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Anti-myeloperoxidase and anti-cathepsin G antibodies in sulphonamide hypersensitivity

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Summary

Background Anti-neutrophil cytoplasmic antibodies (ANCA) are associated with vasculitis in humans. Sulphonamide antimicrobials cause drug hypersensitivity (HS) reactions with some clinical signs that are suggestive of vasculitis.

Objective The purpose of this study was to determine whether sulphonamide HS is associated with anti-neutrophil antibodies, using the dog as a spontaneous clinical model.

Methods Thirty-four sulphonamide-HS dogs, 11 sulphonamide-‘tolerant’ dogs, and nine healthy sulphonamide-naïve dogs were evaluated for anti-neutrophil antibodies using a commercial ELISA against human myeloperoxidase (MPO), a commercial human ANCA Western blot protocol, and immunoblotting against whole canine neutrophils.

Results Using ELISA, anti-MPO antibodies were found with an apparent higher frequency in HS dogs (50%), compared with ‘tolerant’ dogs (18%), which also showed significantly lower absorbances. Among HS dogs, anti-MPO antibodies were significantly more common, with significantly higher absorbances, in dogs that did not survive the HS reaction (78%) compared with survivors (35%). Using immunoblotting, ANCA were detected with similar overall frequencies in HS and ‘tolerant’ dogs. However, one protein targeted by several HS dogs, but no ‘tolerant’ dogs, was identified as cathepsin G.

Conclusion These data indicate that anti-MPO antibodies and anti-cathepsin G antibodies are associated with sulphonamide HS. Anti-MPO antibodies have been shown to be pathogenic both *in vitro* and *in vivo*, leading to vasculitis lesions and vasculitis-like syndromes. The present study therefore suggests that vasculitis might be one mechanism of tissue damage in this sulphonamide HS. Furthermore, the evaluation of ANCA, and its relationship to disease severity and clinical outcome, should be considered in human patients with sulphonamide drug HS.

Keywords adverse drug reaction, dog, sulphadiazine, sulphadimethoxine, sulphamethoxazole
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Introduction

Anti-neutrophil cytoplasmic antibodies (ANCA) have been reported in various vasculitides, to include Wegener’s granulomatosis and Churg–Strauss syndrome [1]. The most frequent and most studied ANCA are anti-myeloperoxidase (MPO) and anti-proteinase 3 (PR3) antibodies. These ANCA have been shown to activate neutrophils and monocytes [2, 3], to be cytotoxic to endothelial cells [4], and to induce vasculitis lesions in rodent models [5]. ANCA are therefore thought to contribute to the pathogenesis of ANCA-associated vasculitis syndromes [6, 7].

ANCA have also been reported in autoimmune disorders, such as systemic lupus erythematosus (SLE) [8] and rheumatoid arthritis [9]. Because ANCA are known to be pro-inflammatory, and because their presence and titers correlate with disease activity, researchers postulate that ANCA also contribute to signs of vasculitis in these autoimmune disorders [9].

Some drug hypersensitivity (HS) reactions are also associated with clinical signs suggestive of a vasculitis syndrome [10]. For example, delayed HS reactions to potentiated sulphonamide antimicrobials, reported in both humans and dogs, may include clinical signs of dermatitis, hepatitis, glomerulonephritis, and pneumonitis, which are

similarly associated with vasculitis syndromes [11, 12]. There is no recent epidemiological study available on sulphonamide HS. However, the incidence is estimated at 1–5% in the HIV-negative population [13], while it can reach 65% in HIV-positive patients [14]. In 1991, the World Health Organization published a report on adverse drug reactions to sulphonamides, reporting a mortality rate of 6.7% [15]. Regarding dogs, the prevalence of sulphonamide HS is harder to establish because drug prescriptions are not tracked. A retrospective study from the Dermatology department of the Veterinary School of Utrecht (the Netherlands) reported an incidence of 0.25% skin HS among dogs receiving sulphonamides [16], while a retrospective in an American veterinary school reported a fatality rate of 21% in dogs hospitalized for a sulphonamide idiosyncratic reaction [12].

In both species, the reactions are thought to be immune mediated, and triggered by a reactive nitroso metabolite of the parent drug (Fig. 1) [17]. Although a consequent amount of data has been published on cellular immunity in sulphonamide HS, the role of humoral immunity, and in particular ANCA, has not been well characterized in this drug HS syndrome. Therefore, the purpose of this study was to determine whether sulphonamide HS is associated with the presence of anti-neutrophil antibodies, using the dog as a spontaneous clinical model.

