Tissue Antigens ISSN 0001-2815

BRIEF COMMUNICATION

Association of hypothyroid disease in Doberman Pinscher dogs with a rare major histocompatibility complex DLA class II haplotype

L. J. Kennedy[1], H. J. Huson[2], J. Leonard[2], J. M. Angles[3], L. E. Fox[4], J. W. Wojciechowski[5], C. Yuncker[2] & G. M. Happ[2]

1 Center for Integrated Genomic Medical Research, University of Manchester, Manchester, UK
2 Institute of Arctic Biology, University of Alaska, Fairbanks, AK 99775, USA
3 Koret Center for Veterinary Genetics and Center for Companion Animal Health, School of Veterinary Medicine, University of California, Davis, CA 95616, USA
4 College of Veterinary Medicine, Iowa State University, Ames, IA 50011, USA
5 Animal Allergy & Dermatology Clinic, Kingston, NH 03838, USA

Key words
DLA; Dogs; hypothyroid disease; MHC

Correspondence
Dr Lorna J. Kennedy
Center for Integrated Genomic Medical Research
Stopford Building
Oxford Road
Manchester M13 9PT, UK
Tel: 44 161 275 7316
e-mail: lorna.kennedy@manchester.ac.uk

Received 5 August 2005; accepted 20 September 2005

doi: 10.1111/j.1399-0039.2005.00518.x

Abstract

Canine hypothyroid disease is similar to Hashimoto's disease in humans, which has been shown to be associated with human major histocompatibility complex (MHC) genes. We have collected 27 Doberman Pinschers affected with primary hypothyroid disease and compared their MHC class II haplotypes with 129 unaffected Doberman Pinschers. Three dog-leucocyte antigen (DLA) genes, DLA-DRB1, DQA1 and DQB1, were characterized by sequence-based typing and assigned to haplotypes for each dog. One rare haplotype was found at an increased frequency in the affected dogs compared to the unaffected dogs (Odds ratio = 2.43, $P < 0.02$). This haplotype has only been found in Doberman Pinschers and Labradors to date.

The dog is by far the leading companion animal worldwide and a major proportion of veterinary healthcare, and its associated costs are directed at its well being. In a recent report by the American Kennel Club, four of the 11 most frequent canine diseases had an immunological basis, including hypothyroid disease, other autoimmune diseases, cancer and allergic dermatitis. Major histocompatibility complex (MHC) encoded genes are likely to be an important factor for such immunologically mediated conditions. Although some breed predisposition to hypothyroid disease has been reported anecdotally, this research potentially applies to all dog breeds. This paper reports on a study that was performed to look for any MHC associations with hypothyroid disease in Doberman Pinscher dogs.

As in other species, variation within the MHC of canids is central to the adaptive immune response, and only through gaining an understanding of this genetic system will we be able to manipulate the underlying mechanisms of response and induce protective immunity against disease. This may come through relating peptide/vaccination design to MHC class II polymorphism or by adopting informed breeding strategies.

In addition to such potential veterinary benefits, there are sound scientific reasons for investigating the regulatory role of immune response by the MHC in the dog. The majority of experimental work relating to MHC gene polymorphisms in open populations has been restricted to humans and to a lesser extent in other primates. Studies of other species have largely focused on laboratory-inbred rodent strains where there is a highly restricted and to some extent contrived range of MHC haplotypes. Little is known about the extent or distribution of MHC alleles and haplotypes in open or wild populations and how this relates to immunity and disease resistance. The genus *Canis* is ideal for conducting these types of studies as it exists both in wild populations (wolves, coyotes, wild dogs, etc.) and in domesticated situations. In the latter, it

can be studied both in restricted relatively inbred groups (pedigree breeds) and open populations (mongrels).

Given the relationship between MHC class II polymorphism and autoantibody production seen in humans, it is also useful to explore this area in canids. Dogs produce a range of autoantibodies, many of which also have counterparts in human disease and therefore provide an ideal opportunity to study the mechanisms underlying these associations.

Autoimmunity is a significant clinical problem in the dog, often resulting in high-titre pathogenic autoantibodies. Autoantibodies such as those directed against thyroglobulin are relatively common and highly clinically relevant, particularly in some dog breeds. Primary hypothyroid disease is a common endocrinopathy in dogs (1, 2) and is often caused by lymphocytic thyroiditis (3). Canine lymphocytic thyroiditis is considered to be an autoimmune-mediated disease on the basis of its clinical and histological similarities to Hashimoto's thyroiditis in man (4), and because of the presence of autoantibodies to thyroglobulin (5).

