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Anti-myeloperoxidase antibodies stimulate neutrophils to damage human endothelial cells

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Anti-myeloperoxidase antibodies stimulate neutrophils to damage human endothelial cells. Anti-myeloperoxidase autoantibodies are found in association with idiopathic necrotizing glomerulonephritis and systemic vasculitis. It is not known if their presence is an epiphenomen or an integral part of the pathogenic process. To further delineate their hypothesized pathogenicity, we studied their ability to stimulate neutrophils to damage human umbilical vein endothelial cells in vitro. Anti-myeloperoxidase antibodies from human, rabbit and mouse sources were utilized. These antibodies stimulated neutrophils to damage endothelial cells as determined by ^{51}Cr release. The effect was dependent on priming the neutrophils with tumor necrosis factor-α, and further enhanced with the addition of endotoxin. The amount of endothelial cell damage was dependent on the dose of anti-myeloperoxidase, the source of the neutrophils, the concentration of TNF, and the presence of endotoxin. Under identical conditions, control antibodies did not stimulate neutrophils to damage endothelial cells. The effect was confirmed by labeling the endothelial cells with ^{3}H-adenine which yielded the same results. These results provide further in vitro evidence that anti-myeloperoxidase autoantibodies may play a significant role in the pathogenesis of idiopathic pauci-immune glomerulonephritis and vasculitis.

Anti-neutrophil cytoplasmic antibodies (ANCA) are autoantibodies directed against constituents of the primary granules of polymorphonuclear leucocytes (PMN) and lysosomes of monocytes. They are found in the sera of patients with pauci-immune necrotizing glomerulonephritis and systemic vasculitis, including Wegener's granulomatosis and polyarteritis nodosa [1]. ANCA that produce an artifactual perinuclear staining by indirect immunofluorescence microscopy on alcohol-fixed PMN (P-ANCA) are usually specific for myeloperoxidase (MPO) [2]. Most ANCA that produce a cytoplasmic pattern by indirect immunofluorescence (C-ANCA) are directed against proteinase 3 [3].

The finding of an autoantibody directed against one of the main cells involved in the acute phase of the immune response has led to the hypothesis that ANCA play a pathogenic role in glomerular and vascular inflammation. The potential pathogenic function of ANCA has been supported by the finding that these autoantibodies stimulate PMN in vitro to degranulate and produce reactive oxygen species [4]. In this study, we investigated whether anti-myeloperoxidase (αMPO) antibodies could stimulate PMN to injure human endothelial cells in vitro.

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Methods

Isolation of endothelial cells

Human endothelial cells (HUVEC) were isolated from umbilical cord veins by collagenase (Sigma Chemical Co., St. Louis, Missouri, USA) digestion based on Gimbrone's method [5]. They were cultured in RPMI-1640 with 20% HI fetal calf serum, 50 μg/ml gentamicin, 200 μg/ml kanamycin, 90 μg/ml heparin (Sigma) and 4.0 ng/ml acidic fibroblast growth factor (R & D Systems, Minneapolis, Minnesota, USA). The cells were grown to near confluence in tissue culture dishes that had been coated with 1% gelatin (Sigma) and then passed with trypsin 0.05%/EDTA 0.02% into 48 well plates (Costar, Cambridge, Massachusetts, USA) for the injury assay. All cells were used at the second or third passage. Endothelial cell phenotype was confirmed by typical cobblestone morphology and the presence of von Willebrand's factor antigen using rabbit anti-human vWF (DAKO, Carpinteria, California, USA). All effects were observed in wells that were 100% confluent. The density of the endothelial cells was 100,000 cells/well.

Isolation of polymorphonuclear leucocytes

Human PMN were obtained from normal human donors. Fifty milliliters of whole blood was drawn into a heparinized syringe and the RBCs sedimented with "Plasmagel" (Cellular Products, Buffalo, New York, USA). The PMN were separated with a Ficoll-Hypaque gradient (Sigma) and the remaining RBCs removed with hypotonic lysis.

Antibodies

Plasma was obtained from patients who had undergone plasmapheresis as part of the treatment of their disease. The diagnoses of the patients were MPO-ANCA associated pauci-immune necrotizing vasculitis and ANCA (−) necrotizing glomerulonephritis. The plasma was used at a dilution of 1:7.

Patient IgG was prepared by DEAE-Sephacel (Sigma) anion absorption of plasma followed by precipitation of the supernatant with 50% ammonium sulfate. The samples were dialyzed against PBS overnight. The two samples were prepared in parallel and had similar LPS concentrations of less than 120 pg/ml. The final concentration of the IgG in the injury assay ranged from 0.5 mg/ml to 2.0 mg/ml. We have previously demonstrated that heterologous antibodies against azurophilic

granule constituents activate PMN in vitro in a totally analogous fashion to PMN activation by ANCA [4, 6]. Therefore in the experiments described below, both autoantibodies (that is, ANCA) and heterologous antibodies specific for MPO were tested as well as negative control antibodies. Mouse monoclonal anti-human MPO, rabbit polyclonal anti-human MPO (R-αMPO), anti-human placental alkaline phosphatase, and anti-human lysozyme were obtained from DAKO. If not otherwise stated, the final concentration of the R-αMPO was 50 μg/ml.

Stimulators

Recombinant human TNF-α was obtained from Genzyme (Boston, Massachusetts, USA). The biologic activity was 50 pg/U as determined by killing of actinomycin D treated murine L929 cells. Endotoxin from *E. coli* 026:B6 was purchased from Difco (Detroit, Michigan, USA). If not otherwise stated, the concentration of TNF-α was 50 U/ml and of LPS was 500 ng/ml. In the LPS dose response experiment, the concentrations used were 0.5, 1.0, 2.5, 5.0, 7.5, and 10.0 μg/ml.