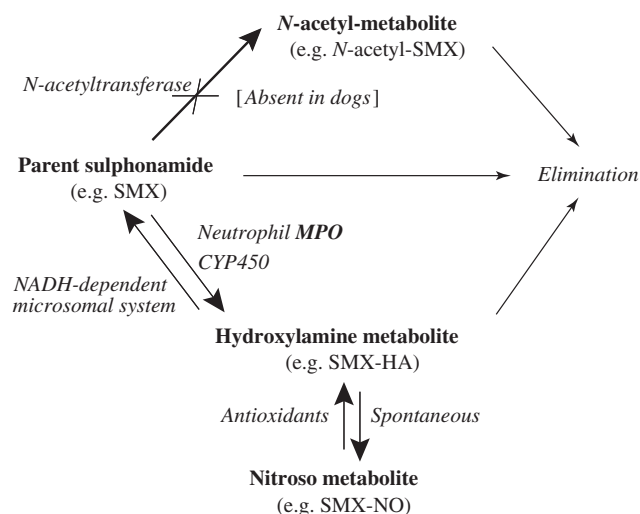


Fig. 1. Metabolism of sulphonamide antimicrobials in humans and dogs and its hypothesized link to drug hypersensitivity. The major elimination pathway for sulphonamide antimicrobials in humans is *N*-acetylation, which is absent in dogs. The parent drug can also be oxidized to its hydroxylamine metabolite by CYP450 or by peroxidases, such as neutrophil myeloperoxidase (MPO). This hydroxylamine spontaneously oxidizes to a nitroso metabolite, which is cytotoxic and can covalently bind to proteins and form haptens. The nitroso metabolite can be reduced by antioxidants such as thiols and ascorbate, while the hydroxylamine can be enzymatically reduced back to the parent drug. SMX, sulphamethoxazole.

Material and methods

Inclusion criteria for dog samples

The HS group consisted of privately owned dogs that developed signs of sulphonamide HS after five or more days of treatment with a potentiated sulphonamide antimicrobial, including trimethoprim/sulphamethoxazole (TMP–SMX), trimethoprim/sulphadiazine (TMP–SDZ), or ormetoprim/sulphadimethoxine (OMP–SDM). A control group comprised privately owned dogs that had received a therapeutic course of potentiated sulphonamides at a standard dosage for at least 10 days, or multiple courses over at least 7 days, without any adverse reaction ('tolerant' group). A second control group included nine healthy pet dogs (naïve group) with no history of sulphonamide antimicrobial administration, blood product transfusion, neutropaenia, or significant inflammatory disease.

To further evaluate whether the parent sulphonamide or its nitroso metabolite [18] could be associated with the development of anti-neutrophil antibodies, sera were also analyzed from four beagle dogs experimentally dosed with SMX-nitroso or vehicle for 2 weeks, followed by TMP–SMX for another 2 weeks [19].

Detection of anti-myeloperoxidase antibodies by enzyme-linked immunosorbent assay

A commercial kit for the detection of human anti-MPO antibodies [Anti-MPO (P-ANCA) kit; Utecht & Lüdemann GmbH, Kalusdorf, Germany] was used to detect anti-MPO antibodies in dog sera. The assay was run according to the manufacturer's instructions with the exception of a 1 : 20 dilution for canine sera, and the use of horseradish peroxidase (HRP)-conjugated anti-dog IgG (H+L) secondary antibody (1 : 5000; Jackson ImmunoResearch Laboratories Inc., Westgrove, PA, USA). All samples were run in duplicate in three independent assays. Absorbance readings were obtained using a μ Quant plate reader at 450 nm (Bio-Tek Instruments, Winooski, VT, USA).

The kit provided human positive controls for anti-MPO antibodies; healthy naïve dog sera were used as negative controls. A serum sample was considered positive for anti-MPO antibodies if the absorbance was >3 standard deviations above the mean absorbance values for naïve canine controls. This cut-off implies less than a 0.5% risk of false-positive results. Serum samples available in insufficient quantity to be used at a 1 : 20 dilution were analyzed at a 1 : 80 dilution, in parallel with samples from six negative controls and four sera found positive at a 1 : 20 dilution, all diluted to 1 : 80.

