

Standard Article

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Antihistone Autoantibodies in Dobermans With Hepatitis

H. Dyggve , S. Meri, T. Spillmann, H. Jarva, and M. Speeti

Background: Immune system involvement is suggested as an underlying cause for Doberman hepatitis (DH) based on female predisposition, lymphocyte infiltration, abnormal hepatocyte expression of major histocompatibility complex class II antigens, and homozygosity for dog leukocyte antigen DRB1*00601.

Objective: To measure serum antinuclear antibodies (ANA) and serum antihistone antibodies (AHA) in Dobermans with hepatitis. To determine whether increased serum ANA or serum AHA could be used to support the diagnosis of Doberman hepatitis (DH).

Animals: Privately owned 25 subclinically and 13 clinically affected DH Dobermans and 17 healthy control Dobermans.

Methods: Case-control study. Indirect immunofluorescence (IIF) microscopy and line blot tests were employed for the ANA pilot studies and an enzyme-linked immunosorbent assay (ELISA) assay for detection of IgG AHA.

Results: Indirect immunofluorescence revealed ANA-positive cases, and line blot showed AHA reactivity. In ELISA, importantly increased concentrations of AHA were found in 92% (23/25) of dogs in the subclinical stage and 84.6% (11 of 13) of dogs in the clinical stage of DH compared with no control dogs (0/17) ($P < 0.0005$). The mean AHA absorbance values of the blood samples obtained from the 25 subclinical DH dogs (1.36 ± 0.60 , mean \pm SD) and the 13 clinically affected dogs (1.46 ± 0.49) were significantly higher than in 17 control dogs (0.51 ± 0.18 ; $P < 0.0001$).

Conclusions and Clinical Importance: As the presence of AHA indicates autoimmune activity, our results favor an autoimmune background as one cause for DH. Antihistone antibody could represent a novel means for screening Dobermans with increased serum alanine transaminase concentrations and suspicion of DH.

Key words: Histone; Autoimmune hepatitis; Autoimmunity; Chronic hepatitis; Dog; Doberman hepatitis.

Hepatitis in Dobermans is a rare chronic liver disease of unknown etiology. It typically affects females and is characterized by increased alanine transaminase (ALT) activity, progressive lymphocyte-predominant inflammation, and increased hepatic copper content that correlates with parenchymal inflammation.^{1,2} Two competing models for Doberman hepatitis (DH) etiology have been proposed. One suggests that DH is a form of copper toxicosis, as Dobermans with hepatitis have had increased copper concentrations in the liver.³ According to the second theory, DH is due to a T-cell-mediated autoimmune response in genetically predisposed individuals.⁴ Involvement of the immune system is

From the Department of Equine and Small Animal Medicine, University of Helsinki, Helsinki, Finland (Dyggve, Spillmann, Speeti); and the Department of Bacteriology and Immunology and Immunobiology Research Program, University of Helsinki and HUSLAB, Helsinki, Finland (Dyggve, Meri, Jarva).

Shared last authorship.

The work was done at the Department of Equine and Small Animal Medicine and the Department of Bacteriology and Immunology and Immunobiology, University of Helsinki, Finland.

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Corresponding author: H. Dyggve, DVM, Department of Equine and Small Animal Medicine, University of Helsinki, P.O. Box 57, 00014 Helsinki, Finland; e-mail: hanna.dyggve@gmail.com.

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Abbreviations:

AHA	antihistone antibody
AIH-1	type 1 autoimmune hepatitis
AIH	autoimmune hepatitis
ALT	alanine transaminase
AMA	antimitochondrial antibody
ANA	antinuclear antibody
Anti-LKM1	liver-kidney microsome type 1 antibody
Anti-LMP	antiliver membrane antibody
CDH	clinical Doberman hepatitis
DLA	dog leukocyte antigen
DH	Doberman hepatitis
ELISA	enzyme-linked immunosorbent assay
HLA	human leukocyte antigen
IIF	indirect immunofluorescence
MHC	major histocompatibility complex
PBC	primary biliary cholangitis
RA	rheumatoid arthritis
RT	room temperature
SDH	subclinical Doberman hepatitis
SLA	soluble liver antigen
SLE	systemic lupus erythematosus