Injury assay

All conditions were run in duplicate using the same HUVEC source and passage, and the same PMN donor in control and experimental conditions unless otherwise stated. Only HUVEC that were in media that contained <0.01 ng/ml endotoxin (QCL-1000 LAL kit, Whitaker Bioproducts, Walkersville, Maryland, USA) were utilized. HUVEC were radiolabeled with 15 μCi/ml ^{51}Cr (NEN, Boston, Massachusetts, USA) overnight in the 48-well plate and incubated with 300 U/ml TNF-α for three hours in order to prime the endothelial cells [7]. The monolayers were then washed and the experiment performed in RPMI-1640 with 1% HI FCS. At time zero the antibodies plus TNF and/or LPS were added with the PMN. The final concentration of the PMN was 2.0×10^6/ml. The final well volume was 750 μl. The wells were sampled over six hours and the sample volume replaced at each time point with media. The sample volumes of 150 μl were withdrawn from the top of the media so as not to disturb the endothelial monolayer.

The ^{51}Cr CPM were determined on an LKB gamma counter (Model 1272). At the end of the experiment, the cells were lysed with 10 N NaOH and the post-lysis ^{51}Cr CPM measured. These results were expressed as a fraction of the post-lysis radioactivity. The ^{51}Cr release at each time point and the post-lysis was adjusted for the amount present at time = 0, the volume of the well, and the amount previously removed. A representative sample of the ^{51}Cr CPM released is presented in Table 1. To confirm that the ^{51}Cr release was due to lysis and not detachment in two experiments the samples were centrifuged at 400 × g for 10 minutes. The differences in ^{51}Cr release between the various conditions were found only in the supernatant and not in the pellet fractions.

To further substantiate the ability of αMPO to stimulate primed PMN to damage HUVEC, the more sensitive indicator of injury, ^{3}H-adenine (NEN), was substituted for ^{51}Cr as the marker of endothelial cell damage [8]. The HUVEC were incubated for 16 hours with 5 μCi/ml ^{3}H-adenine. They were then handled in an identical manner to the ^{51}Cr protocol. The amount of ^{3}H-adenine released was measured in a liquid scintillation counter (Packard Instrument Co., Downers Grove, Illinois, USA; Model #4530).

Table 1. Representative CPM from duplicate samples

Time (HR)	Total CPM in well					
	α-Alk phos		R-αMPO		M-αMPO	
1	1871	2047	1978	1242	1932	1855
2	1659	2046	3440	2364	1916	2657
3	4395	4906	7707	8409	14974	11855
4	6816	6089	13446	12264	24589	23603
5	7945	8537	16814	15494	34495	32744
6	10064	11327	18568	16723	37757	38102
Post-lysis	60813	56088	59914	57051	64622	66480

Duplicate results of ^{51}Cr released from HUVEC injured by PMN stimulated by control and experimental antibodies. The PMN were stimulated with 10 μg/ml mouse monoclonal anti-human myeloperoxidase (M-αMPO), 50 μg/ml rabbit polyclonal anti-human alkaline phosphatase (α-Alk phos), or 50 μg/ml rabbit polyclonal anti-human myeloperoxidase (R-αMPO) in the presence of 50 U/ml TNF-α and 500 ng/ml LPS. The CPM have been adjusted for background, T = 0 CPM, amount of ^{51}Cr previously removed from the well, and the assay volume removed.

The LDH concentration was determined as previously described [9]. LDH activity was measured in an enzymatic test where NAD^+ is reduced by the conversion of lactate to pyruvate. The NADH then reduces FAD^+ which in turn reduces the tetrazolium dye INT, resulting in a formazan that is measured spectrophotometrically at 490 nm.

Day to day variability in the amount of ^{51}Cr release was observed. On certain days, regardless of the stimulator employed no signal could be detected above the spontaneous release. Both technical and biological causes of this phenomenon were investigated.

Adding 100 ng/ml cold chromium to the assay media significantly increased the amount of ^{51}Cr detected from experimental wells but not control wells. Since PMN also take up ^{51}Cr [10], this technique probably decreased the PMN uptake of ^{51}Cr.

To determine if the periodic unresponsiveness was specific for our stimulus the known stimulator of PMN-mediated HUVEC damage, phorbyl myristate acetate (PMA) [11], was evaluated. In six experiments 100% concordance was observed between the ability of PMA (100 ng/ml) and αMPO antibodies to stimulate PMN to injure endothelial cells. Whether or not the entire assay worked on a given day was independent of the stimulus.

To elucidate the variability attributable to the PMN, six normal donors were evaluated. The PMN were isolated simultaneously and the assay was performed with identical reagents on HUVEC derived from the same umbilical cord.

The rate of release of ^{51}Cr, ^{3}H-adenine, and LDH under various conditions was compared using a multivariant linear regression model, and expressed as the slope ± the standard error. Wilcoxon rank sum test was used for comparison of nonparametric data. The statistics were calculated with the SAS System (SAS Institute, Inc., Cary, North Carolina, USA). The "*N*" values represent the number of experiments in which the effect was observed. The figures are from individual experiments that are representative of the aggregate. Experiments were excluded from analysis when the positive control values were not above spontaneous release levels (for example, as

shown above, when PMA failed to stimulate neutrophil mediated endothelial cell damage).

Anti-endothelial cell antibodies

The direct cytotoxic potential of the antibodies on HUVEC was determined with a complement dependent cytotoxicity assay. Using a modification of the method of Leung et al [12], patient plasma, R-αMPO, and mouse monoclonal αMPO were evaluated. ^{51}Cr labeled HUVEC were incubated with the antibodies diluted in Hank's balanced salt solution with 1% BSA for one hour at 37°C. After washing, the cells were incubated for one hour with a 1:10 dilution of baby rabbit complement (Gibco, Grand Island, New York, USA). The supernatent and post-lysis ^{51}Cr CPM were determined and the results expressed as a fraction of the post-lysis CPM. The anti-HLA-A,B monoclonal antibody W6 (Accurate Chemical & Scientific Corp., Westbury, New York, USA) was used as a positive control.

Results

Effect of patient plasma and IgG on ^{51}Cr release

Plasma containing MPO-ANCA stimulated TNF-α and LPS primed PMN to damage endothelial cells. The slope of ^{51}Cr release from the wells with ANCA (+) plasma was 0.072 ± 0.008 versus 0.009 ± 0.008 for ANCA (−) wells ($P < 0.0001$). At the end of four hours 23% of available ^{51}Cr was released from the ANCA (+) wells versus 6% from the ANCA (−) wells.

