti-idiotype antibodies between the different PHIG preparations. The anti-idiotype antibody content in a particular PHIG to an autoantibody depends upon the size of the donor pool, the proportion of dimers and the contribution of privileged donors who have previously developed the relevant autoantibody to the pool [22].

PHIG has been used in several autoimmune conditions [1–

4,7,23–28], although proven benefit through controlled trials has been established in only a few: childhood acute idiopathic thrombocytopenic purpura [26], chronic inflammatory demyelinating polyneuropathy [27] and Kawasaki disease [28]. The mechanisms of action of PHIG are likely to be multiple [2,3]. Fc-dependent mechanisms include blockade of Fc receptors on phagocytic cells [29,30]; suppression of B lymphocyte function and modulation of T cell function [31,32]. There is also evidence that PHIG can modulate the synthesis and release of inflammatory cytokines and cytokine antagonists by monocytes and macrophages [33]; and bind to C3b and C4b, thereby preventing the binding of complement components to its target [34]. The data presented in this study show that idiotypic interactions between PHIG and autoantibodies lead to neutralization of autoantibodies. It is possible that this may in the longer term modulate the autoimmune repertoire through an interaction with PHIG-reactive B and T cells [35,36]. Indeed a decline in autoantibody titres, *in vivo*, along with a clinical reponse has been shown following PHIG treatment in anti-factor VIII disease [4], idiopathic thrombocytopenia [23] and in ANCA-associated systemic vasculitis [11].

In ANCA-associated systemic vasculitis the fall in the circulating ANCA activity was found to correlate with the degree of pretreatment inhibition of ANCA by PHIG *in vitro* [11]. If there is similar correlation between the *in vitro* inhibition of anti-MPO binding to MPO by PHIG and *in vivo* anti-MPO levels as well as disease activity following PHIG therapy, then the variation in the inhibitory capacity of the differing PHIG preparations noted *in vitro* will influence the choice of PHIG used in the treatment of patients with anti-MPO-positive disease. Current prospective trials using PHIG in ANCA-positive vasculitis will allow these correlations to be studied.

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