suggested by female predisposition, the presence of lymphocyte infiltration, an abnormal expression of major histocompatibility complex (MHC) class II antigens by the hepatocytes,⁴ and homozygosity for the risk allele DRB1*00601 of the dog leukocyte antigen (DLA) system.⁵ In early DH, the most prominent feature is mononuclear cell infiltration in the parenchymal and portal areas of the liver. As the disease gradually progresses, further structural changes develop, and the histology has more characteristic features of the human leukocyte antigen (HLA)-associated autoimmune hepatitis (AIH), with more typical interface hepatitis and bridging necrosis changes.^{2,6} The risk of developing DH is hereditary, and

it seems to be a complex trait disease, a condition in which the mode of inheritance involves one or more genes that operate alone or together, in combination with environmental factors.⁵

Autoantibodies are one of the characteristics of autoimmunity. In humans, autoantibodies are screened to diagnose an autoimmune disorder and, in some cases, to monitor disease progression.⁷ While most autoantibodies are not known to be pathogenic, many are linked to the development of autoimmune diseases. Therefore, autoantibodies play a substantial role in the study of autoimmune disease processes.⁸ In general, the immune system has a remarkable capacity to prevent self-antigens from stimulating autoimmune reactivity. Autoreactive B cells are efficiently removed at 2 checkpoints to ensure self-tolerance. The first checkpoint occurs in the bone marrow, where many autoreactive B cells undergo clonal deletion or become anergic as they mature.⁹ The second checkpoint is situated in the periphery, where the mechanisms are thought to be based largely on defective activation signals given to the lymphocyte when it encounters a self-antigen. This phenomenon leads to a state of anergy or apoptosis.⁹ Defects in the first and second checkpoints have been observed in human patients with rheumatoid arthritis (RA)¹⁰ and systemic lupus erythematosus (SLE).¹¹ High levels of antihistone antibodies (AHA) have been described in different autoimmune conditions of humans, such as in primary biliary cholangitis (PBC),¹² induced or spontaneous SLE,¹³ type 1 autoimmune hepatitis (AIH-1),^{14,15} and RA.¹⁶

Our previous findings suggest that DH is a tissue-specific autoimmune disease.^{2,5} To test the hypothesis that Dobermans with DH have autoantibodies, a case-control study was performed. Also, we addressed the question of whether these antibodies could be used for supporting in the diagnosis of the disorder.

Materials and Methods

Study Material

The study material consisted of 25 subclinical DH Dobermans (SDH) (20 females and 5 males) and 13 Dobermans suffering from clinical Doberman hepatitis (CDH) (11 females and 2 males). A suspicion of DH was raised when a Doberman had a >3-fold elevation in serum ALT values (reference limit 18–77 U/L). The mean ALT concentration in the SDH group was 759 U/L (range, 256–1,575 U/L) and in the CDH group 837 U/L (range, 496–1,157 U/L). DH was diagnosed histologically in both groups by a liver biopsy with hematoxylin and eosin staining, demonstrating mononuclear cell infiltration in the parenchymal and portal areas of the liver and distinct degree of fibrosis and necrosis. In the SDH group, 6 dogs had slight fibrosis, and of these dogs, mild bridging necrosis was found in 5 dogs and 1 dog had multifocal apoptotic cells. Clinical Doberman hepatitis dogs showed moderate-to-marked fibrosis and moderate-to-massive necrosis. The CDH dogs were biopsied postmortem. The rubeanic acid stains were positive for copper in all DH dogs, based on granular cytoplasmic staining in the hepatocytes. The degree of copper accumulation was evaluated on a scale 0–5 with a previously characterized classification.¹⁷ In the SDH group, 3 dogs were grade 1, 3 were grade 1–2, 9 were grade 2, 6 were grade 2–3, and 4 were grade 3.