The PMN stimulating activity was found to reside in the IgG fraction of the plasma. At a concentration of 1 mg/ml MPO-ANCA IgG added to the PMN with TNF-α and LPS resulted in significantly more ^{51}Cr release ($P < 0.0001$) than the samples with IgG from disease controls ($N = 3$; Fig. 1). A linear dose response effect was observed between 0.5 mg/ml and 2.0 mg/ml IgG ($r^2 = 0.99$). This phenomenon was further characterized using heterologous polyclonal and monoclonal αMPO.

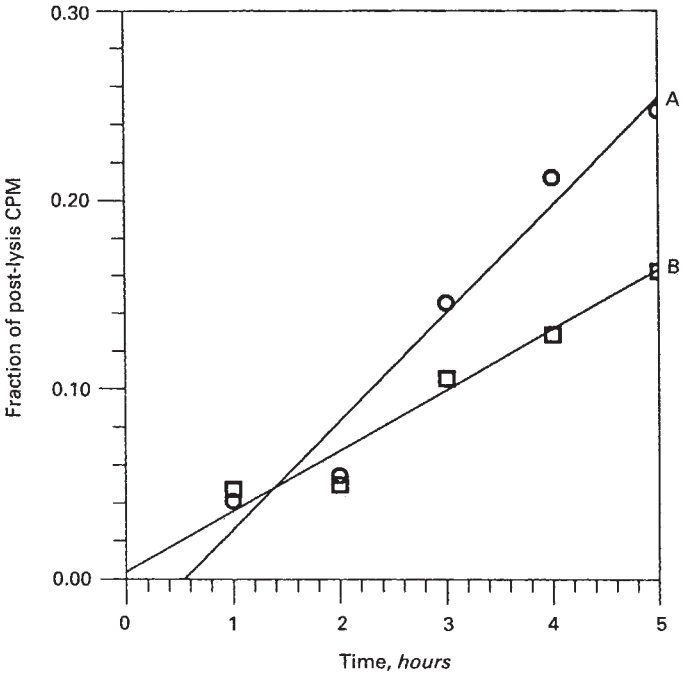

Fig. 1. *Effect of purified patient IgG on PMN injury of HUVEC.* Both contain TNF and LPS; (○) 1.0 mg/ml IgG from ANCA (+) patient (slope = 0.057 ± 0.002); (□) 1.0 mg/ml IgG from patient with α-GBM glomerulonephritis [ANCA (−)] (slope = 0.031 ± 0.002). (○ vs. □: $P < 0.0001$).

Effects of αMPO, TNF-α, and LPS on ^{51}Cr release

The combination of rabbit polyclonal αMPO (R-αMPO), TNF-α and LPS resulted in a significant increase in PMN-mediated ^{51}Cr release from the endothelial cells when compared with TNF-α and LPS without R-αMPO ($N = 14$; $P < 0.0001$; Fig. 2). A dose response effect was observed with the R-αMPO at concentrations of 25 and 50 μg/ml in combination with TNF-α and LPS (Fig. 3). The effect was entirely dependent on priming the PMN. Addition of 25 μg/ml or 50 μg/ml of R-αMPO without other stimulators did not significantly increase the rate of ^{51}Cr release over PMN alone ($N = 2$, $P > 0.05$).

TNF-α alone did not significantly increase ^{51}Cr release over PMN alone ($P > 0.05$, $N = 9$). In combination with R-αMPO, TNF-α produced variable results that were in part concentration dependent. A total of 200 U/ml TNF-α plus R-αMPO resulted in a significant increase in PMN-mediated ^{51}Cr release when compared to R-αMPO alone ($P = 0.0003$) or TNF-α alone ($P = 0.02$; Fig. 4). When the dose was lowered to 50 U/ml inconsistent results were observed. In three experiments, a significant increase in ^{51}Cr release was seen with the addition of R-αMPO ($P < 0.05$). However, in one there was a significant decrease ($P < 0.0001$).

Consistent with published reports, the effect of LPS alone on PMN-mediated ^{51}Cr release from HUVEC was variable [13, 14]. A significant increase in ^{51}Cr release ($P = 0.011$) over PMN alone was seen in one experiment but not in another. When multiple concentrations of LPS ranging from 0.5 to 10.0 μg/ml were evaluated in the absence of R-αMPO significant increases in ^{51}Cr release were seen with 2.5 μg/ml and 10.0 μg/ml ($P < 0.03$) but not with 0.5, 1.0, 5.0 or 7.5 μg/ml. In studies with R-αMPO in combination with LPS the ^{51}Cr slope was not significantly increased over PMN alone ($N = 8$). Thus LPS is not a sufficient primer of PMN to facilitate αMPO stimulated endothelial damage. However, when combined with TNF-α it functioned in a synergistic manner to increase αMPO-stimulated PMN-mediated endothelial damage. R-αMPO and TNF produced a slope of ^{51}Cr release of 0.029 ± 0.005; with the addition of LPS the slope increased to 0.073 ± 0.004 ($P < 0.0001$).

The effect of mouse monoclonal αMPO was of particular interest because it reacts in an identical manner as MPO-ANCA on Western blot analysis [15]. Ten μg/ml of mouse monoclonal αMPO with TNF-α and LPS stimulated PMN to damage HUVEC ($N = 2$). This effect was greater than that observed with R-αMPO ($P < 0.0001$; Fig. 5).

