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Thyroiditis—A Model Canine Autoimmune Disease

GEORGE M. HAPP

Department of Biology, University of Vermont, Burlington, Vermont 05405

I. Introduction and Background

Although low thyroid function has been recognized for over 100 years and hypothyroidism is the most common endocrinopathy of dogs (Feldman and Nelson, 1987; Dunn, 1989; Chastain, 1990; Jeffers, 1990; Panciera, 1990a), definitive diagnosis can be uncertain, especially when clinical signs are vague and results of diagnostic tests are equivocal. Therefore the true incidence of canine thyroiditis remains difficult to establish. Thyroiditis is representative of a large number of autoimmune diseases in which immunological surveillance falters so that self and nonself become confused and the body attacks itself.

In human medicine, autoimmunity is the cause of most common endocrine disorders (Baker, 1992). This appears to be true for dogs as well. A significant measure of the diagnostic ambiguity about canine hypothyroidism stems from our ignorance of the fundamental causes and contributing mechanisms underlying the autoimmune responses of dogs. The development of autoimmune disease generally involves a multistep process: an initiating event produces nonspecific trauma or inflammation that triggers a secondary immune reaction in a genetically predisposed individual. The endocrine gland is progressively destroyed by the autoimmune attack, and as the hormone titers change, clinical symptoms appear (Baker, 1992). This chapter reviews thyroiditis as a model for research on the general features of canine autoimmunity. One aim is to screen for canine alleles of the major histocompatibility complex (MHC) like those that have been associated with autoimmune disease in people (Sinha *et al.,* 1990; Farid, 1991, 1992; Volpé, 1991).

Many clinical symptoms suggest a deficit in the level of circulating thyroxine (T_4) or triiodothyronine (T_3), but no single symptom or combination of symptoms is generally accepted as definitive. Common presentations include impaired growth of the epidermis or its derivatives, lethargy, exercise intolerance, cold intolerance, mental dullness, reproductive inadequacies, myopathy, neuropathy, cardiovascular abnormalities, and behavioral anomalies (Feldman and Nelson, 1987; Dunn, 1989; Jeffers, 1990; Panciera, 1990a). The disease is most frequent in 4–10 year old dogs, and certain breeds appear genetically predisposed (Feldman and Nelson, 1987). The most common early symptom in dogs is a generally poor skin and hair coat with bilateral alopecia, particularly of the flanks and tailhead. In working dogs, there is commonly loss of energy, reduced concentration, and thinness of the footpads, leading to lameness. In greyhounds, low thyroid hormone levels have been correlated with poor racing performance (Taylor and Hauler, 1983), but this initial suggestion was not confirmed in a follow-up study with larger sample sizes (Beale *et al.,* 1992).

The usual thyroid function tests involve a panel of measurements including total (bound and unbound) and free (unbound) T_3 and T_4 concentrations in the blood. While some experts accept serum total T_4 as the most reliable single diagnostic indicator (Panciera, 1990b; Nelson *et al.*, 1991), others believe that accurately measured free T_4 levels are most predictive of thyroid dysfunction (Larsson, 1988; Refsal and Nachreiner, 1993).

More information helps in understanding difficult presentations. Thus, most investigators recommend a complete panel of thyroid measurements including concentrations of circulating autoantibodies to T_3

and T_4 for assessment of clinical patients and genetic screening of breeds or individual dog families at risk for thyroid disease. If the results are unclear, the panel of hormone measurements is followed by a thyroid stimulating hormone (TSH) stimulation test (Ferguson, 1984; Beale, 1990; Jeffers, 1990) and confirmed by rescue with oral administration of levothyroxine given twice daily (synthetic L-thyroxine). Discussions of these tests are available elsewhere (Panciera, 1990b) and are beyond the scope of the present review. Other factors, including the female reproductive cycle (Reimers *et al.*, 1984), age, sex, body size and illness (Jeffers, 1990; Panciera, 1990b; Reimers *et al.*, 1990), glucocorticoids (Panciera, 1990b; Kaptein *et al.*, 1992), and the genetics of the dog, can influence these values. As in any disease of a regulatory system, the problem could lie in signal production, signal transmission, signal reception, or the effector response downstream. The present chapter will focus on primary hypothyroidism, which is due to insufficient production of T_3 or T_4 in the thyroid gland.