Detection of antibodies against human neutrophil cytoplasmic proteins using immunoblotting

Dog samples were also tested for antibodies to a wider range of neutrophil antigens using commercial human cytoplasmic neutrophil immunoblotting strips (ANCA blot kit; Utecht & Lüdemann GmbH). The assay was performed according to the manufacturer's instructions except for the addition of a preliminary blocking step [30 min in 5% milk in phosphate-buffered saline (PBS)-Tween], and the use of alkaline phosphatase-conjugated anti-dog IgG (H+L) secondary antibody (1 : 5000; Jackson ImmunoResearch Laboratories Inc.). Following addition of BCIP/NBT substrate, positive bands were directly visualized as purple precipitates. Human serum positive for anti-MPO antibodies, provided with the kit, was used as a positive control. A pool of the nine healthy naïve dog sera was used as a negative control.

Canine neutrophil isolation

In order to detect antibodies to targets other than cytoplasmic proteins, or antibodies specific to canine proteins, an immunoblotting assay using dog whole neutrophils was developed. To isolate canine neutrophils, 20 mL of blood was collected in heparin from healthy pet dogs. Blood was underlain with an equal volume of Lympholyte-Poly buffer (Cedarlane Laboratories Limited, Burlington, NC, USA) and centrifuged at 500 g for 1 h at RT. After washing the lower layer of cells containing neutrophils with Hanks-EDTA 5 mM, pH 7.4, the cell pellet was re-suspended in ACK lysing buffer (Cambrex, Baltimore, MD, USA) to eliminate red blood cell contamination. Cells were again washed, and re-suspended in PBS. All neutrophil preparations used in the study contained 95–99% neutrophils. A pool of seven normal canine neutrophil samples was aliquoted, stored at -20°C , and used for all subsequent immunoblotting assays.

Detection of anti-canine neutrophil antibodies by immunoblotting

Pooled canine neutrophil lysate (30 µg per lane) was electrophoresed in a 26-well 12.5% Criterion gel (BioRad, Hercules, CA, USA), and transferred to a polyvinylidene difluoride membrane (Pierce Biotechnology, Rockford, IL, USA). The entire membrane was blocked for an hour at room temperature (RT) using StartingBlot T20 Blocking Buffer (Pierce Biotechnology), and then split into semi-membranes (each containing one marker lane and one lane of neutrophil proteins). These were incubated overnight with a 1/100 dilution of dog test serum at 4°C , followed by a 1 : 10 000 dilution of HRP-labelled anti-dog IgG secondary antibody (Bethyl Laboratories, Montgomery, TX, USA) for 1 h at RT. Finally, each semi-membrane

was incubated with ECL substrate (Pierce Biotechnology) and exposed to a digital camera (UVP Inc., Upland, CA, USA). Rabbit anti-human MPO antibody (1 : 5000 dilution; Sigma-Aldrich, St Louis, MO, USA) with HRP-labelled anti-rabbit IgG (H+L) secondary antibody (1 : 10 000 dilution; BioRad) was used as a positive control. All samples were tested in two independent assays.

Identification of potential protein targets

For bands that appeared to be targeted by antibodies in canine sera, selected lanes of the SDS-PAGE gels were not transferred, but were stained with Imperial Blue Coomassie stain (Pierce Biotechnology). Candidate bands of appropriate molecular weight were excised from the gel and digested with trypsin. Peptides were then analyzed by MALDI-TOF mass spectrometry for fingerprint quality (Matrix-Assisted Laser Desorption Ionization – Time of Flight; Bruker Daltonics, Billerica, MA, USA), before MS/MS analysis (Agilent 100 Series LC/MSD Trap SL; Agilent Technologies, Palo Alto, CA, USA) and sequence matching using the Mascot search software (Matrix Science Inc., Boston, MA, USA).

Statistical analysis

All statistical analyses were performed using commercial data analysis software (Prism; GraphPad Software Inc., San Diego, CA, USA), with $P < 0.05$ considered significant. Continuous values were analyzed using a Student *t*-test after log transformation of the data and verification of the Goodness of fit by Shapiro–Wilk's test. Frequencies were compared using a Fisher exact test.