Under identical conditions, control antibodies did not incite PMN to damage endothelial cells. Rabbit polyclonal anti-alkaline phosphatase did not increase the ^{51}Cr release over the TNF-α and LPS alone ($N = 4$, $P > 0.05$). The rate of ^{51}Cr release from TNF-α, LPS and anti-alkaline phosphatase was significantly lower than the samples containing TNF-α, LPS and R-αMPO ($P = 0.03$) or M-αMPO ($P < 0.0001$; Fig. 5). Rabbit anti-human lysozyme and anti-rat albumin produced

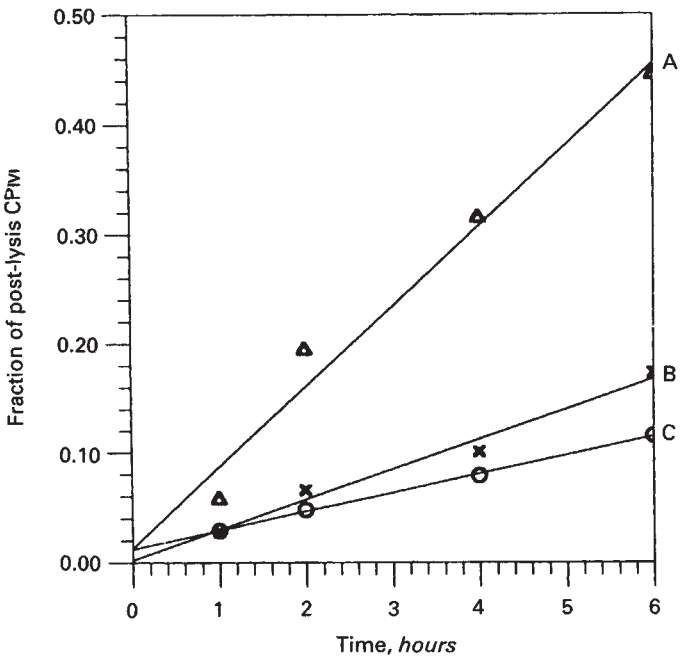

Fig. 2. *Effect of LPS and TNF priming on αMPO stimulated PMN damage of HUVEC.* Symbols are: (△) R-αMPO, TNF and LPS (slope = 0.078 ± 0.004); (X) LPS and TNF (slope = 0.028 ± 0.004); (○) R-αMPO (slope = 0.017 ± 0.004). (△ vs. X or ○: $P < 0.0001$; X vs. ○: $P > 0.05$).

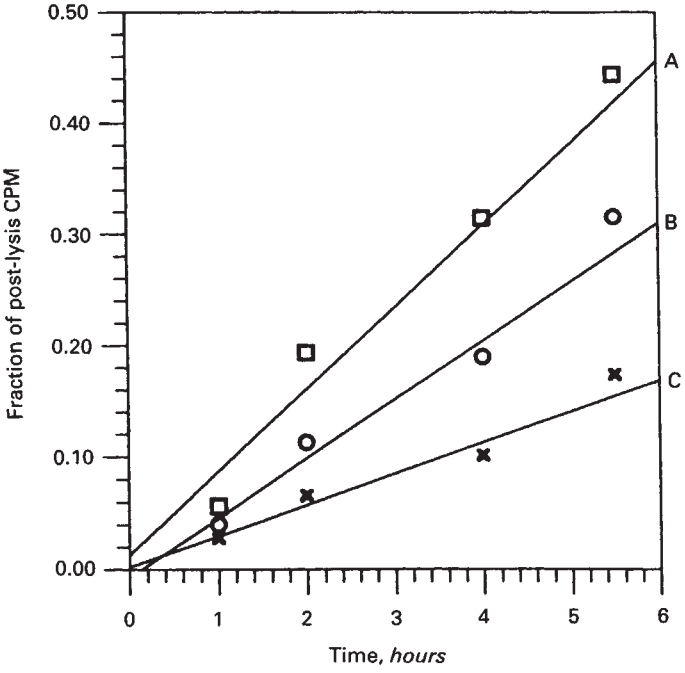

Fig. 3. *Dose effect of R-αMPO.* Symbols are: (□) 50 μg/ml R-αMPO, TNF-α and LPS (slope = 0.078 ± 0.004); (○) 25 μg/ml R-αMPO, TNF-α and LPS (slope = 0.052 ± 0.004). (X) TNF-α and LPS (slope = 0.028 ± 0.004). The slope of each line is significantly different from the other two ($P < 0.0001$).

similar results as the anti-alkaline phosphatase (data not shown).

^{3}H-adenine release is increased by anti-MPO antibodies

In order to test the system with another marker of endothelial cell damage the release of ^{3}H-adenine was investigated. The effects of human MPO-IgG, R-αMPO, M-αMPO, and the negative control antibodies were confirmed in experiments where the HUVEC were labeled with ^{3}H-adenine. Significantly more damage was produced by primed PMN stimulated with human MPO-IgG versus disease control IgG ($P = 0.001$; Fig. 6). The dose response effect of R-αMPO was again demonstrated at 25 and 50 μg/ml (Fig. 7). As seen with ^{51}Cr release the most profound effect was seen with M-αMPO. The slope of ^{3}H-adenine release with M-αMPO plus the primers was 0.250 ± 013, which resulted in 80% of the radiolabel being released at four hours versus 22% for the primers alone (Fig. 7). The addition of the negative control antibody, anti-alkaline phosphatase did not change the slope of ^{3}H-adenine release compared to primers alone.

LDH release

LDH release was investigated as another measure of cell injury, albeit less specific in that it can be released from either HUVEC or PMN. Part of its utility was revealing PMN autotoxicity when the LDH rose but the ^{51}Cr did not. The pattern of LDH release had some similarities to that seen with ^{51}Cr release. R-αMPO did not significantly increase LDH over PMN alone ($N = 2$, $P > 0.05$). TNF-α at concentrations from 10 to 200 U/ml also did not significantly increase LDH release ($N = 4$, $P > 0.05$). However, the combination of TNF-α and R-αMPO resulted in a slope that was significantly higher ($N = 3$; $P < 0.0001$) when compared to TNF-α or R-αMPO alone (Fig. 8).

The pattern of LDH release differed from ^{51}Cr release in regards to the effect of LPS. LPS and R-αMPO resulted in a significant increase in the LDH slope. Over the entire range of LDH concentrations tested, LPS in combination with R-αMPO resulted in a significantly higher LDH slope than R-αMPO or LPS alone ($P < 0.01$). A dose response effect of LPS plus R-αMPO was found. The slope of the sample containing 10.0 μg/ml LPS (slope = 3.23 ± 0.22) was significantly higher than all samples containing 5.0 μg/ml LPS (slope = 2.17 ± 0.22; $P = 0.001$) or less. The addition of higher concentrations of LPS alone never produced the same effect as the addition of R-αMPO.