Canine hypothyroidism has a strong genetic component (Haines *et al,* 1984; Conaway *et al.*, 1985a). This is especially apparent in closely bred lines (Musser and Graham, 1968). In a classic example within the beagle colony of the Argonne National Laboratory near Chicago, 401 animals from two partially inbred lines were examined and scored positive for thyroiditis if there were inflammatory cells in sections of the thyroid gland (Fritz *et al.*, 1970). There was a total of 63 cases of the disease in the laboratory colony. When the pedigrees of the afflicted and healthy dogs were compared, a statistically significant genetic component was obvious. In fact, the eight litters of one line showed 23 of the 30 pups with histological evidence of hypothyroidism. The authors also suggest that thyroiditis was less common in the founder dogs of the colony, which were certainly less inbred.

The incidence of thyroiditis differs according to the breed of dogs. In certain breeds, notably borzois (Conaway *et al.*, 1985a), giant schnauzers (Greco *et al.*, 1991), Doberman pinschers, akitas, cocker spaniels, golden retrievers, Irish setters, Old English sheepdogs, Skye terriers, and Shetland sheepdogs (see Jeffers, 1990; Panciera, 1990a), thyroid disease apparently occurs at high frequency in many lines. It is generally believed that thyroiditis is less common in mongrels and outbred dogs like Alaskan huskies.

We are in the midst of a diagnostic revolution—the application of the techniques of molecular biology to the unequivocal detection of carriers of genetic disease. As this review was in its final draft in late 1993, there appeared a much-heralded report of the identification of the human colon cancer gene. This colon cancer gene is, in fact, a homolog of a previously characterized gene of bacteria and yeast (Fish-

el *et al.*, 1993); its identification illustrates the increasing convergence of human medical genetics with basic research in molecular biology.

Within the past 2 years, candidate genes responsible for leukocyte adhesion deficiency in Holstein cattle (Shuster *et al.*, 1992), hyperkalemic periodic paralysis in quarter horses (Rudolph *et al.*, 1992), porcine malignant hyperthermia (Fuji *et al.*, 1992), and early onset progressive retinal atrophy in Irish setters (Farber *et al.*, 1992) have been identified. Veterinary applications of molecular diagnostics usually employ a human or mouse probe (DNA sequence) derived from genes for clinically similar diseases in humans or mice. These four papers emphasize the importance of a comparative approach to animal medicine and the many commonalities that emerge from studies at the molecular level. In addition, genetic marker bands on DNA fingerprints have been closely linked with the Weaver syndrome (Georges *et al.*, 1993) and with the polled (hornless) gene in cattle and should allow marker-assisted selection to breed out these diseases (Womack *et al.*, 1992). When the carriers of these predisposing genes are identified, it is possible to trace the genes back through ancestors to their origins. For example, the leukocyte adhesion deficiency in Holsteins apparently come mostly from one outstanding bull.

Are there analogous candidate genes to account for genetic canine thyroiditis? One might guess that thyroid dysfunction would be associated with structural abnormalities of thyroid-specific proteins.

The coding sequence for the canine thyrotropin receptor has been determined (Parmentier *et al.*, 1989) and the sequence for a variant has also been reported (Libert *et al.*, 1990). A genetic variant of the TSH receptors was reported in neoplastic canine thyroid tissue (Verschueren *et al.*, 1992). However, this receptor does not seem to be involved in typical cases of canine thyroiditis.

Restriction fragment length polymorphism (RFLP) has been reported in the human thyroid peroxidase gene, but the variants are of unknown physiological or pathological significance (Rose *et al.*, 1991). Rare mutant forms of human thyroid peroxidase are associated with abnormal or absent enzyme function (Mangklabruks *et al.*, 1991; McLachlan and Rapoport, 1992). The wild type thyroid peroxidase gene has been substantially sequenced (Kimura *et al.*, 1987), but no sequence information is available on any mutants. Even when the gene is wild type, the cloned cDNAs for human thyroid peroxidase are of several sizes, apparently reflecting the presence of alternative splicing sites during the maturation of the primary RNA transcript into the final messenger RNA. The physiological or pathological significance of these differences in size of message, at least one of which produces a truncated protein apparently

lacking the 5′ sequence for insertion into membranes, has not been evaluated (Kimura *et al.*, 1987; Nagayama *et al.*, 1990; McLachlan and Rapoport, 1992).

The ability to produce autoantibodies to thyroid peroxidase is inherited as an autosomal dominant (Phillips *et al.*, 1990, 1991). The search for a candidate gene for that relatively rare human condition is under way. The significance of such a gene for canine disease is unclear, since thyroid peroxidase is not a pathogenic antigen in dogs affected with autoimmune thyroiditis (Thacker *et al.*, 1994).

