

ORIGINAL PAPER

Association of drug-serum protein adducts and anti-drug antibodies in dogs with sulphonamide hypersensitivity: A naturally occurring model of idiosyncratic drug toxicity

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Summary

Background Sulphonamide antimicrobials, such as sulphamethoxazole (SMX), provide effective infection prophylaxis in immunocompromised patients, but can lead to drug hypersensitivity (HS) reactions. These reactions also occur in dogs, with a similar time course and clinical presentation as seen in humans.

Objectives Drug-serum adducts and anti-drug antibodies have been identified in sulphonamide HS humans. The aim of this study was to determine whether similar markers were present in dogs with sulphonamide HS.

Methods Thirty-four privately owned sulphonamide HS dogs, 10 sulphonamide-‘tolerant’ dogs, 18 sulphonamide-naïve dogs, and four dogs experimentally dosed with SMX and the oxidative metabolite SMX-nitroso, were tested for drug-serum adducts by immunoblotting, and anti-drug antibodies by ELISA.

Results Sulphonamide-serum adducts were found in 10/20 HS dogs tested (50%), but in no tolerant dogs. Anti-sulphonamide IgG antibodies were detected in 17/34 HS dogs (50%), but in only one tolerant dog; antibody absorbance values were significantly higher in HS dogs. There was a significant association between the presence of sulphonamide-serum adducts and anti-sulphonamide antibodies ($P = 0.009$). Anti-drug antibodies were also found in dogs experimentally dosed with SMX-nitroso followed by SMX, but not in a dog dosed with drug vehicle, followed by SMX. **Conclusion** Similar humoral markers are present in dogs and humans with sulphonamide HS, supporting the use of dogs as a naturally occurring model for this syndrome in humans. These data suggest the potential use of drug-serum adducts and anti-drug antibodies as markers for sulphonamide HS. Preliminary data indicate that anti-sulphonamide antibodies may be triggered by the SMX-nitroso metabolite, not by the parent drug, in dogs.

Keywords anti-drug antibody, drug adduct, drug hypersensitivity, sulphamethoxazole, sulphonamide

Submitted 20 December 2005; revised 10 February 2006; accepted 27 March 2006

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Introduction

Potentiated sulphonamide anti-microbials are the drugs of choice for prevention of secondary infections in immunocompromised patients [1], and for treating Nocardia and atypical Mycobacterial infections in dogs [2]. However, the occurrence of delayed sulphonamide-hypersensitivity (HS) reactions limits the usefulness of these anti-microbials. Sulphonamide HS occurs in both humans and dogs

with a similar clinical presentation. Clinical signs are first noted 5 or more days after starting a course of therapy, and can include fever, skin eruptions, eosinophilia, hepatopathy or blood dyscrasias (thrombocytopenia, neutropenia), with arthropathy also common in dogs [3, 4]. These idiosyncratic drug reactions are thought to be immune mediated, and are likely triggered by a reactive metabolite of the parent drug (Fig. 1) [5]. Sulphonamides generate hydroxylamine metabolites in both humans and dogs [6, 7]; these hydroxylamine metabolites further oxidize to nitroso derivatives, which can haptenize tissue proteins and lead to antibody and/or cell-mediated immunity

Funding: This study was funded by a grant from the National Institutes of Health, GM 61753.