Results

Patient characteristics

Sera from 34 privately owned dogs with a history of sulphonamide HS reactions were available for study. As described previously, clinical signs included dermatitis, hepatopathy, arthropathy, protein-losing nephropathy, pneumonitis, keratoconjunctivitis, and thrombocytopenia; seven dogs also showed neutropaenia (defined as < 3000 cells/µL) [12]. Sera were also available from 11 'tolerant' dogs that had received therapeutic courses of potentiated sulphonamide antimicrobials. Dosage, treatment duration, and time between treatment and blood collection were not statistically different between HS and tolerant groups.

In both groups, dogs were from various mixed and pure breeds. Females were less numerous in the tolerant group (36%) than in the HS group (62%). However, this difference was not statistically significant ($P = 0.097$). Similarly, there was no statistical difference in age between tolerant

(median of 7 years old; range 0.3–12) and HS animals (median of 6 years old; range 1–14) ($P=0.80$).

Anti-myeloperoxidase antibodies detected by enzyme-linked immunosorbent assay

Absorbance values using the anti-MPO ELISA were significantly higher in HS dogs (1.048, range 0.288–3.551) compared with both the naïve group (0.646, range 0.329–0.882; $P=0.002$) and the 'tolerant' group (0.689; range 0.395–1.699; $P=0.040$) (Fig. 2a). Using the pre-defined cut-off value, 17 out of 34 sulphonamide HS dogs (50%) were positive for anti-MPO antibodies, compared with 2 out of 11 'tolerant' dogs (18%) (Fig. 2b). This difference showed a trend towards a higher frequency of anti-MPO antibodies in HS dogs, but did not quite reach significance ($P=0.06$).

Positive results did not correlate with age (ranging from 0.3 to 12 years old with a median of 5 in the negative group; ranging from 1 to 14 years old with a median of 7

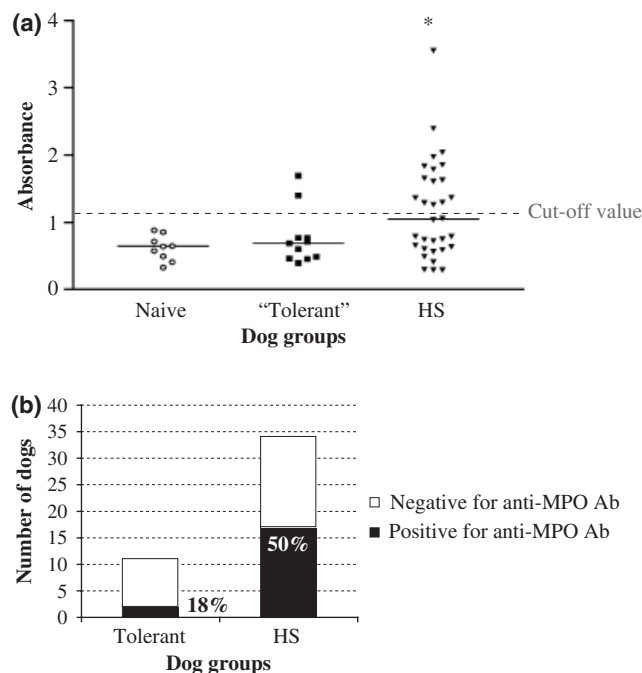


Fig. 2. Detection of anti-myeloperoxidase (MPO) antibodies by ELISA in sulphonamide-hypersensitive (HS) dogs. Using a commercial ELISA kit for anti-human MPO antibodies, sera were tested from naïve, 'tolerant' (dogs without an adverse reaction to a therapeutic course of sulphonamide antimicrobials), and HS dogs. (a) Scatter plots of ELISA absorbance values for nine naïve dogs, 11 'tolerant' dogs, and 34 HS dogs. Each point represents the mean absorbance of three independent assays for each sample. Overall mean absorbance values were higher in HS dogs compared with tolerant and naïve dogs ($*P=0.038$). The dotted line represents the cut-off value for determining a positive test result, or three standard deviations above the mean absorbance for the naïve group. (b) The frequency of anti-MPO antibodies was higher in HS dogs compared with tolerant dogs, but did not reach significance ($P=0.06$).

for the positive group; $P=0.066$) or gender (eight males and 11 females among positive dogs; 12 males and 14 females among negative dogs; $P=0.23$).