Neutrophil donor variability

In order to evaluate the day to day variability in the assay PMN from six donors were tested simultaneously on identical HUVEC with TNF-α, LPS, and R-αMPO. As demonstrated in Figure 9, there was a broad range of response from the six donors when R-αMPO was added. When compared to the donor specific control without R-αMPO, two of the donors' PMN demonstrated a significant increase with R-αMPO (Table 2). With PMN from two of the non-responders, the slopes of ^{51}Cr release with R-αMPO were not significantly different than the others without R-αMPO. At t = 1 hour, the LDH concentrations from these two non-responders were significantly

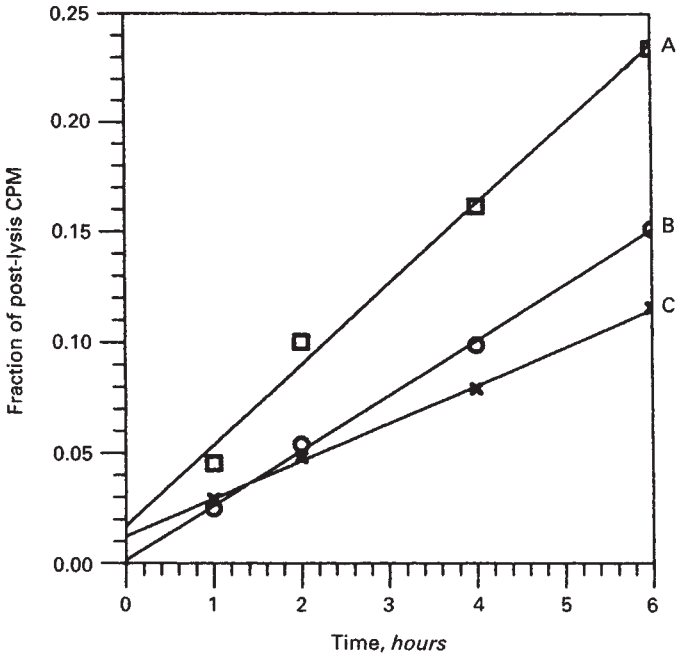

Fig. 4. *Effect of TNF-α priming on αMPO-induced PMN HUVEC damage as measured by ^{51}Cr release.* Symbols are: (□) 220 U/ml TNF-α and R-αMPO (slope = 0.037 ± 0.004); (○) 200 U/ml TNF-α (slope = 0.025 ± 0.004); (X) R-αMPO (slope = 0.017 ± 0.004). (□ vs. ○: $P = 0.02$; □ vs. X: $P = 0.0003$; ○ vs. X: $P > 0.05$).

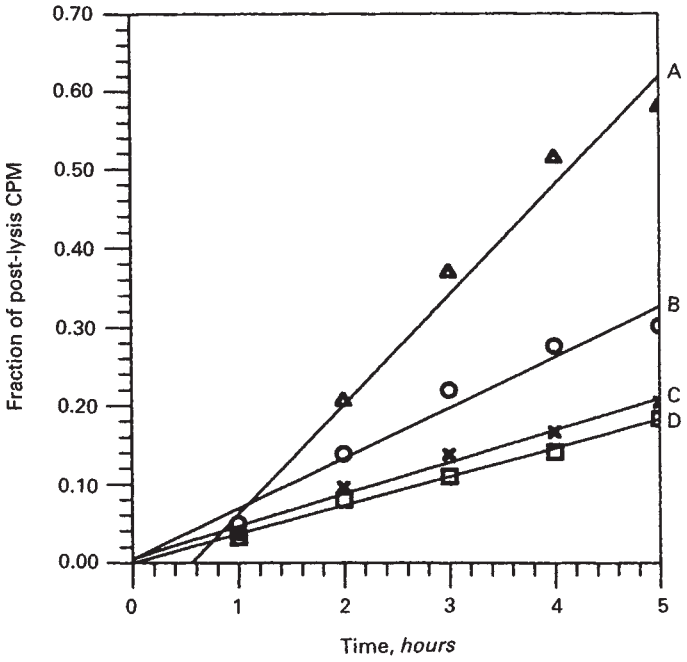

Fig. 5. *Effect of M-αMPO, R-αMPO, and the negative control antibody R-α-alkaline phosphatase on PMN injury of HUVEC.* Symbols are: (△) 10 μg/ml M-αMPO, TNF-α and LPS (slope = 0.140 ± 0.007); (○) R-αMPO, TNF-α and LPS (slope = 0.064 ± 0.007); (X) TNF-α and LPS (slope = 0.041 ± 0.007); (□) 50 μg/ml R-αalkaline phosphatase, TNF-α and LPS (slope = 0.037 ± 0.007). (△ vs. ○, X, or □: $P < 0.0001$; ○ vs. X or □: $P < 0.03$; X vs. □: $P > 0.05$).

higher ($P < 0.0001$) than the four other donors using a Wilcoxon rank sum test.

Since ^{51}Cr is a more sensitive measure of HUVEC damage [16] the LDH increase was attributed to PMN autotoxicity. The hypothesis was tested that PMN overstimulation was the cause of the PMN destruction without endothelial cell damage. The PMN from one of the two non-responding donors were tested with reduced amounts of TNF-α and LPS. Neither 10 nor 50 U/ml of TNF-α alone significantly increased ^{51}Cr release over untreated PMN. TNF-α and R-αMPO resulted in a significant increase in the ^{51}Cr release compared to untreated PMN ($P = 0.0002$) and PMN treated with TNF alone ($P = 0.018$). However, when 10 U/ml TNF-α and 100 ng/ml LPS were added without R-αMPO a significant increase in ^{51}Cr release was observed ($P = 0.009$). Adding R-αMPO increased the slope further, however, the slope was not significantly different than that obtained with the TNF-α and LPS alone ($P > 0.05$). Because these neutrophils were more sensitive to the synergistic effect of LPS and TNF-α substantial HUVEC damage occurred without R-αMPO. Therefore, the HUVEC damage was not further increased upon the addition of R-αMPO. These results confirmed that PMN from this donor were more sensitive to the priming agents. The day to day variability seen in the assay was at least in part attributable to the source of the PMN.