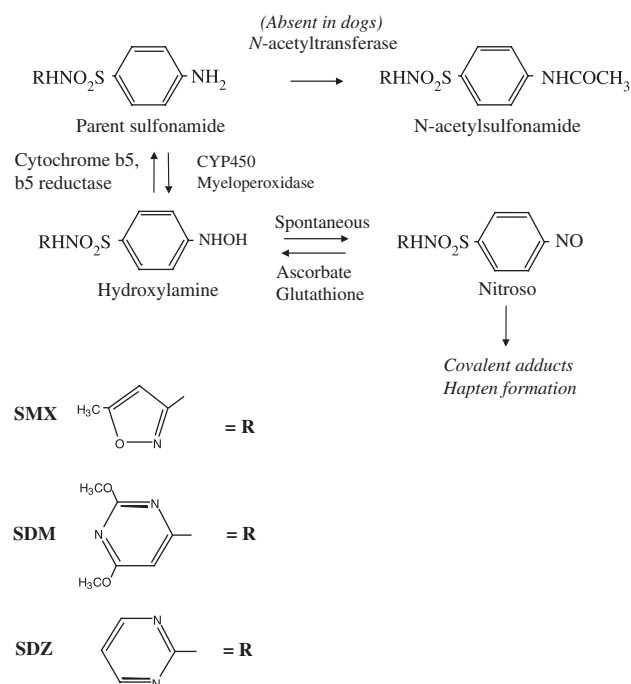


Fig. 1. Metabolism of arylamine sulphonamide anti-microbials in humans and dogs. *N*-acetylation of the parent drug is the major elimination pathway in humans; this detoxification pathway is absent in dogs [38], and is impaired in approximately 50% of Caucasians [39]. Parent drug is oxidized to its hydroxylamine metabolite by CYP450 (CYP2C9 in humans) [7,40], or by myeloperoxidase [41]. The hydroxylamine spontaneously oxidizes to the nitroso metabolite, which leads to protein haptenization [16]. The nitroso metabolite is reduced by the antioxidants ascorbic acid and glutathione; [42,43] the hydroxylamine by cytochrome *b*₅ and its reductase [44,45].

[8, 9]. Human patients with sulphonamide HS show drug-serum protein adducts, as well as anti-drug antibodies [10–13]. Similar immune markers in dogs with sulphonamide HS have not been explored, but could provide insight into the role of anti-drug antibodies in the pathogenesis of these reactions.

The purpose of this study was to characterize the presence of drug-serum protein adducts and anti-drug antibodies in a naturally occurring syndrome of sulphonamide HS in dogs, and to compare these findings with healthy dogs and dogs tolerating therapeutic courses of these drugs without adverse effects. In addition, anti-drug antibodies were assayed in a group of dogs experimentally dosed with the nitroso metabolite of sulphamethoxazole, followed by challenge with sulphamethoxazole [14].

Methods

Inclusion criteria for dog samples

Privately owned pet dogs were included in the study if they developed signs of sulphonamide HS after 5 or more days of treatment with a potentiated sulphonamide antimicrobial

(HS group), to include trimethoprim/sulphamethoxazole (TMP–SMX), trimethoprim/sulphadiazine or ormetoprim/sulphadimethoxine [3]. Sera for antibody determinations were collected from dogs after recognition of the adverse reaction, and were frozen at -20°C until analysis. In addition, sera were collected from privately owned pet dogs that had been administered a therapeutic course of potentiated sulphonamides at standard dosages, without adverse reaction ('tolerant' group). Sera from healthy dogs without a history of sulphonamide administration were also collected as negative controls (naïve group).

To further determine whether the parent drug or its nitroso metabolite was associated with the development of anti-sulphonamide antibodies, sera were analysed from four beagle dogs experimentally dosed in a pilot study with sulphamethoxazole–nitroso (SMX–NO: 1, 3 or 10 mg/kg/day; or drug vehicle) for 2 weeks, followed by TMP–SMX (50 mg/kg/day) for another 2 weeks, as described previously [14]. This protocol was approved by the Institutional Animal Care and Use Committee at the University of Wisconsin–Madison.

Materials and reagents

Flat-bottom 96-well high binding capacity plates were obtained from Costar (Cambridge, MA). Sulphamethoxazole (SMX), sulphadiazine (SDZ), sulphadimethoxine (SDM), human serum albumin (HSA) and *o*-phenylenediamine dihydrochloride were from Sigma-Aldrich (St Louis, MO, USA). Peroxidase-linked anti-dog IgG (class specific) antibody was obtained from Bethyl (Montgomery, TX, USA) and peroxidase-linked anti-rabbit IgG from Santa Cruz laboratories (Santa Cruz, CA, USA). Tween 20 was purchased from Bio-Rad (Hercules, CA, USA), and PVDF membranes and ECL substrate were from Pierce-Perbio (Rockford, IL, USA).

Sulphonamide–protein conjugation and rabbit immunization

SMX, SDM and SDZ were conjugated to HSA for use as fixed antigens in an ELISA protocol, and to keyhole limpet hemocyanin (KLH) for use as soluble antigens for rabbit immunization, according to a previously published procedure [15]. Drug–protein conjugation was confirmed by spectrophotometry, and for SMX–HSA and SMX–KLH, by reactivity with rabbit anti-SMX antibody, kindly provided by Dr Alastair Cribb.

SMX–KLH and SDM–KLH were used to immunize rabbits using standard protocols with complete Freund's adjuvant, followed by incomplete Freund's adjuvant (Panigen Inc., Branchardville, WI, USA). The reactivity of anti-sera was tested against SMX–HSA, SDM–HSA and SDZ–HSA, by both ELISA and immunoblotting. Control antibodies to SDZ–KLH were not generated; however, SMX anti-sera detected SDZ bound to HSA.