In the CDH group, 3 were grade 1, 8 were grade 2–3, 1 was grade 3, and 1 was grade 4. In the SDH dogs, the copper was associated with inflammation focally in the centrilobular region. The copper was mainly associated with inflammation in the periportal and bridging necrosis areas in the CDH dogs. Quantitative copper was performed for 11 SDH dogs with a median of 792 µg/g dwt (dry weight liver) (range 430–1,886 µg/g dwt) and 7 CDH dogs with a median of 1,490 µg/g dwt (range 630–2,430 µg/g dwt). The median age at diagnosis in the SDH group was 4.9 years (range, 1.9–8.6 years). In the CDH group, the age distribution was 2.5–10.3 years, with a median age of 6.2 years at diagnosis.

The control group comprised 17 apparently healthy Dobermans (10 females and 7 males). The health status of the control dogs was evaluated based on history (no clinical signs of liver or other systemic disease), a complete physical examination, and hematology, full chemistry profile, and urinalysis. All dogs had normal ALT values. These dogs were examined at the University Small Animal Hospital, Faculty of Veterinary Medicine, University of Helsinki, Finland. Four control dogs were biopsied, and no hepatic changes were revealed in the histological analysis. The age distribution of the controls was 2.7–13 years, with a median age of 8.2 years.

The study was approved by the National Ethics Committee for Animal Experiments in Finland (ESLH–2009–08997/Ym–23). All dogs were privately owned Finnish Dobermans, and the owners provided written consent for participation. The samples were gathered during 1981–2010 and stored at –20°C. For the assays, the samples were thawed and analyzed on the same day. None of the dogs received immunosuppressive medication.

Methods

An indirect immunofluorescence (IIF) test was carried out to screen for possible autoantibodies in sera. This test was followed by a line immunoassay (INNO-LIA antinuclear antibody [ANA] Update) for detecting different antinuclear antibodies in a smaller number of cases and controls, as described below. Finally, we performed an enzyme-linked immunosorbent assay (ELISA) for AHA on all cases and controls.

Determination of Autoantibodies by IIF

The presence of antibodies in the sera was analyzed by IIF on mouse liver cryostat sections. We collected serum samples from 8 SDH (7 females and 1 male) and 2 CDH (1 female and 1 male) dogs in the case group and 10 healthy control Dobermans (6 females and 4 males). These samples were diluted to 1 : 10, 1 : 40, and 1 : 160 in 1% BSA/PBS (w/v; 0.5 g bovine serum albumin and 50 mL of phosphate-buffered saline, pH 7.4). Thirty microliters of the diluted sera was applied to the sections and incubated for 30 minute at room temperature (RT). The liver sections were then rinsed with PBS. The primary rabbit anti-dog IgG antibody^a was diluted at 1 : 200 in BSA/PBS, and the sample slides were incubated for 30 minute at RT. The sections were then washed with PBS and treated with Alexa-fluor 488 donkey anti-rabbit IgG^b at 1 : 400 dilution for another 20 minute at RT. After incubation, the slides were again washed with PBS. All incubations were made in a humid chamber to prevent excess drying. The stained samples were then examined under an Olympus fluorescence microscope. (Olympus, Hamburg, Germany).

Line Blot Analysis of Antinuclear Autoantibodies

To detect the presence of antibodies against nuclear and cytoplasmic autoantigens, a commercial INNO-LIA ANA Update test^c was used. The test can detect antibodies against 13 different

nuclear and cytoplasmic antigens in human sera. In this assay, recombinant antigens (SmB, RNP-70k, RNP-A, RNP-C, SSA/Ro52, SSB/La, Cenp-B, Topo-1/Scf-70, Jo-1), synthetic peptides (SmD and ribosomal P antigen), and natural proteins (SSA/Ro60 and histones) are attached to separate lines on a membrane. In addition to the autoantigens, 1 control line is present on each strip. We employed the test for antibodies in dogs as previously described¹⁸ and used the diluents, buffers, and color developers provided by the manufacturer. Nine SDH (7 females and 2 males) and 1 female CDH Doberman were included in the case group and 10 healthy Dobermans (6 females and 4 males) in the control group. The serum specimens were diluted 1 : 200, and the strips were then incubated in diluted serum for 1 hour at RT. After this, the blots were washed and incubated with a 1 : 2,000 diluted primary rabbit antidog poly-Ig antibody for 1 hour at RT. After the second wash, the secondary antibody (anti-rabbit) labeled with alkaline phosphatase and diluted to 1 : 2,000 was added for 1 hour at RT. After the last wash, a color substrate was added for antibody detection and left for 1 hour at RT. The color reaction was then stopped with the provided sulfuric acid. The bands were detected by visual interpretation by the same nonblinded examiner. The control strip was without serum overlay, but was otherwise handled the same way as the other strips in the assay.