Definitive diagnosis of ambiguous cases is difficult, since good clinical diagnostic tests are not available. The disease presents with obesity, lethargy, alopecia and reproductive abnormalities (6). Neutered dogs, of either sex, seem to have a significantly higher risk of hypothyroid disease compared with sexually intact dogs (6). The disease is characterized by low levels of the thyroid hormones, tri-iodothyronine (T3) and thyroxine (T4), plus low levels of thyroid-stimulating hormone (TSH). It is becoming clear that these hormones may be lowered by a variety of different mechanisms, but true primary autoimmune hypothyroid disease is usually characterized by the presence of autoantibodies to thyroglobulin (7). Since about 50% of dogs with low thyroid hormone levels have autoantibodies to thyroglobulin (8), any group of dogs with 'hypothyroid disease' will probably be heterogeneous, adding to the difficulty in detecting an MHC genetic association.

There is a clear genetic component to canine thyroiditis, particularly in closely inbred lines (2), while several breeds are thought to be especially susceptible to the disease. Such breeds include Doberman Pinschers and Golden Retrievers (6) and also Borzois, Giant Schnauzers, Akitas, Irish Setters, Old English Sheep dogs, Skye Terriers and Shetland Sheep dogs (2). An increased incidence of thyroiditis has also been reported in Beagles (9), Great Danes (10) and English Cocker Spaniels (10), although it can develop in dogs of any breed. It is generally believed that thyroiditis is less common in mongrels. This suggests that genetic factors may be important in contributing to the aetio-pathogenesis of thyroiditis and hypothyroid disease. However, studies in humans have demonstrated that MHC gene polymorphisms can be highly significant risk factors for certain autoimmune conditions.

Canine thyroiditis (or lymphocytic thyroiditis) is very similar to Hashimoto's disease in humans. HLA associations have been reported for Hashimoto's disease (11). The MHC in most species consists of three regions of tightly linked genes (class I, II and III), the first two of which are involved in regulation and the presentation of self and non-self antigens to the immune system. In dogs, the MHC is referred to as the DLA system and molecular characterization has only recently begun. The DLA system is known to contain class I, II and III genes, although the precise number of genes and their relative positions has not yet been determined. Genes within the class I and II region appear to be highly polymorphic, but the full extent of this polymorphism has again not been determined. We have previously investigated DLA-DRB1, DQA1 and DQB1 polymorphism in the dog and set up molecular-based methods suitable for routine DLA genotyping (12–15). To date, 67 DRB1, 21 DQA1 and 54 DQB1 alleles have been identified and named by the International DLA Nomenclature Committee (under the auspices of the International Society for Animal Genetics), which is presently co-ordinated by Dr L.J. Kennedy (16, 17). It is likely that further DLA-DQ and DLA-DR polymorphisms will be identified, especially as more breeds are examined.

Previous studies have already demonstrated that considerable differences exist in the distribution of DLA class II specificities in many of the breeds examined (18, 19). It has also been demonstrated that highly conserved DLA-DQ-DR extended haplotypes exist and that these are overrepresented in some breeds (18–20). In the first study of a canine autoimmune disease, it was shown that there is a frequently detected and associated DLA-DRB1 epitope in dogs with autoimmune rheumatoid arthritis (RA) (21). Indeed, this is the same epitope that has been shown to be associated with susceptibility to RA in humans, suggesting a shared pathology and genetic susceptibility for RA between humans and dogs.

Variation in MHC class II allele and haplotype frequencies may explain why individual dogs or certain breeds are good or poor responders to particular antigens or why certain dogs are prone to autoimmunity and the development of autoantibodies (22). We now wish to examine whether the risk of developing canine autoimmune hypothyroid disease and/or thyroglobulin autoantibodies is correlated with specific canine MHC gene polymorphisms. This could give insights into this clinically important condition and could lead to strategies for its long-term reduction in certain breeds that are predisposed to the disease.

For this study, we collected blood samples from 364 purebred Doberman Pinscher dogs at the National dog

show in Sacramento and dog kennels in Florida. All samples were stored in EDTA and were collected within the guidelines for such sampling in the USA. Genomic DNA was extracted using the Qiagen QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) and stored in deionized water. Phenotypic and clinical data were also collected, including sex, age and full hypothyroid panel test results (TGAA%, TT4, TT3, FT4, FT3, autoT4, autoT3 and CTSH). The definition for affected dogs was essentially the convention used by Michigan State, namely titres of anti-thyroglobulin antibodies (TGAA) of >190%. Using this definition, 27 affected dogs with hypothyroid disease, and 129 unaffected dogs were selected for MHC testing.

DLA-DRB1, DQA1 and DQB1 alleles were identified by sequence-based typing and by direct sequencing of purified PCR products.

Forward and reverse primers used for DLA-DRB1, DQA1 and DQB1, respectively, were:

DRBF: GAT CCC CCC GTC CCC ACA G and
DRBR3: CGC CCG CTG CGC TCA (previously unpublished)
DQAin1: TAA GGT TCT TTT CTC CCT CT and
DQAin2: GGA CAG ATT CAG TGA AGA GA (23)
DQB1B: CTC ACT GGC CCG GCT GTC TC and
DQBR2: CAC CTC GCC GCT GCA ACG TG (19, 23).