Defective alleles of the human gene coding for the thyroid hormone receptor have been cloned. The defect produces an autosomal dominant disease termed "generalized resistance to thyroid hormone" (Nagaya *et al.*, 1992). To our knowledge, an analogous condition has not been reported in dogs.

RFLP analysis on human DNA, probing with a repetitive sequence from the 5′ end of the thyroglobulin gene, has identified a complex DNA fingerprint in man that is of little significance in detecting polymorphisms in thyroglobulin itself (Gérard *et al.*, 1990). We subjected the DNA of hypothyroid and euthyroid dogs to RFLP with human probes from within the thyroglobulin coding region. Although there was variation among individuals, we found no consistent correlation of RFLP pattern and thyroid health (Happ, unpublished observations).

The sequence of the canine thyroglobulin gene promoter has been reported (Donda *et al.*, 1991), and the effects of mutations in this region of human, canine, bovine, and rat promoters have been tested in a transient expression assay in primary cultures of dog thyrocytes (Donda *et al.*, 1993). No variants of this promoter region have been clearly linked to thryoid disease in dogs.

Since physiologically important defects in human and animal thyroid structural genes appear to be quite rare, it seems unlikely that a single candidate gene for a thyroid-specific protein, like thyroid peroxidase or thyroglobulin, would account for genetic canine thyroiditis. In the search for alleles correlated with thyroiditis, we believe that the emphasis should be placed on canine *immuno*genetics.

Autoimmune thyroiditis is but one of many canine autoimmune diseases (Halliwell, 1978; Bennett, 1984; Gorman and Werner, 1986a,b,c). These diseases include autoimmune hemolytic anemia, thrombocytopenia, von Willebrand's disease, pemphigus vulgaris, systemic lupus erythematosus, rheumatoid arthritis, Addison's disease, myositis, and many others. There is widespread suspicion that some conditions may be linked, due to common underlying defects in the immune system. In human medicine, commonality is designated as autoimmune diathesis

(Rose and Burek, 1991) or polyglandular autoimmunity (Fisher *et al.*, 1987). In dogs, von Willebrand's disease has been associated with hypothyroidism (Dodds, 1988) and a cluster of immune-related diseases have been reported in Old English sheepdogs in North America (Dodds, 1988) and Western Australia (Day and Penhale, 1992). In dogs as in people, autoimmune reactions are likely to be linked with particular genes of the immune system (Day and Penhale, 1987). These strong parallels suggest many experimental approaches to analyze disease mechanisms, to develop screens for genetic predisposition, and to devise strategies for immunotherapy.

This chapter reviews the molecular biology underlying autoimmune responses, discusses briefly animal models of autoimmune disease, evaluates the techniques that might detect canine genes predisposing toward autoimmune thyroiditis, and summarizes the causes and development of autoimmune thyroiditis in human and dogs. Finally, it suggests future trends and priorities for research on autoimmune diseases.

II. Molecular Basis of Autoimmunity—The Failure of Self-Tolerance

Autoimmunity is a family of complex phenomena with multiple causes (Sinha *et al.*, 1990; Carson,1992; Rose and Mackay, 1992). It can involve both T-lymphocyte and B-lymphocyte effector mechanisms. When self-tolerance breaks down, the immune system confuses self-antigens and foreign antigens and turns upon the body. An understanding of autoimmunity must begin with a brief review of the genes involved in self-recognition.

A. The MHC and T-Cell Receptor Complexes

The key proteins involved in recognition and thus the tolerance of self are: (1) the *immunoglobulin receptors* on B-lymphocytes, (2) the *T-cell receptors* (TCR) on T-lymphocytes, and (3) the protein *products of the major histocompatibility complex* (MHC) *genes*. The B-cell immunoglobulin receptors, unlike the TCR and the MHC products, can exist in soluble form as circulating antibodies. Both immunoglobulins and TCR proteins are coded for by gene sequences that have undergone somatic DNA rearrangement and mutation. The MHC antigens are of two groups: MHC class I receptors, found on the surfaces of all nucleated cells, and MHC class II receptors, found on cells that take up

extracellular antigens, partially digest them, and then present the fragments from digestion on their surface receptors. Fragments from *intra*cellular antigens are bound to MHC class I molecules, and fragments from *extra*cellular antigens are bound to MHC class II molecules. The MHC receptors and the antigenic fragments they bear are recognized by the TCRs (Fig. 1).