In HS dogs with a history of neutropaenia ($n=7$), MPO ELISA absorbance values (0.638, range 0.293–2.93) and the frequency of anti-MPO antibodies (43%) were not significantly different from HS dogs without neutropaenia ($n=27$; absorbance of 1.058, range 0.288–3.551; 48% positive).

In HS dogs that did not survive the HS reaction, either due to death or due to elective euthanasia following poor response to supportive care ($n=9$), MPO ELISA absorbance values were significantly higher (1.608, range 0.566–3.551), compared with sulphonamide HS survivors ($n=23$; 0.793, range 0.288–2.042; $P=0.014$) (Fig. 3a). Anti-MPO antibodies were significantly more prevalent overall in non-survivors (78%), compared with survivors (35%; $P=0.049$) (Fig. 3b).

Sulphonamide HS reactions are hypothesized to be due to an immune response triggered by the nitroso metabolite of the parent drug [18]. However, research dogs experimentally dosed with the nitroso metabolite of SMX, and challenged with the parent drug at a therapeutic dose, did not develop anti-MPO antibodies at any time-point of the dosing experiment (data not shown).

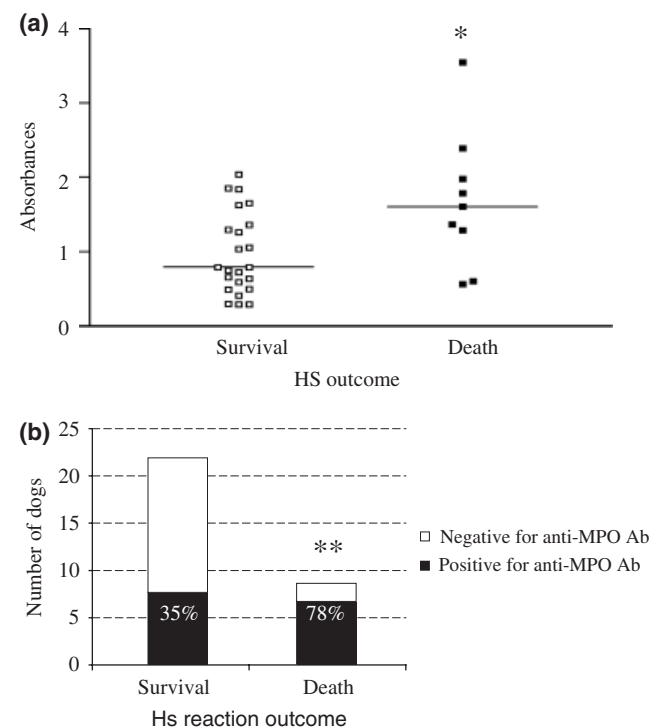


Fig. 3. Anti-myeloperoxidase (MPO) antibodies in hypersensitive (HS) dogs with sulphonamide hypersensitivity, by survival outcome. (a) Mean absorbance for the anti-MPO ELISA assay was significantly higher in dogs that did not survive their sulphonamide HS reactions (Death) compared with survivors ($*P=0.040$). (b) The prevalence of anti-MPO antibodies was significantly higher in sulphonamide HS dogs that died from their reactions, compared with survivors ($**P=0.049$).

Table 1. Detection of anti-neutrophil cytoplasmic antibodies (ANCA) in canine sera using a human commercial immunoblotting kit

Bands (estimated kDa)	Presumptive target	% positive in HS dogs (n = 32)	% positive in 'tolerant' dogs (n = 11)
130–150	MPO	22	18
45	HLE	13	36
40	Unknown	3	9
25	PR3	10	18
20	Unknown	16	18
15	Lyso	16	18
10	Unknown	31	9
	Any protein	56	64

Presumptive targets were determined based on co-migration with bands identified with human control sera positive for anti-MPO antibodies, and comparison with controls for other ANCA from the manufacturer's website (see also Fig. 3).

HS, hypersensitive; MPO, myeloperoxidase.

Anti-neutrophil antibodies detected by immunoblotting

Several human cytoplasmic neutrophil antigens were recognized by canine sera using the commercial kit for human ANCA detection (Table 1). The frequency of ANCA-positive samples overall was similar between sulphonamide HS dogs (18 out of 32 tested; 56%) and 'tolerant' dogs (seven out of 11; 64%). Seven proteins appeared to be targeted by positive canine sera. Compared with the ANCA kit positive controls, four of the detected bands co-migrated with MPO (~130–150 kDa), elastase (HLE, ~30 kDa), PR3 (~25 kDa), and lysozyme (~15 kDa). The molecular weights of the other three targeted proteins were estimated to be ~40, ~20, and ~10 kDa.