Determination of Antihistone Autoantibody Levels by ELISA

After reactivity with the histone antigen had been noted in the line blot, we decided to confirm this finding with a second method. Therefore, an in-house ELISA assay for the detection of AHA was set up. Altogether, 38 sera from DH cases and 17 sera from healthy controls were analyzed. A calf thymus histone preparation^d was dissolved in carbonate buffer (pH 9.6) at a concentration of 1 µg/mL and coated onto Maxisorp microtiter plates^e overnight at +4°C. The plate was next washed 5 times with 0.05% Tween 20/PBS (PBS/Tween). Serum overlay in duplicate (dilution at 1 : 600 in PBS) was incubated on the plates for 1 hour at RT, and then the wells were washed 5 times with PBS/Tween. Rabbit antidog IgG was used as the primary antibody in 1 : 5,000 dilution in BSA/PBS and incubated for 1 hour at RT. The plates were then washed 5 times with PBS/Tween. An HRP-conjugated goat anti-rabbit IgG antibody^f was applied as a secondary antibody for 1 hour at RT in a 1 : 2,000 dilution in BSA/PBS. After 5 PBS/Tween washes, the enzyme activity was determined by adding 100 µL of 8 mg OPD (*o*-phenylenediamine dihydrochloride^g) diluted in 12 mL milliQ water and 5 µL H₂O₂ (30%). The obtained reaction was stopped by adding 100 µL of 1 M H₂SO₄. The optical density (OD) was measured at a wavelength of 492 nm.

One histone-reactive DH sample was chosen as a designated positive control sample and was analyzed at the same location on each plate. The obtained results were adjusted toward this sample for result comparison. The mean absorbance value (the ratio of the absorbance of the tested sample over the absorbance of the positive control sample) for healthy control dogs +2 SD (0.87) was used as a cutoff value for seropositivity. Control sera were stored in -20°C in aliquots and thawed just before use. All measurements were repeated twice.

Statistical Analysis

The statistical analyses were performed with a commercially available statistical program (PASW 18.0 software for Windows^h) with both Student's *t*-test and Fisher's exact test on each ELISA sample group. For normally distributed data, the results were expressed as mean ± SD, and differences with *P*-values < 0.05 were considered significant. The normality assumption was

satisfied by visual interpretation of the Q-Q plots. Graphical data presentation was performed with GraphPad Prism.ⁱ

Results

Indirect Immunofluorescence Detection of Antinuclear Autoantibodies

The immunofluorescence analysis of DH samples using mouse liver sections revealed a typical staining for ANA. In a pilot test of 10 DH cases, 5 serum samples were positive in the ANA test. Two sera (1 SDH and 1 CDH Doberman) showed an ANA titer of 1 : 40, 2 subclinical dogs had a titer of 1 : 160, and 1 subclinical dog had a titer of >1 : 160. All ANA-positive cases showed a homogeneous nuclear pattern (Fig 1). In the control group, 1 of 10 sera showed a detectable ANA at a titer of 1 : 10, and the remainder of healthy controls were seronegative.

Line Blot Analysis of Antinuclear Autoantibodies

Four of 10 DH serum samples (3 SDH and 1 CDH dog) showed clear antihistone reactivity. None of the tested control dog sera reacted with histones. Anti-RNP-A was present in 1 SDH and 1 CDH case as well as in 1 healthy control. Also, 1 subclinical dog had anti-SmD and another anti-SmB reactivity. Additional minor reactivity was seen mostly in the case samples. Positive reactivity was observed toward SSA/Ro60 in all study samples (Fig 2). The presence of AHA was also tested by Far-Western blotting with a calf histone preparation,⁴ and the results were similar to those of the ELISA analysis (data not shown).