Since the MHC class I molecules bind fragments from the digestion of proteins made inside cells, they can reveal the presence of intracellular viruses and thus alert the T-cells to the presence of infection. Although some autoimmune diseases have been linked to the class I

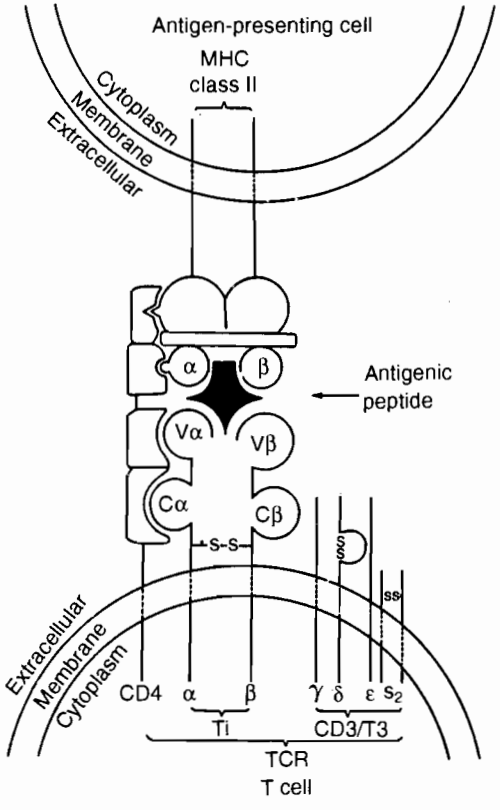

FIG. 1. TCR-antigenic peptide–MHC complex. Antigen is processed within the antigen-presenting cell. The resulting peptide fragments are attached to the MHC and transported to the cell surface. If a TCR (with its accessory CD3 and CD4 proteins) recognizes that complex, the T-cell is activated (from McGregor, 1992). Reprinted with permission of Oxford University Press.

genes, most of the strongest associations are with the class II genes (Nepom and Erlich, 1991). As a general rule, class II molecules are found on cells specialized for the presentation of antigen, such as B-cells, macrophages, and dendritic cells. However, cytokines, such as γ-interferon, can induce other cells, including thyroid epithelia, to produce class II molecules (Todd *et al.*, 1985). Every antigen-presenting cell contains about 200,000 class II molecules. Only 1% (200–300) of the possible MHC-peptide complexes are sufficient to activate a T-cell.

Because naturally occurring autoantibodies occur in the sera of normal individuals, it is clear that B-cells that react to self-antigens can exist without pathogenic consequences. Yet often these B-cells are not activated to become plasma cells that secrete large amounts of antibody. B-cells will become activated only under the influence of helper-T-cells. The critical actors in self-tolerance are the T-cells that regulate the B-cells.

T-cells comprise 70–80% of peripheral blood lymphocytes. The surface of each T-cell is studded with 10,000 to 20,000 TCRs. Every mature TCR is a heterodimer, consisting of two peptide chains, linked to one another by a single disulphide bond and embedded in the plasma membrane. The peptides have four domains: two extracellular, one transmembrane, and one cytoplasmic. Like the immunoglobulins, the TCR peptide has constant and variable regions. The variable regions come close together at the apex of the molecule, farthest from the surface of the cell, to form a shallow groove that binds antigen.

Like B-cells, T-cells originate in the bone marrow, but T-cells undergo an additional maturation stage in the thymus. All T-cells enter the thymus with the same genetic information for making T-cell receptors. Within the thymus, the variability of the TCRs is generated by the rearrangement of the variable (V), diversity (D), and joining (J) elements and by random events that occur at the Vβ–Dβ–Jβ junctions so that each clone produces a TCR with unique specificity (Toyonaga *et al.*, 1985). Once their receptors are functional, the T-cells undergo a two-stage selection. The first stage is a positive selection—T-cells that recognize the particular MHC receptors in that individual survive; those that fail this recognition test would be unproductive and are eliminated. The second stage is a negative selection—T-cells that recognize self-antigens are deleted. The T-cells in the final repertoire have gone through both positive and negative selection. This adaptive process accomplishes tolerance of self.

Organ-specific self-antigens are not present in the thymus, and they may fail to participate in the selection process. For thyroglobulin, which is normally confined to the thyroid follicle, or for myelin-basic

protein, which does not pass the blood-brain barrier, negative selection for T-lymphocytes is often incomplete. One consequence of that incomplete process can be an autoimmune reaction and autoimmune disease. For more complete discussions of the breakdown of tolerance, see Rose and Mackay (1992).