In this assay, 22% of HS dogs were positive for anti-MPO antibodies, while 50% were found positive using the ELISA.

To identify antibodies targeting non-cytoplasmic proteins, or potential antibodies specific for canine proteins, an immunoblotting assay was developed using whole canine neutrophils. This assay gave many background bands in negative controls, but three out of 11 'tolerant' dogs (27%) and 10 out of the 33 HS dogs tested (30%) showed bands absent in healthy naïve controls (Table 2). Two out of these 13 positive samples (both HS dogs) were negative in both human-specific protocols, suggesting a canine-specific target.

One canine neutrophil protein, with an estimated molecular weight of approximately 23 kDa, was targeted by positive sera from six HS dogs, but no sera from 'tolerant' dogs. This band was adequately separated from other proteins and was present in sufficient amounts to be analyzed by mass spectrometry. This analysis revealed a match to a canine cathepsin G precursor, with sequence coverage of 35% and an ion score of 98.

Table 2. Detection of antibodies against whole canine neutrophils in canine sera

Bands (estimated kDa)	Protein target	% Positive in HS dogs (n = 33)	% Positive in 'tolerant' dogs (n = 11)
69	MPO subunit	0	0
60	Unknown	0	9
51	MPO subunit	0	0
45	Unknown	3	0
36	MPO subunit	3	0
26	Unknown	6	9
23	Cathepsin G	18	0
20	Unknown	6	18
17	Unknown	9	0
	Any protein	30	27

The identity of MPO subunit targets was confirmed using a commercial anti-human MPO antibody. The cathepsin G precursor was identified by mass spectrometry.

HS, hypersensitive; MPO, myeloperoxidase.

Discussion

ANCA are thought to contribute to the pathogenesis of immune-mediated vasculitis. HS reactions to sulphonamide antimicrobials present with clinical signs that are suggestive of vasculitis, to include dermatitis, glomerulonephritis, arthropathy, and uveitis [11, 20]. Furthermore, sulphonamide HS is thought to be immune-mediated, and markers of humoral immunity have been detected in sulphonamide HS individuals, including anti-drug antibodies with drug-serum adducts [21, 22], anti-platelet antibodies [23] (and unpublished data in dogs), and anti-liver antibodies [24] (and unpublished data in dogs). However, the presence of ANCA in sulphonamide HS individuals had not been evaluated. The purpose of this study was therefore to determine whether ANCA could be detected in sulphonamide HS, using the dog as a model.

Using an ELISA, anti-MPO antibodies were detected in 50% of HS and 18% of 'tolerant' dogs treated with sulphonamide antimicrobials. MPO has been shown to oxidize the parent sulphonamide to its hydroxylamine metabolite in both human and dog neutrophils [25]. This hydroxylamine is known to spontaneously oxidize to a nitroso metabolite, which readily forms protein adducts, both *in vitro* [22, 26] and *in vivo* [18, 19]. In the case of MPO-mediated generation of the sulphonamide hydroxylamine, the subsequently formed nitroso could easily bind to the closest protein available, MPO. The nitroso-modified MPO, whether released as part of neutrophil degranulation or secondary to neutrophil necrosis, could then be presented to the immune system and recognized as a foreign protein. This could lead to the formation of anti-MPO antibodies (Fig. 4). The lower prevalence of anti-MPO antibodies by immunoblotting (22%), compared