Detection of sulphonamide-serum protein adducts by immunoblotting

Dog sera were initially analysed for drug-serum adducts using standard immunoblotting. However, high background under the conditions of the assay made interpretations difficult. Therefore, a dot immunoblotting method was adopted [11]. Serum from each dog (1 : 3 dilution in methanol; 3 µL) was applied directly to a PVDF membrane. The membrane was then blocked for 1 h at room temperature with 5% milk in PBS-Tween 0.01%. After two washes, the samples were incubated overnight with α -SMX or α -SDM rabbit anti-sera (1 : 500), followed by HRP-labelled mouse anti-rabbit IgG antibody (1 : 5000), for 1 h. Adducts were detected using ECL substrate and visualized with a Biospectrum AC Imaging System (UVP BioImaging Systems, Upland, CA). Each dot immunoblot was repeated independently three times. SMX-HSA, SDM-HSA and SDZ-HSA (1 µg) diluted in methanol were used as positive controls; HSA (1 µg) and sera from sulphonamide-naïve dogs (1 : 3 dilution; 3 µL) were used as negative controls. To detect SDZ adducts, α -SMX rabbit anti-sera was used.

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

Ninety-six well microtitre plates were coated overnight at 4 °C with 10 µg of SMX-HSA, SDM-HSA, SDZ-HSA or HSA. Plates were blocked for 2 h with 2.5% milk in PBS-Tween 20. Dog sera (1 : 100 dilution) were added to plates and incubated overnight at 4 °C. HRP-linked anti-dog IgG (1 : 5000 dilution) or anti-rabbit IgG (1 : 5000) secondary antibody was incubated for 1 h at room temperature. The presence of anti-sulphonamide antibody was detected using *o*-phenylenediamine dihydrochloride as the HRP substrate, with absorbance readings at 450 nm using a microplate reader (Microquant, Bio-Tek Instruments, Winooski, VT, USA). Rabbit anti-sera to SMX and SDM (1 : 1000, followed by anti-rabbit secondary antibody 1 : 5000) were used as positive controls, while sera from healthy sulphonamide-naïve dogs (1 : 100) served as negative controls. Rabbit antibodies to SMX cross-reacted with SDZ-HSA in the ELISA, as for the immunoblots.

ELISA results are expressed in optical density units after blanking for absorbance in HSA-coated wells; results are the mean of two independent assays, each run in duplicate. A result was considered positive for anti-sulphonamide antibodies if the blanked absorbance was >2 SD above the mean blanked absorbance for the sulphonamide-naïve control group.

To confirm the drug specificity of antibodies, sera from dogs with positive antibodies to each drug were re-tested by ELISA following pre-incubation with the drug of exposure (500 µg/mL), HSA (100 µg/mL), or drug-HSA

conjugate (100 µg/mL), for 2 h at 4 °C. In addition, sera from dogs positive for anti-SMX antibodies were also competed with SMX-NO (500 µg/mL), before repeat ELISA. To determine cross-reactivity among sulphonamides, all sera positive for antibodies were also tested against the other two sulphonamides, using ELISA.

Statistical analysis

All data are reported in medians with ranges, and were compared between or among groups using Wilcoxon's rank sum or Kruskal-Wallis, as appropriate. Frequencies were compared using χ^2 , with confirmation by Yate's correction. All statistics were performed using a commercial software program (Statview, Abacus Concepts, Inc., Berkeley, CA, USA), with $P < 0.05$ considered significant.

Results

Dog characteristics

Serum samples from 34 dogs, including 11 males and 23 females from various mixed and pure breeds [3], with ages ranging from 8 months old to 9.5 years old (median 6.0 years old) (Table 1), and clinical signs consistent with delayed sulphonamide HS, were available for study. Clinical signs included fever, thrombocytopenia, neutropenia, hemolytic anemia, hepatic necrosis, arthropathy, skin eruptions, protein-losing nephropathy or pneumonitis [3]. Twenty dogs had been treated with TMP-SMX, eight with ormetoprim-SDM and six with TMP-SDZ (Table 1). HS dogs received a median dose of 44.2 mg/kg/day of potentiated sulphonamide over a median period of 10 days. The median time from drug initiation to the onset of clinical sign of drug HS was 10 days, with a range of 5–45 days.

The time between drug initiation and serum collection was a median of 19 days, while the time from onset of clinical signs of HS to serum collection was a median of 5 days. Two dogs were sampled several years after their HS reactions (3.5 and 7 years, respectively); all other dogs were sampled 1–138 days from their HS reactions.