The T-cells that emerge from the thymus are of several varieties, distinguishable by their behavior and by the proteins they carry on their surfaces. Each TCR is associated with other membrane-bound glycoproteins, either CD3 and CD4 (Fig. 1) or CD3 and CD8. Helper T-cells, which bear a CD4 surface marker, secrete lymphokines, promote the differentiation of B-cells into plasma cells that secrete large quantities of soluble antibody, and trigger the maturation of CD8-positive T-cells into cytolytic or suppressor T-cells. T-cells are the gatekeepers. The efficiency and accuracy of recognition of self depends upon both the presentation system (the MHC molecule) and the recognition of peptide-MHC complex by the complementary TCR. The search for genes predisposing toward autoimmune disease has focused on those of the MHC complex and those coding for TCRs (e.g., Marcadet *et al.*, 1985; Todd *et al.*, 1987; Beall *et al.*, 1989, 1993; Nepom and Concannon, 1992; Roman *et al.*, 1992).

B. Polymorphism in HLA and TCR Genes

The human MHC class I molecules (termed HLA-A , B, and C) consist of a heavy chain and a β_2-microglobulin molecule (Nepom and Concannon, 1992). These are major transplantation antigens. They are highly polymorphic, and multiple allelic variants exist for each of the three loci. There are 14 MHC class II loci, clustered in three regions termed HLA-DR, DQ, and DP. The genes are tightly linked, and thus the antigens within the MHC are usually inherited as a block, called the haplotype. Each locus codes for at least one α- and one β-chain. The α-chains are polymorphic and the β-chains are highly polymorphic. For example, the DQA1 gene has 13 known alleles, DQB1 has at least 19 alleles, and the DRB1 gene has at least 68 alleles (Marsh and Bodmer, 1993).

Linking particular alleles with etiologically complex diseases is challenging. A variety of techniques have nonetheless drawn such relationships. At least 76 diseases are associated with particular serologically defined HLA specificities (Tiwari and Terasaki, 1985). Molecular techniques have demonstrated the existence of several alleles within a single HLA serological specificity, and thus the genetic analysis must proceed to a more detailed level. The usual analysis of HLA-

disease connections proceeds in three steps: (1) a serological specificity or a particular restriction fragment on a DNA fingerprint is associated with a disease; (2) haplotypes that carry that marker are defined; and (3) individual genes within that haplotype are evaluated (Nepom and Concannon, 1992). There is a high concordance for multiple sclerosis in monozygotic twins (McFarland *et al.*, 1984), suggesting genetic predisposition is an important factor in this disease. The disease incidence is correlated with an increased frequency of HLA class II haplotypes DRw15, DQw6, Dw2 in Caucasians (Marcadet *et al.*, 1985; Olerup *et al.*, 1989). Likewise, the HLA-DR3(Dw3) specificities, made up of the linked DQ alleles DQB1*0201, DQA1*0501, and DRB1*0301, are linked to autoimmune diseases such as Graves' disease, myesthenia gravis, and type I diabetes. It is not clear which of these genes is responsible for any of the diseases. In fact, the responsible gene might be elsewhere within the haplotype, linked to the DQ-DR cluster (Nepom and Concannon, 1992).

It is useful to define as many loci as possible in the MHC haplotypes on both chromosomes because of the linkages and also because their products might interact in the phenotype. There are intriguing recent reports of MHC class II heterodimers that form between the α chain of one locus and the β chain of another. NZB $\times$ NZW hybrid mice make such heterodimers, and the F_1 animals show a lupuslike syndrome. The heterodimer, the product of a mixed haplotype, plays a key role in the development of autoimmunity (Nygard *et al.*, 1993).

Each HLA receptor accepts a particular set of antigen fragments in its peptide-binding cleft at the end of the molecule. The alleles that predispose to autoimmune disease often include specific amino acid polymorphisms for autoantigenic peptides. The MHC-antigen complex then triggers autoreactive T-cells to initiate an autoimmune reaction. Critical amino acid sequences in the MHC receptor have been identified in rheumatoid arthritis. Several different DRB alleles are linked to rheumatoid arthritis and all code for DRβ molecules, which carry a specific amino acid sequence in the region of amino acids 67 to 71. That sequence is a T-cell recognition element (Nepom and Concannon, 1992).