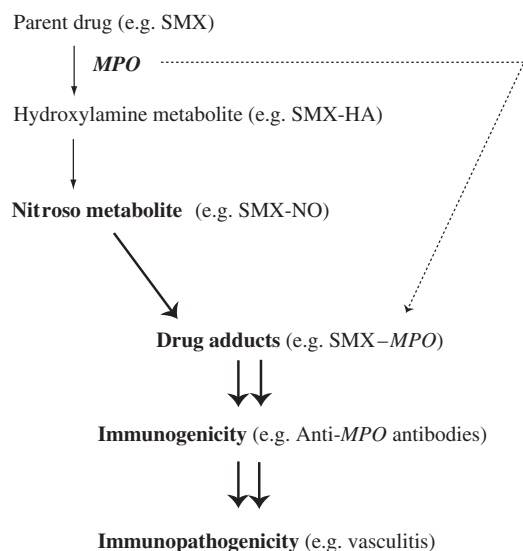


Fig. 4. Potential connection between sulphonamide-nitroso metabolite, anti-neutrophil antibodies, and clinical signs of sulphonamide hypersensitivity (HS). Neutrophil myeloperoxidase (MPO) can oxidize sulphonamides to their hydroxylamine metabolite, which will further auto-oxidize to a nitroso compound. This latter metabolite could then haptenize neutrophil proteins, such as MPO. These drug-protein adducts are potentially immunogenic, and could explain the formation of antibodies against the corresponding neutrophil proteins. Anti-neutrophil antibodies, such as anti-MPO antibodies, are known to trigger vasculitis lesions. Such vasculitis could explain some clinical signs seen in sulphonamide HS such as dermatitis, hepatitis, or glomerulonephritis. SMX, sulphamethoxazole.

with ELISA (50%), may have been caused by denaturation of target proteins or epitopes during sample processing.

The apparently higher frequency and the significantly higher anti-MPO antibody absorbance values in HS compared with 'tolerant' dogs could be secondary to an imbalance in the generation or detoxification of SMX-nitroso. For example, increased oxidation of the parent sulphonamide, and/or decreased clearance of the nitroso or its precursors, could be present in HS subjects. Alternatively, there could be insufficient degradation or elimination of MPO-sulphonamide adducts, leading to enhanced immunogenicity in HS individuals.

Interestingly, research dogs dosed systemically with SMX-NO and challenged with TMP-SMX [19] did not develop anti-MPO antibodies. These dogs did not develop any clinical signs of drug HS during this study, they could therefore be considered as 'tolerant'. It is noteworthy that in the present study, 18% of the 'tolerant' dogs were positive for anti-MPO antibodies while these four research dogs were all negative. This apparent discrepancy could be explained by a difference in genetic background (research inbred beagles vs. miscellaneous mixed and purebred pets), in environmental background (research confinement vs. family and outdoor life), or finally in clinical history (<1.5-year-old research dogs vs. dogs of

various ages; no history of any diseases vs. heterogeneous pathological history).

Although this was a small pilot study, the absence of anti-MPO antibodies in these SMX-NO-dosed dogs could indicate either acquired tolerance or that the nitroso metabolite itself is insufficient to trigger the formation of anti-MPO antibodies. However, the nitroso metabolite may need to be generated locally within neutrophils in order to haptenize MPO; when administered systemically, this reactive metabolite may not have reached sufficient levels in neutrophils to form adducts.

Agranulocytosis (severe neutropaenia) is a well-characterized and potentially fatal idiosyncratic response to a number of drugs [27, 28]. Some drug-induced neutropaenias are thought to be immune-mediated; for example, anti-neutrophil antibodies have been identified with neutropaenia induced by anti-thyroid drugs in both humans [29] and cats [30]. Neutropaenia was present as a component of sulphonamide HS in seven dogs in this study. However, there was no correlation between neutropaenia and the presence of anti-neutrophil antibodies overall. Sulphonamide-associated neutropaenia may instead be T cell mediated, or due to an antibody-mediated event against antigens present in granulocytic precursors but not in circulating neutrophils [31, 32]. It is also possible that sulphonamide-associated neutropaenia is not immune-mediated, and is instead the result of direct drug- or metabolite-induced cytotoxicity.

Although, anti-MPO antibodies were not associated with neutropaenia, they were significantly associated with a poorer prognosis. Indeed, anti-MPO antibodies were more frequent in HS dogs that did not survive their adverse drug reaction compared with dogs that survived, and ELISA absorbance values were higher in HS non-survivors compared with survivors. These findings are consistent with observations in humans with vasculitis syndromes, in which the presence of ANCA is associated with refractory disease and an increased risk of relapse [33–35]. ANCA have been reported in HS reactions to other drugs, to include minocycline [36] and allopurinol [37]. However, no correlation between ANCA and outcome has been evaluated for other drug reactions.