Sera from 18 healthy sulphonamide-naïve dogs, including seven males and 11 females from various breeds, with ages ranging from 1 to 11 years old (median of 4.3 years old), were collected to serve as negative controls. Sera were also available from 10 'tolerant' dogs with a history of therapeutic course of potentiated sulphonamides without adverse reactions. This group consisted of five males and five females of various breeds, with ages ranging from 4 months old to 14 years old (median, 7.0 years old) (Table 1). These 'tolerant' dogs included five dogs given TMP-SMX, four given OMP-SDM and one given TMP-SDZ. Tolerant dogs received a median dose of 29.1 mg/kg/day over a median period of 14 days (Table 1). Blood samples from these sulphonamide-tolerant dogs

Table 1. Clinical characteristics of dogs with hypersensitivity reactions to potentiated sulphonamides (HS dogs) and dogs taking potentiated sulphonamides without adverse reactions ('tolerant' dogs)

	HS dogs	'Tolerant' dogs
Total number	<i>n</i> = 34	<i>n</i> = 10
Gender (male, female)	11 M, 23 F	5 M, 5 F
Age (years)	6.0 (0.67–9.5)	7.0 (0.33–14.0)
Sulphonamide received (# dogs)		
Sulphamethoxazole/trimethoprim	20	5
Sulphadimethoxine/ormetoprim	8	4
Sulphadiazine/trimethoprim	6	1
Dose (mg/kg/day)	44.2 (23.4–71.0) ^a	29.1 (24.8–50.0) ^a
Duration of treatment (days)	10 (5–45) ^b	14 (10–28) ^b
Time from first exposure to HS reaction (days)	10 (5–45)	Not applicable
Delay from first exposure to sampling (days)	19 (7–2555) ^{c*}	14 (10–300) ^c
Delay from onset of HS reaction to sampling (days)	5 (1 to 2543) [*]	Not applicable

*Ranges include two outlier dogs sampled 3.5 and 7 years after HS reactions. Medians and ranges excluding these two dogs were 17 days (7–150 days) from exposure to sampling, and 5 days (1–138 days) from HS reaction to sampling. ^{a,b,c}Pairs of data with identical superscripts are not statistically different. HS, hypersensitivity.

were collected a median of 14 days from the time of first exposure to the drug. None of these parameters (dosage, treatment duration, delay before blood collection) were statistically different between HS and tolerant dogs.

Drug-serum protein adducts

Sera from 20 HS dogs and nine tolerant dogs that were sampled within 1 month of exposure to sulphonamides were selected for testing for drug–protein adducts. Sulphonamide–protein adducts were detected in 10 of 20 HS dogs (50%; Fig. 2). No sulphonamide–serum protein adducts were found in the sulphonamide-tolerant dogs, nor in any of 15 healthy sulphonamide-naïve dogs tested. In dogs with adducts compared with dogs without adducts, neither the median dosage of sulphonamide (39.6 vs. 40.9 mg/kg/day), the median duration of treatment (10.0 vs. 10.5 days), the median time from first drug exposure to blood collection (17 vs. 15 days), nor the time from drug HS (and drug discontinuation) to blood collection (6 vs. 7 days) differed significantly.

Drug-specific antibodies

Sera from the 34 HS dogs, 10 tolerant dogs and 18 naïve dogs were assayed by ELISA for antibodies against SMX, SDM or SDZ, as appropriate for individual drug of exposure. (Naïve dogs were tested against all three sulphonamides). Seventeen of the 34 HS dogs had detectable

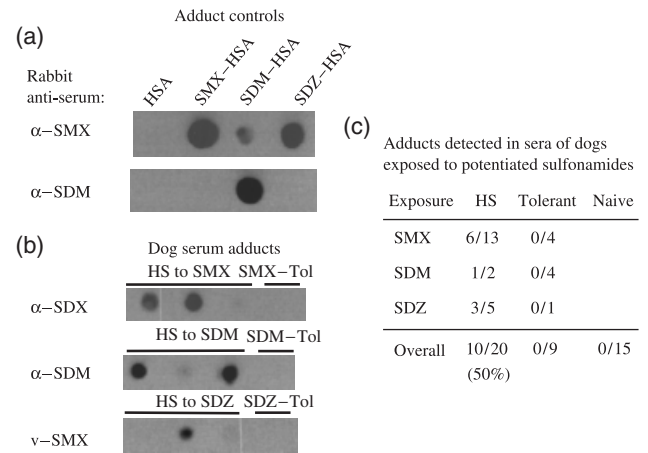


Fig. 2. Detection of sulphonamide–serum protein adducts. (a): dot immunoblots of rabbit anti-sera to sulphonamides (SMX) or sulphadimethoxine (SDM), against control adducts comprised of human serum albumin (HSA), SMX–HSA, SDM–HSA and sulphadiazine (SDZ)–HSA. (b): dot immunoblots for drug–protein adducts in sera from dogs either hypersensitive (HS) or tolerant (Tol) to SMX, SDM, or SDZ (representative blots shown from three HS and one tolerant dog exposed to each drug). (c): frequency of drug–serum adducts in sulphonamide-hypersensitive and -tolerant dogs.