Polymorphism in the TCR genes has also been linked to autoimmune disease. They encode two forms of receptor—an $\alpha\beta$ form on over 90% of the T-cells and a $\gamma\delta$ form on the remainder. There is immense diversity in the antigen-binding sites of the TCRs for two reasons. First, there is the genomic diversity in T-cell genes, and second, there are many combinational variants introduced as the V, D, J, and constant (C) gene segments are spliced together in a process like that used for the pro-

duction of antibodies in the B-cells. For humans, with an estimated 100 alternative Vα segments and 61 Jα segments for the TCR-α chain, there are 6100 combinations (Roman-Roman *et al.*, 1991). The adjacent region, that coding for TCRβ, has an estimated 80 Vβ gene segments, two Dβ segments, and 13 functional Jβ segments (Toyonaga *et al.*, 1985). Additional variation is introduced by the variations and insertions at the splice sites. For people, there is the theoretical potential for more than 2×10^{20} different combinations of the αβ peptide chains of a TCR.

Polymorphism in the germline TCR repertoire is thought to affect susceptibility to autoimmune disease. The sequencing of all the bases in the human TCR complex is still in progress. The available data indicate that there are many subfamilies of the gene segments. Eighty different Vβ segments fall into 24 subfamilies, while the Vα segments are divisible into 29 subfamilies (references in Nepom and Concannon, 1992). Most of that polymorphism has been detected by RFLP. Beall and coworkers (1989) used RFLP of genomic DNA to survey TCR-Vβ alleles in 40 patients with multiple sclerosis and compared the results with 100 normal individuals. The authors concluded that a multiple sclerosis susceptibility gene may be located in the TCR β-chain gene complex. Seboun *et al.* (1989) used sibling pairs to show TCR-Vβ haplotypes were more likely to be similar in siblings with multiple sclerosis than between afflicted and unafflicted siblings. However, other studies have failed to confirm that association with multiple sclerosis (Hillert *et al.*, 1992). A similar set of conflicting data exists for TCR alleles and insulin-dependent diabetes mellitus (IDDM); the conflicts may be due to genetic heterogeneity of the patient populations (Hibberd *et al.*, 1992).

The lack of consistency in careful studies in different research laboratories may reflect the complexity of the interactions between the MHC products and TCR. Recently, Beall and coworkers (1993) reported that the association of multiple sclerosis with particular TCR Vβ haplotypes is significant only in HLA-DR2+ individuals. If confirmed, this result suggests that gene complementation between HLA class II and TCR Vβ genes predisposes to multiple sclerosis. The important event could be either the positive selections in the thymus for the T-cell repertoire, or much later the actual presentation of the antigen fragment to the TCR. In experimental allergic encephalitis of rodents, an animal model of multiple sclerosis, the response is T-cell mediated and the early determinants in myelin basic protein have been identified. However, additional determinants become immunogenic in later phases of the disease, suggesting that T-cell autoimmune response

spreads from one determinant to others. (Lehmann *et al.,* 1992). As noted by Nepom and Concannon (1992) "the study of TcR gene polymorphism and its impact on autoimmunity is in its infancy."

III. Screening for Canine Genes That Might Predispose toward Thyroiditis

A. Canine Molecular Immunogenetics

The extensive use of dogs as experimental subjects in organ transplantation experiments has sustained research in canine histocompatibility typing. The nomenclature for the canine MHC complex is summarized well in the reports of the Third International Workshop on Canine Immunogenetics. Class I alleles, designated as types DLA-A, B, C, code for serologically defined antigens (Bull *et al.,* 1987). Class II alleles, designated as types DLA-DR, DQ, DP, and DO, code for antigens defined by reactivity in a microlymphotoxicity test (Deeg *et al.,* 1986). As of the Third International Workshop, there were 5 DLA-A antigenic specificities, 4 DLA-B antigenic specificities, 3 DLA-D antigenic specificites, and 10 DLA-D homozygous typing cell specificities. Depending on whether it is homozygous or heterozygous at these loci, any given dog expresses one or two alleles of the α-chain of each class I gene and β-microglobulin and one or two alleles for the α- and β-chains of the class II alleles. This information can also be used for canine paternity testing (Bull and Gerlach, 1992).

An important series of recent papers by Ulla Sarmiento, Rainer Storb, and their coworkers at the University of Washington, Seattle, has demonstrated the allelic polymorphism of the DLA genes and begun to specify the base sequences of the individual alleles. By RFLP analysis, with dog DNA exposed to a human HLA probe, Sarmiento and Storb (1989) report that there are at least eight class I genes including the canine homologs of HLA-A, B, and E genes. When RFLP analysis was applied to canine MHC class II genes, they too were demonstrated to be polymorphic (Sarmiento and Storb, 1988b).