Anti-endothelial cell antibodies

If the MPO-ANCA plasma had cytotoxic anti-endothelial cell antibodies, the increase in ^{51}Cr release could be unrelated to PMN activation by ANCA. In a complement mediated assay, normal pooled sera released 6.7 ± 1.5% (mean ± SD) of the available ^{51}Cr from unstimulated HUVEC and 7.8 ± 2.0% from TNF-α stimulated HUVEC. The positive control anti-HLA antibody caused a significant increase with both unstimulated (32.2%; $P < 0.0001$) and stimulated HUVEC (35.8%; $P < 0.0001$) using a z test. MPO-ANCA released 7.6% of available ^{51}Cr from the unstimulated HUVEC and 6.8% from the stimulated HUVEC. These were not significantly different than normal pooled sera ($P > 0.05$). R-αMPO and M-αMPO were compared to buffer control. Without TNF-α stimulation of the HUVEC, the percent release by buffer, R-αMPO, and M-αMPO was 11.6%, 13.5%, and 8.2%, respectively; with TNF-α stimulation it was 16.1%, 13.0% and 6.4%, respectively. Thus it is unlikely that the damage to endothelial cells is a consequence of direct antibody cytotoxicity.

Discussion

This study has demonstrated several new properties of αMPO antibodies. In addition to activating PMN in vitro, they are also capable of stimulating PMN to damage target cells. Priming of the PMN is essential for endothelial cell damage to occur. The amount of endothelial damage is dependent upon the dose of αMPO present. The effect was demonstrated with αMPO antibodies from four sources: 1) MPO-ANCA plasma, 2) purified patient IgG, 3) rabbit polyclonal αMPO, and 4) mouse monoclonal αMPO. Endothelial cell damage did not occur with control antibodies.

The demonstration of ANCA-stimulated PMN-mediated damage of HUVEC in vitro provides further evidence in support of the hypothesized pathogenic role of these autoantibodies. The association between these antibodies and vasculitis

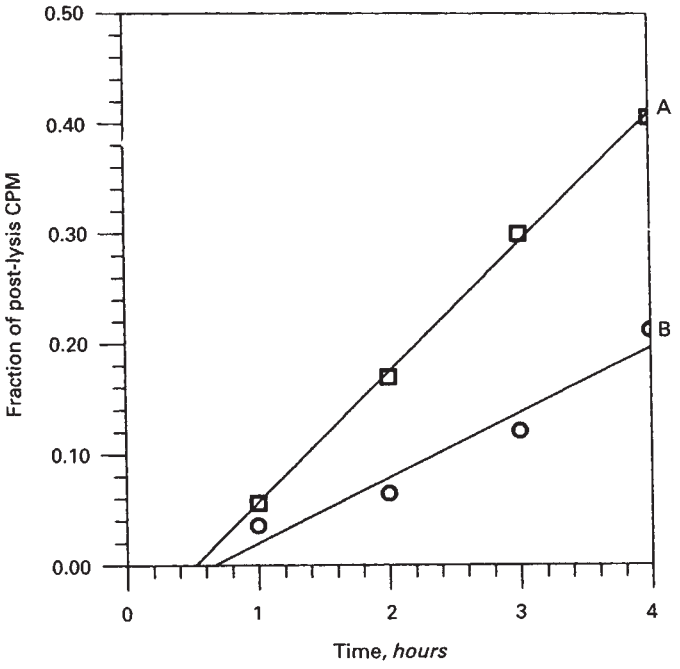

Fig. 6. *Effect of purified patient IgG on PMN injury of HUVEC as measured with ^{3}H-adenine release.* All contain TNF and LPS. Symbols are: (□) 1.0 mg/ml IgG from ANCA (+) patient (slope = 0.118 ± 0.013), (○) 1.0 mg/ml IgG from patient with α-glomerular basement membrane glomerulonephritis [ANCA (−)] (slope = 0.059 ± 0.013). (□ vs. ○: P = 0.002).

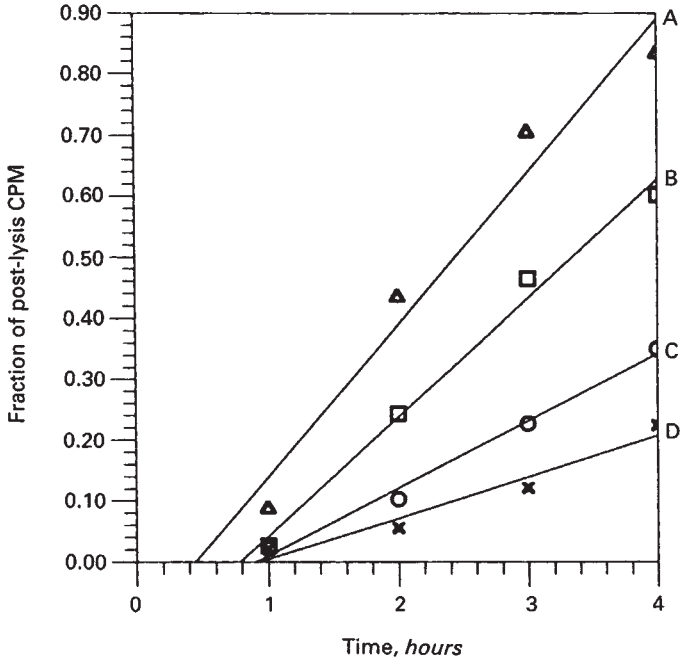

Fig. 7. *Effect of M-αMPO and R-αMPO on PMN injury of HUVEC as measured with ^{3}H-adenine release.* All contain 10 U/ml TNF and 100 ng/ml LPS (△) 10 μg/ml M-αMPO (slope = 0.250 ± 0.013); (□) 50 μg/ml R-αMPO (slope = 0.194 ± 0.013); (○) 25 μg/ml R-αMPO (slope = 0.111 ± 0.013); (**X**) TNF and LPS (slope = 0.067 ± 0.013). (△ vs. ○ or **X**: P < 0.0001; △ vs. □: P = 0.003; □ vs. ○ or **X**: P < 0.0001; ○ vs. **X**: P < 0.02).