IgG antibodies against the sulphonamide they received (13/20 SMX-HS, 3/8 SDM-HS and 1/6 SDZ-HS). Positive results for anti-drug antibodies were found in only one tolerant dog. The prevalence of anti-sulphonamide antibodies was significantly higher in HS dogs (50%) compared with tolerant dogs (10%) ($P = 0.025$). One 'naïve' dog with no recorded history of sulphonamide administration had positive antibodies to both SMX and SDM. However, at the time of blood sampling the owner did not report that this dog had been adopted from South Africa, and sulphonamide exposure before adoption could not be completely ruled out.

The median ELISA absorbance (blanked for HSA control) for anti-drug antibodies in sulphonamide-tolerant dogs (0.102, range – 0.293 to 0.534) was not significantly different from sulphonamide-naïve dogs (0.070, range – 0.594 to 0.549; $P = 0.53$). However, the median absorbance in the sulphonamide HS group (0.442, range – 0.340 to 1.454) was significantly higher than in both the naïve and tolerant groups ($P = 0.0001$ and $P = 0.011$, respectively; Fig. 3a). Dogs with positive serum antibodies did not differ from dogs with negative antibodies with respect to the time between initial drug exposure and serum collection, onset of clinical signs and serum collection, or daily dosage or duration of therapy. These parameters were similarly not correlated with ELISA antibody absorbance values. There were no significant associations between the presence of antibodies or adducts, and clinical signs of either hepatopathy ($n = 10$), blood dyscrasias ($n = 24$) or fever ($n = 12$), although numbers in each subgroup were relatively small.

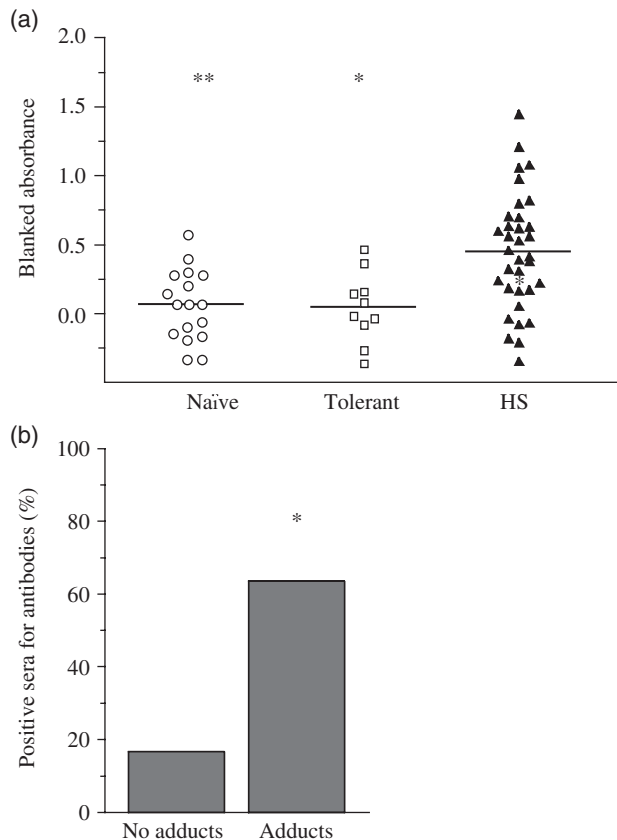


Fig. 3. Anti-sulphonamide antibodies and drug-serum adducts. (a): enzyme-linked immunosorbent assay absorbance values (blanked for human serum albumin) in dogs that were sulphonamide-naïve ($n = 18$; median values shown for assays against each of three sulphonamides), sulphonamide tolerant ($n = 10$), and sulphonamide hypersensitive (HS) ($n = 34$). Median absorbance for the HS group (0.442, range -0.340 to 1.454) was significantly higher than naïve controls (0.070, range -0.594 to 0.549 ; $**P = 0.0001$) and tolerant dogs (0.102, range -0.293 to 0.534 ; $*P = 0.011$). (b): dogs with drug-serum adducts had a significantly higher prevalence of anti-drug antibodies (64%) compared with dogs without adducts (17%; $*P = 0.009$). Both tolerant and HS dogs were included ($n = 29$).

Antibody absorbance values in dogs with drug-serum adducts were significantly higher (0.639, range -0.061 to 1.216) compared with dogs without serum adducts (0.170, range -0.340 to 1.454 ; $P = 0.007$). Furthermore, there was a significant positive association between the presence of drug-serum adducts and the presence of anti-drug antibodies across tolerant and HS dogs ($n = 29$; $P = 0.009$; Fig. 3b).