Nine restriction endonucleases were used to digest the DNA from peripheral blood leukocytes of 23 dogs known to be homozygous for the nine DLA-D types, as defined by leukocyte reactions. After separation of the fragments by agarose gel electrophoresis, they were exposed to human HLA probes for DRB, DQB, DPB, and DOB gene sequences. It was possible to discriminate among the nine homozygotes using only two restriction enzymes and a probe for a gene coding for a HLA-DPβ

chain. When a dog DLA-DRB probe is applied to the DNA from dog families, the RFLP patterns assort with the DLA-DRB specificities assigned by mixed leukocyte cultures (Burnett *et al.*, 1994). In a parallel study, Sarmiento and Storb (1988a) detected five α-chain genes.

Starting with dogs known to be homozygous for the DLA-DRBs, the Seattle group sequenced the cDNAs for nine DRB alleles. According to cluster analysis, the nine alleles are subdivided into three major groups that resemble the canine analogs of the human supertypic groups (Sarmiento and Storb, 1990; Sarmiento *et al.*, 1990). Sequencing of DQA and DQB genes revealed more alleles than previously reported by DNA fingerprinting. There now appear to be at least four DQA alleles and four distinct DQB alleles (Sarmiento *et al.*, 1992, 1993). It would not be surprising to have more loci and alleles discovered with increasing study of the canine genome.

In all mammals, the TCR on the cell surface is complexed with the membrane protein CD3, composed of five constant chains. The sequence of the canine CD3ε subunit has been determined (Nash *et al.*, 1991). Preliminary work with human TCR probes suggested that the canine TCRB gene, like its human homolog, has two constant regions (Chaganati *et al.*, 1992). A more recent paper provides the first sequence information on the dog T-cell receptor itself (Ito *et al.*, 1993) The investigators isolated messenger RNA from peripheral leukocytes of a dog, synthesized the corresponding cDNAs, and preferentially amplified the cDNAs for the TCRα and TCRβ chains by adding V gene universal forward primers and reverse primers for either the TCRA or the TCRB gene. From the clones isolated, only one α-chain and one β-chain sequence were found, both of which showed strong similarity to other mammalian TCRs.

B. Screening for MHC Genes

The keen interest in the HLA complex has led to development of many protocols for detecting histocompatibility types. The molecular techniques developed for human HLA typing should be quite applicable to DLA typing (Bull and Gerlach, 1992). These powerful methods will complement classical typing techniques using serology or leukocyte reactions, as described by Bull *et al.* (1987) and Deeg *et al.* (1986) for DLA class I and DLA class II, respectively. They were developed in part as supplements to the serological typings. In a well-characterized and ethnically homogenous population like the Swedes, the assignments are fairly reliable, but much less so in more heterogeneous populations (Olerup *et al.*, 1993).

1. Restriction Fragment Length Polymorphism (RFLP)

Southern blotting is a powerful technique to study target sequences of DNA in a mixture of many fragments that are separated by size. Using multilocus probes, RFLP is widely applied to identify individuals; in that context, it is known as DNA fingerprinting (Jeffreys *et al.*, 1985). It was first used for investigation of HLA class II polymorphism by Wake and coworkers (1982). For MHC typing, RFLP begins with isolation of DNA (generally from blood cells), digestion with one or more restriction endonucleases, and separation of the fragments by size on agarose gels. The DNA fragments are transferred to a solid support (usually nitrocellulose or nylon) by capillary or vacuum blotting, and finally, the blot is immersed in a solution containing single-stranded-DNA sequences (probes) that bind to complementary target sequences in the DNA. The bound probes are usually visualized on X-ray film blackened by their radioactivity or chemiluminescence. The usual result is a stack of short bands, much like a bar code.

With application of many restriction enzymes and probes, it is possible to demonstrate MHC gene polymorphism, much as Sarmiento and Storb (1988b, 1989) have done for DLAs. Meticulous attention to technical details is required for consistency. For the results to be comprehensible and unambiguous, the RFLP patterns must not be too complex and the probes must not cross-react with several loci, or else the alleles become difficult to distinguish from one another. With a very precise choice of restriction enzyme and probe, it is possible to accurately determine the genotype of many HLA-DR and DQ specificities. To minimize cross-reactions with unrelated sequences, short exon-specific probes are now utilized (Bidwell *et al.*, 1993.

Two advantages of this technique are: (1) that many alternative heterologous probes can be used by adjusting the stringency of the hybridization reactions, and (2) that one can demonstrate polymorphism even if one knows relatively little of the actual base sequences in the target DNA. Since the restriction enzyme cuts are often outside the actual coding region of the MHC genes, polymorphism may be found to be of no functional relevance, and furthermore, some polymorphism within the MHC exons is likely to be missed. Great care must be taken in applying specific protocols developed for Caucasian populations to non-Caucasians (Bidwell *et al.*, 1993).