Enzyme-Linked Immunosorbent Assay Analysis of Antihistone Antibodies

Overall, samples from 38 DH-positive Dobermans (25 SDH and 13 CDH) and sera from 17 clinically healthy control Dobermans were analyzed. The AHA result was above the cutoff value in 23 of 25 SDH dogs (92%) and in 11 of 13 CDH dogs (85%). All 17 controls were seronegative. These results showed a significant association between AHA IgG and DH (*P* < 0.0005). The antibody results showed a greater variation in the case groups. When comparing the mean absorbance values, SDH (1.36 ± 0.60, mean ± SD) and CDH (1.46 ± 0.49) absorbance values were both significantly higher than the values for controls (0.51 ± 0.18; *P* < 0.0001). The difference between the subclinical and clinical case groups was not significant (*P* = 0.29) (Fig 3). One subclinical dog was an outlier with a higher value, and the statistical tests were carried out with and without this result. The resulting outcome remained the same even when the outlier was included in the analysis.

Thus, in total, 34 of 38 dogs (89%) with either subclinical or clinical disease had IgG AHA at a dilution of 1 : 600. The ELISA assay had a sensitivity of 89.5% (95% confidence interval [CI] from 75.20 to

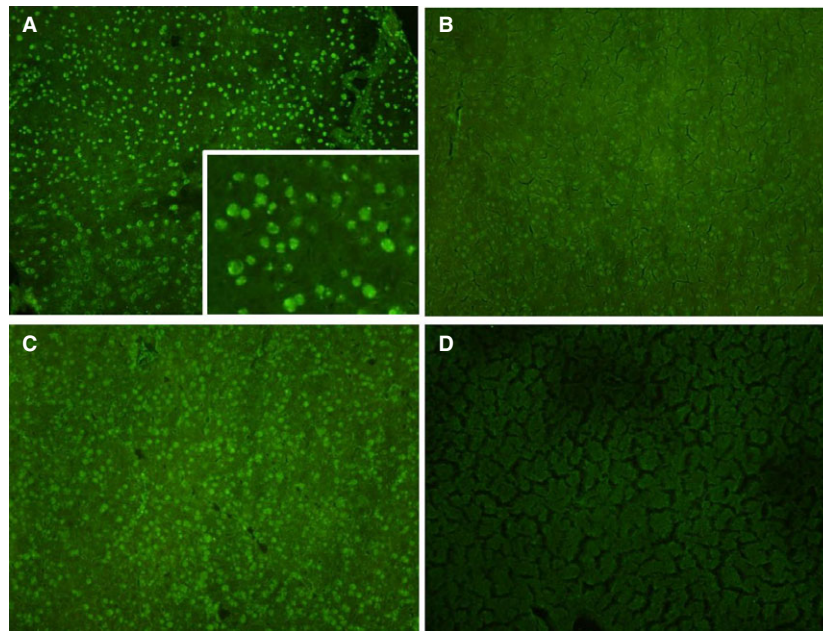


Fig 1. Indirect immunofluorescence analysis of autoantibodies at 400 \times magnification. The sera were diluted 1 : 10, 1 : 40, and 1 : 160 and incubated on mouse liver slides for detection of antinuclear antibodies. In the case group, 5 of the 10 samples were positive in the antinuclear antibody (ANA) test. (A) ANA titer of 40 was seen in 2 of the 10 samples (inset at 1,000 \times magnification). (B) Two of 10 samples had an ANA titer of 160. (C) One of the case group samples had a titer of >160. (D) A sample with no detectable ANA, dilution 1 : 10. Scale bar 25 μ m. Representative data are shown.