The apparently higher frequency of anti-MPO antibodies and the significantly higher absorbances in HS dogs compared with 'tolerant' dogs, along with the correlation between anti-MPO antibodies and decreased survival, supports a possible pathogenic role for anti-MPO antibodies in sulphonamide HS. Experimental and clinical data suggest that anti-MPO and anti-PR3 antibodies are directly pathogenic in ANCA-associated vasculitis syndromes. These ANCAs have been shown to activate granulocytes and macrophages *in vitro* [2, 3]. This activation triggers degranulation and an oxidative burst, with release of reactive oxygen-producing enzymes (e.g. MPO), reactive oxygen species, and pro-inflammatory cytokines

[38]. This activation has also been shown *in vivo* in human patients with ANCA-associated vasculitides [39]. *In vitro*, anti-MPO and anti-PR3 IgG have been shown to mediate accelerated apoptosis in neutrophils and to disrupt their proper removal by macrophages [40, 41]. Anti-MPO and anti-PR3 antibodies also stabilize neutrophil interactions with endothelial cells *in vitro* [42], and neutrophils activated by these antibodies are cytotoxic to endothelial cells [4, 43].

Consistent with these findings, anti-MPO and anti-PR3 antibodies have been shown to reproduce vasculitis lesions such as glomerulonephritis in rodent models [5]. ANCA-mediated vasculitis could therefore contribute to the pathogenesis of similar lesions in sulphonamide HS (Fig. 4). Interestingly, no vasculitis lesions were identified in any of the four dogs for which a necropsy was conducted, but this represents only a very limited number of the HS dogs studied.

In the dogs in this study, ANCA against many protein targets other than MPO were detected in sera immunoblotted to neutrophils. While anti-MPO and anti-PR3 antibodies are the most commonly found and studied anti-neutrophil antibodies in humans, other neutrophil targets have been identified, such as azurocidin [44], elastase [45], lactoferrin [46, 47], lysosyme [48], cathepsin G [33, 47], HMG 1 and 2 (High Mobility Group) [49], EEA1 (Early Endosomal Antigen) [50], and actin [51]. However, *in vitro* or *in vivo* pathogenic effects remain to be established for ANCA targeting antigens other than MPO and PR3.

In the present study, anti-neutrophil antibodies were identified in some 'tolerant' dogs. This suggests that anti-neutrophil antibodies are themselves not sufficient to trigger antibody-mediated lesions; any pathogenic events associated with anti-neutrophil antibodies might require other co-stimulatory molecules or 'danger signals' [52, 53]. In ANCA-associated vasculitis syndromes, the development of lesions depends on a number of individual factors, such as coagulation state, blood flow, endothelial cell activation state, degree of inflammation, and probably genetic predisposition [7, 10, 54].

Interestingly, however, anti-cathepsin G antibodies were detected in several sulphonamide HS dogs but not in any 'tolerant' dogs. Anti-cathepsin G antibodies have previously been detected in humans with various immune-mediated disorders, such as inflammatory bowel disease, in which they can be associated with a refractory form of ulcerative colitis [33, 45]. Cathepsin G, like PR3, is a serine protease, found in both neutrophils and monocytes. Cathepsin G exerts antimicrobial activity, but also appears to play an important role in the regulation of inflammation. Cathepsin G has been shown to activate chemerin, a chemoattractant for antigen-presenting cells [55], to increase levels of soluble TNF- α [56], and to inhibit the uptake of apoptotic neutrophils by

macrophages [57]. However, cathepsin G has also been shown to down-regulate inflammation by decreasing complement-mediated neutrophil function [58]. These multiple roles for cathepsin G make it difficult to predict the pathological consequences of anti-cathepsin G antibodies. However, the finding that anti-cathepsin G antibodies were only found in HS dogs, but not in any 'tolerant' dogs in this small survey, suggests that anti-cathepsin G antibodies may contribute to the pathogenesis of sulphonamide HS. Additional studies in a larger number of subjects are warranted to address this question.

In conclusion, anti-MPO antibodies were present in the sera from dogs with sulphonamide HS, at an apparently higher frequency and with significantly higher absorbance values, compared with dogs that tolerated sulphonamides without HS. These anti-MPO antibodies were significantly associated with poor survival among HS dogs. In addition, anti-cathepsin G antibodies were detected in some sulphonamide HS dogs, but not in 'tolerant' dogs. These data are consistent with the hypothesis that ANCA-mediated vasculitis may play a role in the pathogenesis of systemic signs of sulphonamide HS. The detection of such ANCA in human patients with sulphonamide HS, and an evaluation of the *in vitro* pathogenic effects of these ANCA, would further address this hypothesis.

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