All the primers are intronic and locus specific. The product sizes were 303 bp for DLA-DRB1, 345 bp for DLA-DQA1 and 300 bp for DLA-DQB1.

DLA-DRB1 and DLA-DQB1 PCR reactions were performed with 50–100 ng DNA in a 50 μl reaction containing ×1 PCR buffer as supplied by Qiagen (with no extra magnesium), Q solution (Qiagen), final concentrations of 0.1 μM for each primer, 200 μM each dNTP and with 2 U of Taq polymerase (Qiagen HotStarTaq; Qiagen, Valencia, CA). DLA-DQA1 PCR reactions were performed with 50–100 ng DNA in a 50 μl reaction containing ×1 PCR buffer, 200 μM each dNTP, final concentrations of 2.5 mM of $MgCl_2$ and 0.2 μM for each primer, with 2.5 U AmpliTaq polymerase (Applied Biosystems, Foster City, CA). A negative control containing no DNA template was included in each run of amplifications to identify any contamination.

A touchdown PCR protocol was used for DLA-DRB1 amplifications, which consisted of an initial 15 min at 95°C, 14 touchdown cycles of 95°C for 30 s, followed by 1 min annealing, starting at 62°C and reducing by 0.5°C each cycle and 72°C for 1 min. Then 20 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 1 min plus a final extension at 72°C for 10 min. The PCR protocol for DLA-DQA1 was 94°C for 4 min, then 30 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 45 s, followed by 72°C for 10 min. The PCR protocol for DLA-DQB1 was 95°C for 15 min, then 30 cycles of 94°C for 30 s, 64°C for 30 s, and 72°C for 1 min, followed by 72°C for 10 min.

Table 1 DLA alleles and haplotypes identified in this study

DRB1*	DQA1*	DQB1*	Number of homozygotes	Number of heterozygotes
00201	00901	00101	8	13
00601	00401	01303	67	68
01501	00901	00101	–	6
00601	005011	00701	1	12
01201	00101	00201	6	47
00601	005011	01303	–	2

All PCR samples were purified using the Qiagen QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Cycle sequencing was performed using Big Dye Terminator V3 (Applied Biosystems). Sequence purification was performed with Sephadex G-50 DNA grade and the samples sequenced on an Applied Biosystems 3100 Genetic Analyzer. Sequencing data were analysed using Sequencher (GeneCodes), SeqScape, MatchTools and MatchTools Navigator (Applied Biosystems).

Four haplotypes were initially identified in homozygous dogs (Table 1). These haplotypes were then assigned in heterozygous dogs, and another two haplotypes were identified. One haplotype, DLA-DRB1*00601/DQA1*00401/DQB1*01303, was very common, found in 135 of 156 (86.5%) dogs. The frequencies for each haplotype were calculated for the affected and control dogs (Table 2). One haplotype, DLA-DRB1*01201/DQA1*00101/DQB1*00201, is almost twice as frequent in affected dogs compared to unaffected dogs, 55.56 *vs* 29.46% (Odds ratio = 2.43, confidence limits = 1.19–7.61, $P < 0.02$). This is a rare haplotype in the general dog population, found in only 30 out of 3014 dogs typed in Manchester (L.J. Kennedy, unpublished data), and, to date, only found in Labradors and Dobermans.

While it is clear that this MHC haplotype is found in association with hypothyroid disease in Dobermans, it is unlikely that the same rare haplotype will be found to be associated with hypothyroid disease in other dog breeds. We expect that in other breeds, different haplotypes will be

Table 2 Numbers of affected and control dogs with each DLA haplotype

DRB1*	DQA1*	DQB1*	Number of affected dogs ($n = 27$) (%)		Number of control dogs ($n = 129$) (%)	
00201	00901	00101	3	(11.11)	18	(13.95)
00601	00401	01303	25	(92.59)	110	(85.27)
01501	00901	00101	1	(3.70)	12	(9.30)
00601	005011	00701	0		2	(1.55)
01201	00101	00201	15[a]	(55.56)	38	(29.46)
00601	005011	01303	0		6	(4.65)

[a] Odds ratio = 2.43, confidence limits = 1.19–7.61, $P < 0.02$.

found to be associated with hypothyroid disease, and that these haplotypes may have alleles in common with the predisposing haplotype found in Dobermans. It seems possible that there may also be other genes in the MHC region that are linked to hypothyroid disease, and that the MHC is a marker for these hypothyroid genes.