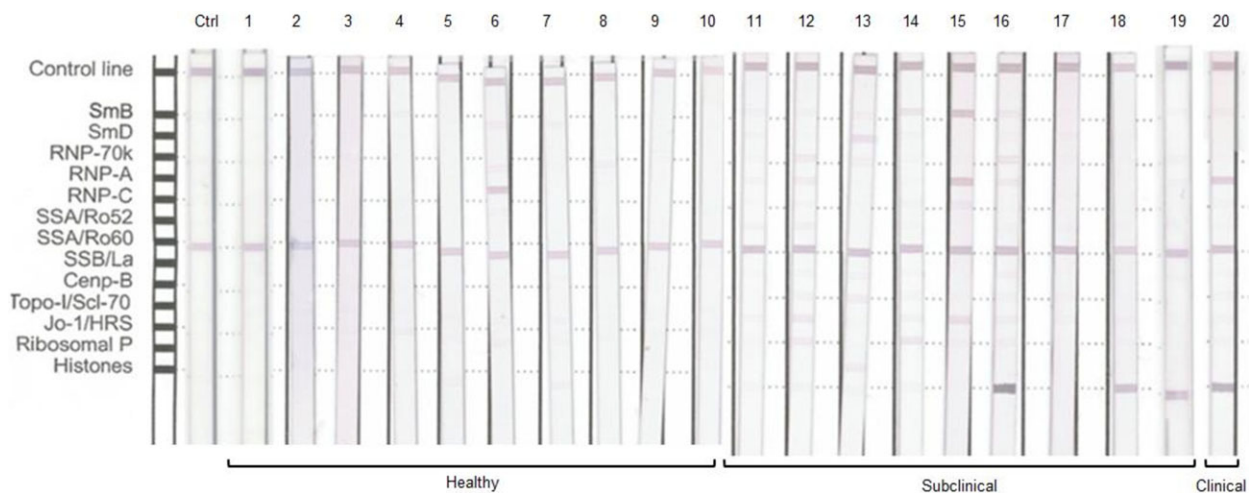


Fig 2. Line blot analysis of antinuclear antibodies in 10 controls (1–10) and 10 DH cases (11–20). Altogether 4 cases (3 of 10 subclinical and 1 of 10 clinical) showed antihistone reactivity in the line blot. Anti-RNP-A was present in 1 subclinical and 1 clinical case as well as in 1 healthy control. In addition, 1 subclinical dog had anti-SmD, and another had anti-SmB reactivity. All strips, including the negative control, reacted toward SSA/Ro60. The control strip (Ctrl) was incubated without serum but with buffer, and otherwise handled the same way as the other strips.

97.06%) and a specificity of 100% (95% CI from 80.49 to 100.00%) for detecting subclinical or clinical DH.

Discussion

The main goal of this study was to gather evidence that autoimmunity can be one cause of DH. We were

able to demonstrate significantly increased levels of AHA in both SDH and CDH Dobermans. Moreover, the healthy control Dobermans were all seronegative for AHA measured by line blot analysis and ELISA.

An autoimmune disease is a condition in which tissue injury is caused by an abnormal T cell or antibody reaction to self. The modified Witebsky's postulates^{19,20} characterize the different types of experimental criteria

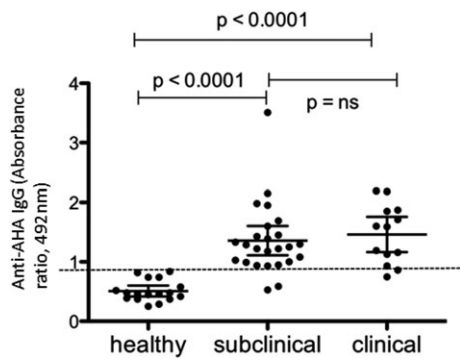


Fig 3. Enzyme-linked immunosorbent assay results for IgG antibodies against histone comparing DH patients with control dogs in 1 of 600 dilution. A cutoff value of 0.87 for positivity is indicated with a dotted line. Subclinical DH cases ($n = 25$, mean \pm SD: 1.36 ± 0.6) and clinical DH cases ($n = 13$, 1.46 ± 0.49) were both significantly higher than values for controls ($n = 17$, 0.5 ± 0.18 ; $P < 0.0001$). A statistical difference was not noted between the case groups ($P = 0.29$).