Specificity and cross-reactivity of anti-sulphonamide antibodies

In order to determine the specificity of anti-drug antibodies, positive sera from five HS dogs, exposed to either SMX ($n = 2$), SDM ($n = 2$) or SDZ ($n = 1$), were subjected to competition with the drug of exposure, drug-HSA conjugate, or HSA alone. Sera from dogs with anti-SMX

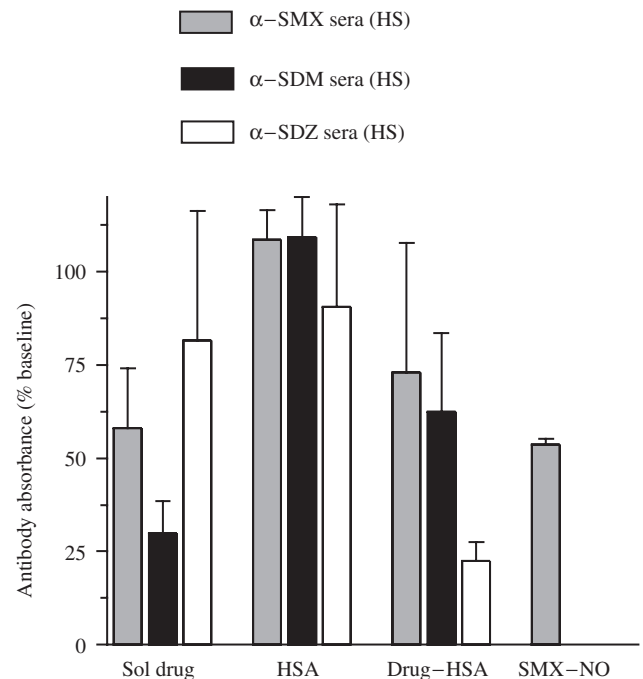


Fig. 4. Competition of positive antibodies by soluble drug, bound drug, or nitroso metabolite. Sera from HS dogs with positive antibodies to sulphamethoxazole (SMX), sulphadimethoxine (SDM) or sulphadiazine (SDZ), incubated with the drug of exposure (Sol drug; $500 \mu\text{g/mL}$), human serum albumin (HSA) ($100 \mu\text{g/mL}$), drug-HSA conjugate ($100 \mu\text{g/mL}$) or SMX-nitroso ($500 \mu\text{g/mL}$), for 2 h at 4°C , before enzyme-linked immunosorbent assay (ELISA). Percent inhibition of ELISA absorbance is shown relative to baseline positive absorbance (run concurrently in the presence of drug vehicle only). Competition assays were each run in duplicate in two independent assays; five dogs total were evaluated.

antibodies were also competed with SMX-NO. Binding of anti-drug antibodies was inhibited by free drug, drug conjugated to HSA, or SMX-NO metabolite, while HSA alone did not compete (Fig. 4).

To further evaluate the cross-reactivity of anti-drug antibodies to other anti-microbial sulphonamides, we tested each positive serum in an identical ELISA against the other two non-exposed sulphonamides. Among the 17 HS dogs with positive antibodies to the drug of exposure, nine (53%) exhibited antibodies against at least one of the two other sulphonamides tested, with six (35%) dogs showing positive results against all three sulphonamides tested. The single sulphonamide-tolerant dog with anti-drug antibodies against SMX (the drug of exposure) was negative for antibodies to SDM or SDZ.

Anti-SMX antibodies in dogs experimentally dosed with SMX-NO and TMP-SMX

To determine whether the parent sulphonamide or its nitroso metabolite led to the formation of anti-drug antibodies in dogs, we tested sera from four beagle dogs dosed experimentally with a 2 week course of SMX-NO,

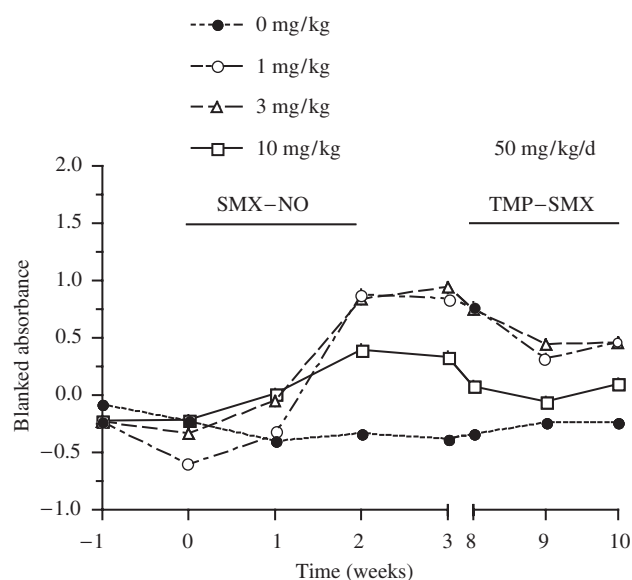


Fig. 5. Time-course of anti-sulphamethoxazole (SMX) antibodies in dogs experimentally dosed with SMX-nitroso (NO) metabolite or trimethoprim (TMP)-SMX. Dogs ($n = 4$) were dosed with SMX-NO (1–10 mg/kg, or drug vehicle) IP over 2 weeks [14]. All dogs were then challenged with TMP-SMX at 50 mg/kg/day per os, for an additional 2 weeks. Serum antibodies were determined by enzyme-linked immunosorbent assay.