Acknowledgments

This work was supported in part by grants from the Morris Animal Foundation (97CA09), the Doberman Pinscher Foundation of America, the National Institutes of Health (2 P20 RR016466) and the National Science Foundation (0346770), and the American Kennel Club (No: 2447). We thank Dr Raymond Nachreiner at Michigan State University for the thyroid-panel assays. We thank Lisa Boehmer for samples from the UCLA Doberman colony.

References

1. Chastain CB, Panciera. Hypothyroid diseases. In: Ettinger SJ, Feldman EC, eds. *Textbook of Veterinary Internal Medicine, 4th edn. Philadelphia*: *W.B. Saunders*, 1995, 1487–500.
2. Happ GM. Thyroiditis – A model canine autoimmune disease. *Adv Vet Sci Comp Med* 1995: **39**: 97–139.
3. Lucke VM, Gaskell CJ, Wotton PR. Thyroid pathology in canine hypothyroidism. *J Comp Pathol* 1983: **93**: 415–21.
4. Beierwaltes WH, Nishiyama RH. Dog thyroiditis: occurrence and similarity to Hashimoto's struma. *Endocrinology* 1968: **83**: 501–8.
5. Mizejewski GJ, Baron J, Poissant G. Immunologic investigations of naturally occurring canine thyroiditis. *J Immunol* 1971: **107**: 1152–60.
6. Panciera DL. Hypothyroidism in dogs – 66 cases (1987–92). *J Am Vet Med Assoc* 1994: **204**: 761–7.
7. Vajner L. Lymphocytic thyroiditis in beagle dogs in a breeding colony: findings of serum autoantibodies. *Vet Med (Praha)* 1997: **42**: 333–8.
8. Dixon RM, Mooney CT. Canine serum thyroglobulin autoantibodies in health, hypothyroidism and non-thyroidal illness. *Res Vet Sci* 1999: **66**: 243–6.
9. Benjamin SA, Stephens LC, Hamilton BF et al. Associations between lymphocytic thyroiditis, hypothyroidism, and thyroid neoplasia in Beagles. *Vet Pathol* 1996: **33**: 486–94.
10. Haines DM, Lording PM, Penhale WJ. Survey of thyroglobulin autoantibodies in dogs. *Am J Vet Res* 1984: **45**: 1493–7.
11. Farid NR. Understanding the genetics of autoimmune thyroid disease – still an elusive goal. *J Endocrinol Metab* 1992: **74**: 495A–496A.
12. Kennedy LJ, Carter SD, Barnet A et al. Nine new dog DLA-DRB1 alleles identified by sequence based typing. *Immunogenetics* 1998: **48**: 296–301.
13. Kennedy LJ, Carter SD, Barnes A et al. DLA-DRB1 polymorphisms in dogs defined by sequence specific oligonucleotide probes (SSOP). *Tissue Antigens* 1999: **53**: 184–9.
14. Kennedy LJ, Carter SD, Barnes A et al. DLA-DQA1 polymorphisms in dogs defined by sequence specific oligonucleotide probes (SSOP). *Tissue Antigens* 2000: **55**: 257–61.
15. Kennedy LJ, Hall LS, Carter SD et al. Identification of further DLA-DRB1 and DQA1 alleles in the dog. *Eur J Immunogenet* 2000: **27**: 25–8.
16. Kennedy LJ, Altet L, Angles JM et al. Nomenclature for factors of the dog major histocompatibility system (DLA), 1998: first report of the ISAG DLA nomenclature committee. *Tissue Antigens* 1999: **54**: 312–21.
17. Kennedy LJ, Angles JM, Barnes A et al. Nomenclature for factors of the dog major histocompatibility system (DLA), 2000: second report of the ISAG DLA nomenclature committee. *Tissue Antigens* 2001: **58**: 55–70.
18. Kennedy LJ, Carter SD, Barnes A et al. Interbreed variation of DRB1, DQA1 alleles and haplotypes in the dog. *Vet Immunol Immunopathol* 1999: **69**: 101–11.
19. Kennedy LJ, Barnes A, Haap GM et al. Evidence for extensive DLA polymorphism in different dog populations. *Tissue Antigens* 2002: **60**: 43–52.
20. Kennedy LJ. Canine major histocompatibility complex polymorphism. *PhD Thesis*. University of Manchester, Manchester, 2000.
21. Ollier WER, Kennedy LJ, Thomson W et al. Dog MHC alleles containing the 'human RA shared epitope' confer susceptibility to canine rheumatoid arthritis. *Immunogenetics* 2001: **53**: 669–73.
22. Tizard IR. Vaccination and vaccines. In: Tizard IR, Schubot RM, eds. *Veterinary Immunology, 5th edn. London*: *W.B. Saunders*, 1996, 265–83.
23. Wagner JL, Burnett RC, DeRose SA, Storb R. Molecular analysis and polymorphism of the DLA-DQA gene. *Tissue Antigens* 1996: **48**: 199–204.