used to assess whether a disease is of autoimmune origin. These criteria are divided into direct proof and indirect and circumstantial evidence. The direct proof proposes that an autoimmune reaction is reproduced in a normal recipient by transferring a pathogenic antibody. The indirect evidence requires that a similar disease is reproduced in animals kept under experimental settings. Many disease processes are described by the word “autoimmune” even though they neither meet direct nor indirect criteria but have strong circumstantial evidence for support. Suspicion of an autoimmune disorder can be raised when affected patients have a statistical association with a particular MHC class II haplotype, an abnormal expression of MHC class II proteins on the affected organ, a lymphocytic infiltration of the target organ, increased levels of autoantibodies, a favorable response to immunosuppression, a positive family history of the disease, or an association with another autoimmune disease in the same individual or their family. Female predisposition is also typical for many autoimmune diseases.⁹

Many of these aforementioned circumstantial mechanisms and elements underlying autoimmune responses have been identified in humans. The most thoroughly described predisposing genetic factor in the development of nearly all autoimmune diseases is the MHC class II association.²¹ In the development of T-cell tolerance, these MHC class II molecules present self-antigens on the surface of professional antigen-presenting cells to naïve T cells. The self-antigen and MHC class II molecule complex guides T cells to survive or perish. In this way, these genes have a central role in the development of T-cell tolerance.²² The expression of MHC proteins can also be induced in endothelial cells by inflammatory conditions in the target organs of autoimmune diseases. An abnormal hepatocyte expression of MHC class II is seen, for example, in the cholestatic autoimmune diseases PBC and chronic sclerosing cholangitis.^{23,24} It has been proposed that autoimmune diseases have common

genetic factors predisposing to autoimmunity, as a familial clustering of the same autoimmune illness or occurrence of multiple autoimmune diseases in the same individual or the family is recognized.²⁵ Patients with AIH may have associated autoimmune features or a family history of other autoimmune diseases such as autoimmune thyroiditis,²⁶ inflammatory bowel disease, type 1 diabetes, and arthritis.²⁷ Except for diabetes mellitus, human autoimmune diseases are more common in females, but the reasons for this sex bias remain mostly unknown. X-chromosomal and hormonal factors, or lack of protective Y-chromosomal factors, have been suggested to play a role.²⁸

The typical histological characteristics of human AIH include interface hepatitis with portal and periportal monocellular infiltrates, and, usually, clusters of plasma cells. Fibrosis is featured in all but the mildest variants of the disease.⁶ An important part of the diagnostic process is the detection of autoantibodies in patients with suspected autoimmune disease. People with AIH have increased levels of autoantibodies that can be used to diagnose and differentiate the condition from other forms of chronic hepatitis. Autoimmune hepatitis can be further subclassified as AIH-1 and AIH-2 according to different antibody patterns. The standard antibody pattern for the diagnosis and classification of AIH-1 requires the identification of ANA,²⁹ smooth muscle antibodies,³⁰ and antibodies to soluble liver antigen (SLA).³¹ Anti-SLA is detected in 15–30% of AIH-1 patients and is associated with a more severe disease course. Thus, anti-SLA antibodies can be of prognostic value.³² The more serious form of AIH, AIH-2, is usually characterized by antibodies against liver-kidney microsome type 1 (anti-LKM1).³³ Antibodies to histones, although not pathognomonic, are present in 35% of ANA-positive patients with AIH.¹⁴

A suspicion for an immune-mediated background for DH was raised based on similar characteristics to autoimmune diseases in humans. The Doberman breed is at increased risk of the development of a severe form of chronic hepatitis and cirrhosis, and affected dogs exist in the same litter and lines of descent.^{1,34} Hepatitis in Dobermans mainly affects females.^{1,35} An association of many different DLA class II alleles and haplotypes with immune-mediated diseases in disease susceptibility has been previously established in dogs. In DH, homozygosity for the DLA allele DRB1*00601 of the DLA system genotype was significantly associated as a risk factor for DH.⁵ In early DH, the most prominent feature in histology is mononuclear cell inflammation in the parenchymal and portal areas of the liver. Also, an aberrant expression of MHC class II antigens on hepatocytes that correlated with the degree of inflammation was noted.⁴

Our aim was to investigate the presence of autoantibodies in DH and to find more proof for the suspected autoimmune background. Two studies have previously reported autoantibodies in DH. One study assessed the presence of circulating autoantibodies with IIF in 3 DH Dobermans.³⁶ One DH dog had been ANA-positive at a titer 1 : 10 with a granular nuclear fluorescent staining. However, antiliver membrane antibodies (anti-