followed by a 2 week course of TMP-SMX [14]. These dogs had previously been tested negative in an ELISA detecting anti-SMX antibodies, using a 1 h incubation with primary sera [14]. However, as anti-SMX IgG in humans can have a low affinity for drug [10], we repeated these assays using our modified ELISA protocol which includes an overnight incubation with patient serum. Using this assay, the three dogs that were dosed with SMX-NO showed rising antibody ELISA absorbance values during the SMX-NO dosing phase, while the dog that received drug vehicle had no detectable antibodies (Fig. 5). During the TMP-SMX dosing phase, dogs previously dosed with SMX-NO had persistent detectable anti-SMX antibodies, while the dog that received the vehicle control during the first dosing phase remained negative during TMP-SMX treatment.

Discussion

In the present study, drug-protein adducts were detected in the sera of dogs with a history of sulphonamide HS, but not in dogs given a therapeutic course of sulphonamides without adverse effects. This expands upon what has been shown in smaller groups of human patients, in which sulphonamide-serum protein adducts are more common in hypersensitive vs. tolerant patients [13]. The absence of adducts in tolerant dogs could not be attributed to a longer delay from sulphonamide exposure to sampling, as these lag times were similar between hypersensitive and tolerant groups. While none of the tolerant dogs had

detectable serum adducts, our sample size of 10 dogs is too small to conclude that adduct detection is exclusive to hypersensitive dogs. In humans, one immunoblotting study identified serum-drug adducts in two of two SMX HS patients, but not in the single SMX-tolerant patient tested [11]. Another study in humans reported serum-sulphonamide adducts in six out of eight HS human patients, but only in one of four tolerant patients [13]. Larger scale studies are needed to determine whether such drug-serum adducts could be used as reliable markers of sulphonamide HS risk in either humans or dogs.

Adducts result from the metabolite SMX-NO [16, 17], and the higher prevalence of adducts in HS human patients and dogs may reflect increased generation or impaired clearance of SMX-NO or its immediate precursor, SMX-hydroxylamine (Fig. 1), or impaired clearance of the adducts themselves. Serum adducts could contribute to the pathogenesis of HS by providing a depot for prolonged drug exposure. Serum adducts may also reflect the presence of additional drug adducts in target tissues that could then trigger humoral or cell-mediated immunity [16, 18, 19]. Because standard immunoblots for drug adducts (with electrophoresis) had persistently high background levels, we were not able to identify specific targets in dog sera. In humans, 30 or 40 kDa serum adducts have been described, but these proteins have not been characterized [11, 13]. Albumin (~70 kDa) is poorly reactive with SMX metabolites [20], making it an unlikely target.

Significantly more HS dogs (50%) had anti-sulphonamide antibodies than did tolerant dogs (10%). Furthermore, ELISA absorbance values were significantly higher in HS dogs. In humans, anti-sulphonamide antibodies have also been demonstrated, using a similar ELISA methodology, in patients with sulphonamide HS, either with a higher prevalence than in patients tolerant of SMX [12], or with a similar prevalence [10]. Anti-drug antibodies can also be absent in some human patients with sulphonamide HS [13]. These results together suggest that anti-drug antibodies could be potential markers of sulphonamide HS, but that used alone, they may not be sufficiently sensitive or specific. We detected anti-drug antibodies in two dogs 3.5–7 years after their adverse drug reaction. These two dogs had high ELISA absorbance values despite the delay between HS reaction and serum collection. This indicates that the humoral response to sulphonamide drugs in dogs can be long-lived. In humans, patients sampled beyond 1 year of sulphonamide HS reactions have been found to be negative for anti-drug antibodies [21], although only a few patients have been tested.

Sulphonamide anti-microbials themselves are considered too small to elicit a humoral response [22]. In rodents, SMX-NO, but not SMX itself, elicits anti-drug antibodies [19, 23]. This is consistent with our preliminary findings in dogs experimentally dosed with SMX-NO or drug vehicle, followed by TMP-SMX, in that only dogs dosed

with SMX-NO developed anti-drug antibodies. This suggests that conversion to an oxidative metabolite may also be important for antibody generation by SMX in dogs, a species affected by clinical HS. However, experiments in additional dogs, followed for a longer period of time, are necessary to confirm this.