was first made by Davies in 1982 [17]. We [18] and others [19] have defined further the correlations between various ANCAs and idiopathic glomerulonephritis. The first data that suggested a pathogenic role for these autoantibodies were reported in 1990. We found that ANCA can stimulate cytokine primed and to a lesser extent unprimed PMN to degranulate and produce reactive oxygen species. Cytokine priming translocates the intra-granular antigens to the cell surface, thereby making them available to interact with ANCA [4]. These observations have been extended by the demonstration that a range of heterologous antibodies directed against different constituents of the primary granules also induce neutrophil activation [6]. Keogan et al have also reported similar observations [20].

The current findings add further support to the contention that ANCA are pathogenic. In order to support ANCA's presumed role in causing vasculitis it was crucial to demonstrate endothelial cell damage. It does not necessarily follow that a substance that activates PMN will also stimulate them to damage endothelial cells. Varani et al [21] found that immune complexes, activated complement, and phorbol esters activated PMN and stimulated them to damage endothelial cells. However, platelet activating factor and formyl-methionyl-leucyl-phenylalanine (fMLP) while stimulating the production of reactive oxygen species and protease release did not stimulate PMN mediated endothelial cell injury. MPO-ANCA not only cause neutrophil activation but also promote the more complex pathophysiological phenomenon of endothelial cell injury.

ANCA and heterologous antibodies to MPO are an addition to the list of agents that are able to stimulate PMN to injure endothelial cells in vitro. C5a and immune complexes are the only other physiologic factors identified that induce endothelial damage by PMN [21, 22]. The known pharmacologic inciters of PMN-mediated endothelial cell damage are fMLP [23] and PMA [11]. Various groups have found conflicting results with C5a [22, 24] and fMLP [25] in regards to their ability to stimulate PMN-mediated endothelial cell injury. These differences are probably attributable to contamination with LPS [23] and the concentration of serum used in the assay [24]. It has previously been demonstrated that LPS amplifies the effect of fMLP stimulation of PMN [26] and increases their ability to damage HUVEC [23]. Heterologous αMPO combined with TNF-α is also a stimulator of PMN whose effect is enhanced by the presence of LPS. However, in contrast to fMLP, LPS was not necessary for the endothelial damage to occur, but its presence amplified the effect.

There are interesting correlations between the clinical presentation of ANCA associated glomerulonephritis and this in vitro system. In general ANCA titers correlate well with disease activity, but some patients retain high titers despite clinical quiescence [27]. Analogous to this, αMPO alone was not found to be a sufficient stimulus for PMN to damage endothelial cells in vitro. Priming factors, such as cytokines with or without LPS, must be present. This priming dependency of the system is probably due to the need for the expression of PMN ANCA antigens [4] and adhesion molecules [28, 29] on the PMN cell surface. Another clinical finding that suggests the cytokine dependency of this disease in vivo is that patients often experience a "viral prodrome" before the onset

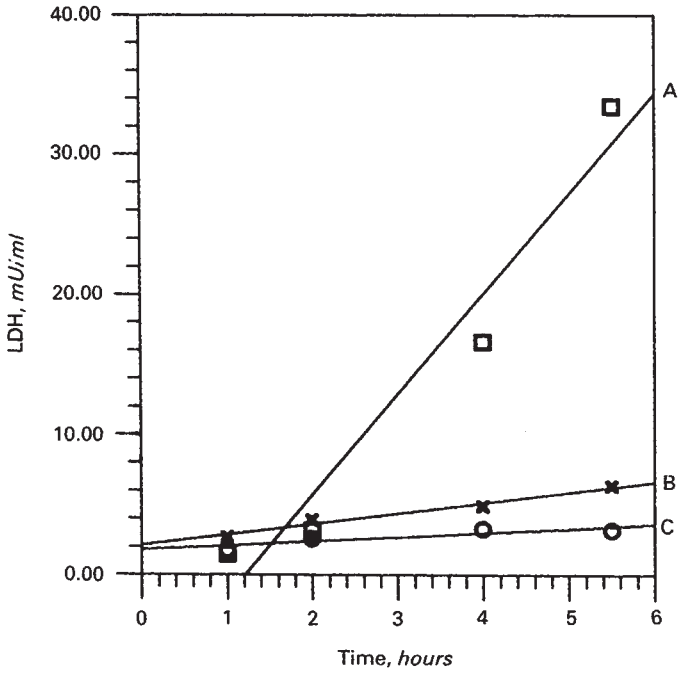

Fig. 8. *Effect of R-αMPO and TNF-α on LDH release.* Symbols are: (□) R-αMPO and 200 U/ml TNF-α (slope = 7.16 ± 0.92); (X) R-αMPO (slope = 0.76 ± 0.92); (○) 200 U/ml TNF-α (slope = 0.31 ± 0.92). (□ vs. X or ○: $P < 0.0001$; X vs. ○: $P > 0.05$).