LMP), smooth muscle antibodies, and antimitochondrial antibodies (AMA) were not present. No details concerning these dogs' medical records were given.³⁶ The other study found anti-LMP in connection with chronic hepatitis in a study on 21 canine patients of different breeds, including 4 Dobermans. Two of the Dobermans had high anti-LMP titers, while the other 2 were anti-LMP-negative. The latter 2 were on corticosteroid treatment.³⁷

Previous work in dogs has reported AHA to be common in dogs suffering from SLE.³⁸ Besides having a nuclear function, recent studies have indicated a significant toxic or pro-inflammatory effect of histones when released into extracellular space by damaged and activated cells.³⁹ Histone release has been suggested to play a part in autoimmune disease,⁴⁰ not just acting as a direct autoantigen, but also magnifying the destructive autoimmune processes.⁴¹

In the present study, significantly increased levels of AHA in both SDH and CDH Dobermans were detected. This result provides supporting evidence for the earlier suggestion that the immune tolerance has failed in DH, a key feature seen in autoimmune disorders. Furthermore, we wanted to determine whether these antibodies could aid the disease diagnostics. Based on our results, an increased AHA titer in conjunction with a high ALT concentration in a Doberman with or without clinical signs of hepatitis supports the diagnosis of DH. A negative AHA result does not completely rule out DH. The difference between the 2 case groups SDH and CDH was not statistically significant (Fig 3), suggesting that AHA is not a suitable biomarker for disease progression. The gold standard for diagnosing DH is histopathological evaluation of liver tissue for typical histopathological findings and to analyze the copper content. Antihistone antibody screening could be a valuable tool in DH diagnostics in the subclinical and especially in the clinical stage of DH, if taking a liver biopsy is not performed due to potential significant risk of bleeding or due to client concerns or constraints. In this case, an aspirate and cytology could be considered to attempt to detect hepatic copper.⁴²

Anti-RNP-A was found in 1 SDH and 1 CDH case as well as in 1 healthy control. This reactivity does not fulfill the criteria for RNP autoantibodies. Nonspecific positive reactivity was observed toward SSA/Ro60 (Fig 2) in the line blot analysis, and, apparently, this was due to the reactivity of the secondary antibody. The differences in the female/male ratio between the DH and control groups may have had an influence on the data. This is, however, unlikely due to the result that also males were positive among the SDH and CDH dogs. A limitation of our study was that there was not enough serum for some cases to make all the different assays and the World Small Animal Veterinary Association's (WSAVA) standardized evaluation of histopathology could not be performed for all the older DH samples. The grading of histopathological findings for chronic hepatitis was based on Knodell's method.^{2,43} Also, the histopathological evaluation was not available for all of the control dogs. Nonetheless,

these dogs were considered healthy at the sampling, and their health status was followed until 2 years after sampling with no elevation of ALT. One limitation was that copper was not quantified in all DH dogs; however it was scored and found in association with inflammation in DH dogs.

Conclusions and Prospects

Our findings provide novel information about dogs suffering from DH. To date, the etiology and pathogenic mechanisms of DH remain unknown. Our autoantibody findings, combined with previous observations in DH, support the theory that DH has an autoimmune origin. From a clinical standpoint, AHA may be a useful biomarker that could be used to improve the currently difficult early diagnosis of DH in Dobermans with increased ALT concentrations. Further studies are needed to reveal the link between autoimmunity, liver damage, and abnormal copper retention in DH.

Footnotes

^a Bethyl Laboratories Inc, Montgomery TX, USA

^b Invitrogen, Carlsbad, CA, USA

^c Innogenetics N.V., Ghent, Belgium

^d Sigma, Deisenhofen, Germany

^e Nunc, Roskilde, Denmark

^f Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA

^g Dako, Denmark

^h SPSS, Inc, Beijing, China

ⁱ GraphPad Software, San Diego, CA, USA

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Conflict of Interest Declaration: Authors declare no conflict of interest.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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