In dogs with drug-serum adducts compared with those without circulating adducts, anti-drug antibodies were significantly more common, with higher absorbance values. This supports the hypothesis of a mechanistic relationship between drug adduct formation and anti-drug antibodies. Dogs with antibodies but without detectable serum adducts ($n=3$) may have cleared adducts before sampling; alternatively, haptenized proteins other than those in the serum, such as cellular proteins, could have triggered the generation of drug-specific antibodies. In human patients undergoing SMX desensitization, only those with detectable serum adducts developed cutaneous HS; however, only eight patients were studied [13]. In addition, one patient undergoing SMX prophylaxis developed serum adducts without HS. Therefore, the relationship between drug adducts and anti-drug antibodies remains to be established in humans.

In competition assays, free parent drug or drug-HSA conjugates inhibited the binding of anti-sulphonamide antibodies in dogs, but HSA alone did not. SMX-NO also competed out anti-SMX antibodies with the same efficiency as SMX. NO metabolites differ from the parent drug only in their reactive nitrogen group (which forms the adduct), and should not be recognized as different from the parent molecule once an adduct is formed [24]. In a clinical setting, the presence of the drug, its metabolite or serum protein conjugates in the blood stream could potentially influence binding of anti-drug antibodies to haptenized cells. The application of these results to other subjects with sulphonamide HS is limited by the relatively few sera tested by competition, and relatively large standard deviations for some experiments. However, these competition results are supported by the results of cross-ELISAs run on all dogs with positive antibodies. Interestingly, 53% (nine of 17) of sulphonamide HS dogs with positive antibodies against the drug of exposure also showed cross-reactivity towards at least one other sulphonamide anti-microbial, with 35% (six of 17) reacting against all three sulphonamides tested. These results in dogs parallel one study in human patients that demonstrated apparent cross-reactivity between SMX and SDZ in four individuals [12].

It is not yet clear whether anti-drug antibodies play a direct role in the pathogenesis of sulphonamide HS. Drug-specific T cell clones have been demonstrated in a few individuals with sulphonamide-associated skin reactions [25, 26], suggesting that these skin lesions are T cell-mediated [5,23]. Antibodies could therefore be a simple epiphenomenon of a cell-mediated immune reaction,

useful as a diagnostic marker. Alternatively, antibodies could contribute to the pathogenesis of drug HS in several ways. First, antibodies, like adducts, can serve as drug carriers [27, 28], acting as a depot to prolong drug exposure over time. Secondly, antibodies can serve as modulators of a cell-mediated response by enhancing the presentation of antigens to T cells [29, 30]. Anti-drug antibodies could also be directly pathogenic, triggering complement-dependent lysis or phagocytosis of cells adducted with drug, or leading to immune-complex organ damage. Drug-dependent anti-platelet antibodies are found in patients with sulphonamide-associated thrombocytopenia [31], and antibodies targeting liver microsomal proteins have been reported in patients with sulphonamide-associated multi-organ failure [21, 32]. A pathogenic role for these antibodies is supported by the finding of complement-dependent lysis of leukocytes or platelets *in vitro* by patient sera [33, 34], and by reports of clinical responses to intravenous immunoglobulin [35–37].

Neither anti-drug antibodies nor drug-specific T cells may be sufficient, however, for sulphonamide HS to develop. In humans, as in dogs, anti-sulphonamide antibodies can be found in tolerant individuals [10], albeit with a lower incidence than in patients with HS [12]. Antibodies were found in our pilot study in dogs experimentally dosed with SMX-NO, without clinical signs [14], and rodents dosed with SMX-NO develop both humoral and cell-mediated responses, again without clinical HS [18, 19, 23]. As dogs develop sulphonamide HS naturally, and can also be studied under controlled conditions, they represent a unique species in which to study this idiosyncratic drug reaction. The data presented herein support the use of the dog to further explore the mechanisms of sulphonamide HS as a model for humans. Work is underway in our laboratory to understand the accompanying factors, such as antioxidant depletion, that may influence the development of overt drug HS in the setting of a cellular or humoral immune response.

Acknowledgements

The authors would like to thank Dr Alastair Cribb for generously providing rabbit α -SMX sera used in initial experiments, Drs Ronald Schultz and Charles Czuprynski for advice on ELISA and immunoblotting assays, Dr Erica Behling-Kelly for valuable discussions and Ms Jacquie Leighton (Panigen Inc., Blanchardville, WI) for generating anti-SMX and anti-SDM antibodies.

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