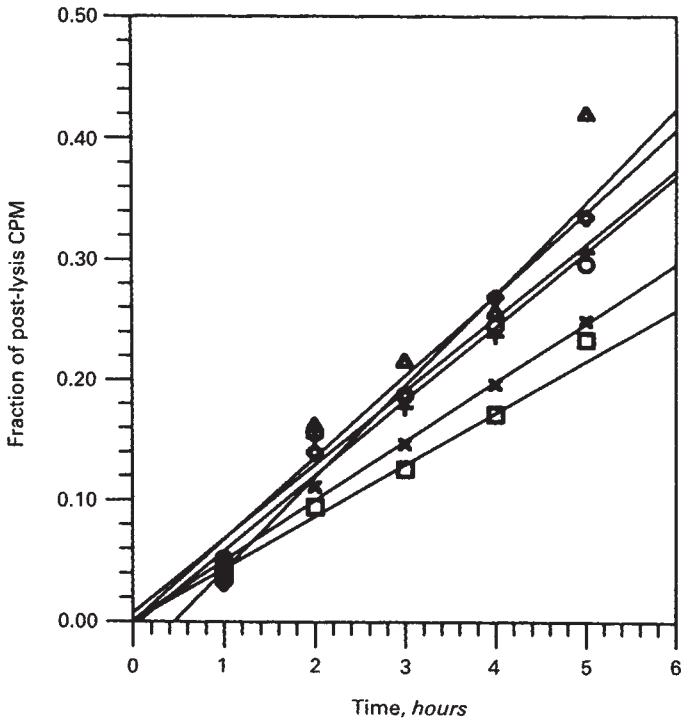

Fig. 9. *Donor variability in regards to response to αMPO.* When six donors were tested simultaneously the slope of the highest responder was significantly greater ($P < 0.01$) than the slopes of the two lowest responders. The response of the other three donors fell in between and was not significantly different from any of the others.

or exacerbation of nephritic symptoms [27]. Presumedly, in vivo cytokine stimulation initiates translocation of PMN intragranular antigens thereby making them available to the ANCA leading to PMN activation and vascular inflammation.

The variation seen between PMN from different donors may also help explain the wide clinical spectrum of ANCA associated diseases. When primed PMN from six donors were evaluated simultaneously, three patterns of response were distinguished. PMN from two donors significantly increased HUVEC damage when αMPO was added. Samples that contained PMN from two of the four donors that did not demonstrate increased HUVEC damage with αMPO had significantly higher LDH concentrations after one hour of incubation with stimulators without αMPO. We concluded that our usual concentrations of primers resulted in PMN activation before attachment to HUVEC and/or PMN autotoxicity. Either one of these events would account for the absence of HUVEC damage.

Overstimulation of the PMN as the cause of the lack of HUVEC damage was confirmed. PMN from one of these donors were tested with decreased levels of primers and a significant increase in HUVEC damage was demonstrated with αMPO. Donor variability is not unique to this system, the effect has been demonstrated previously with fMLP stimulated PMN adherence to HUVEC [30]. These results demonstrate the paradoxical nature of the HUVEC damage. The level of PMN priming must be sufficient enough to translocate the intracellular ANCA antigens to the cell surface. However, if the PMN are overstimulated, they either are able to injure HUVEC without αMPO or die upon the addition of αMPO before injuring the HUVEC.

The pronounced effect seen with the mouse monoclonal αMPO is attributable to two factors. First, it would be expected that the titer of specific αMPO antibodies would be substantially higher than in the rabbit polyclonal or purified human MPO-ANCA IgG preparations. Second, we have found that the immunoreactivity of this monoclonal to MPO is identical to MPO-ANCA [15]. In contrast, rabbit polyclonal αMPO reacts with several MPO epitopes.

Table 2. Detectable response to anti-myeloperoxidase is dependent on the PMN donor

Donor #	Slope of ^{51}Cr release (−)R-αMPO	(+)R-αMPO	% change	*P* value
1	0.043	0.076	75.9	0.003
2	0.035	0.062	75.6	0.01
3	0.043	0.061	43.1	0.09
4	0.035	0.043	24.2	NS
5	0.040	0.049	22.7	NS
6	0.061	0.068	10.7	NS

Comparison of the slopes of ^{51}Cr release from six donors evaluated simultaneously. The PMN were primed with TNF-α and LPS. With #1 and #2 a significant increase in ^{51}Cr release was observed with the addition of R-αMPO. In contrast PMN from donors #4 and #5 with R-αMPO resulted in ^{51}Cr release that was not significantly different from all cases without R-αMPO. The lack of a significant change was due to inability to increase HUVEC damage over baseline. The slope obtained with PMN without R-αMPO from donor #6 was not significantly different than all of the cases with R-αMPO and was significantly higher ($P < 0.05$) than #2, #4, and #5 without R-αMPO. The lack of a significant increase was attributed to a high baseline ^{51}Cr release.

There are several potential sources of bias in our experimental system. First, reagents may be contaminated with LPS. This was controlled for through several means. When possible, as in the case of tissue culture media, only reagents with <10 pg/ml LPS were used. However, stimulating effects of LPS have been reported at the 3 pg/ml level [26], which is below the detection level of commercial assays. Thus we prepared reagents in parallel with matched levels of LPS, as in the case of the patient IgG preparations. There are also several findings that argue against LPS being responsible for the effect observed with αMPO. First, the degree of damage we report with αMPO antibodies has never been observed by us or others [14] with LPS as the sole stimulant. Second, the pattern of LDH release was distinctly different between LPS and αMPO. At all concentrations tested, LPS never caused a significant increase in LDH release. However, at the lowest LPS concentration tested, addition of αMPO significantly increased LDH release.

A second source of potential bias with this experimental system is anti-endothelial cell antibodies. Auto-antibodies directed against endothelial cells have been found in patients with Kawasaki's disease [12], hemolytic uremic syndrome [31], systemic lupus erythematosus [32], and other forms of vasculitis [33] including Wegener's granulomatosus. We tested our MPO-ANCA patient plasma for anti-endothelial cell antibodies using a complement-mediated injury assay and found no cytotoxic activity when compared with normal serum or buffer.

In summary, these findings further support a role for MPO-ANCA in the pathogenesis of pauci-immune glomerulonephritis and vasculitis. They build on our previous report by demonstrating that primed PMN activated by MPO-ANCA are able to damage endothelial cells, which are a major site of injury in ANCA-associated glomerulonephritis and vasculitis. The cytokine dependency of this effect correlates with the clinical finding that patients with ANCA may remain asymptomatic until there is unrelated activation of the immune system as occurs with viral infections. These results support the hypothesis that ANCA cause idiopathic glomerulonephritis and vasculitis by activating primed PMN leading to tissue destruction.

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