2. Polymerase Chain Reaction / Sequence-Specific Oligonucelotide (PCR-SSO)

The technique of amplification of specific genes or portions of genes with the polymerase chain reaction (PCR) has led to enormous innova-

tions in all subfields of molecular biology, including histotyping. HLA types can be determined without separation of restriction fragments by using probes that are specific for particular alleles. DNA is purified from blood and the HLA genes are amplified by PCR. Aliquots from the reaction mixture are spotted on nitrocellulose or nylon supports, either in a slot blot or a dot blot, and the membrane is immersed in a solution of labeled oligonucleotides. The sites of nucleotide binding are visualized by the usual techniques.

The first stage of discrimination is the PCR, where judicious choice of primers will determine the possible pool of products. As a general rule, the primers are chosen to amplify a sequence of about 300 bases coding for the NH_2-end of the B gene, the highly variable segment that codes for the terminal antigen-binding portion of the receptor's β chain. In a typical set of protocols for human DRB/DQB/DPB typing, one set of generic DR primers and six more sets of specific primers are utilized (Tiercy *et al.,* 1993) to resolve 53 DRB1, 3 DRB3, 3 DRB5, 17 DQB1, and 22 DPB1 alleles with a total of 67 SSO probes. With the first set of primers and 15 oligonucleotide probes, HLA-DQB1 alleles can be distinguished; a second set of primers and 18 probes allow identification of the HLA-DPB1 alleles, and a third set of amplifications and 14 probes allow the identification of all major HLA-DR groups and some specific alleles within them. When the HLA-DR assignments are ambiguous at this point, more specific primers are utilized to distinguish among the DR alleles.

The PCR-SSO techniques are very effectively exploited in human clinical laboratories for typing of leukemic or kidney transplant patients and for volunteer bone marrow donors. The power of this method is impressive. Its principal disadvantages are: (1) the requirement for a large number of SSO probes and very precise optimization of the hybridization and posthybridization washing conditions for each, (2) the considerable investment in time and facilities to be efficient about typing, and (3) the fact that the protocols, as developed for Caucasian patients, are sometimes ambiguous with non-Caucasians, requiring the application of additional probes. Considerable new information about canine base sequences followed by judicious evaluation of the many alternative SSO probes will be required before PCR-SSO can be useful for canine DLAs.

3. Polymerase Chain Reaction / Restriction Fragment Length Polymorphism (PCR-RFLP)

Like PCR-SSO, PCR-RFLP begins with amplification of the base sequence coding for the highly variable amino terminal region of the β-chain. Following amplification, the aliquots of the PCR reaction mix-

ture are digested with a panel of restriction enzymes and subjected to electrophoresis on acrylamide or agarose. The proper choice of allele-specific endonucleases is critical and is made much easier by evaluating the target sequence for each enzyme against the banks of sequenced HLA alleles. Alleles are differentiated from one another on the basis of the size of the digestion fragments.

The PCR-RFLP techniques were originally developed for homozygotes, with distinct primers for each HLA class (Maeda *et al.*, 1990; Uryu *et al.*, 1990; Salazar *et al.*, 1992). As originally proposed for HLA-DRB and -DQB typing, this technique employed five restriction enzymes to distinguish 16 patterns characteristic of HLA-DR and -Dw homozygous serotypes (Maeda *et al.*, 1990). The addition of heterozygotes to the test pool clouded the results with incomplete digestion products (Olerup, 1990). Various modifications of the techniques have been proposed to address these difficulties. Codigestion with two restriction enzymes improves the discrimination among some alleles (Sawitzke *et al.*, 1992). Addition of a constant restriction site in one of the primers offers an internal digestion control (Mercier *et al.*, 1992). A recent improvement is the use of "more informative enzymes," which have a single recognition site in some alleles and none in other alleles in the amplified regions (Inoko and Ota, 1993). The use of 29 enzymes allows 93 different alleles to be distinguished in homozygotes or heterozygotes in a Japanese population, such that genotypes can be defined simply by determining whether the amplified DNA is digested. Additional information can be obtained by amplifying and digesting noncoding as well as coding sequences (Limm *et al.*, 1993; Simons *et al.*, 1993).

Inoko and Ota (1993) argue forcefully for the superiority of PCR-RFLP as compared with PCR-SSO. The advantages include: (1) simplicity (29 commercially available enzymes and no radioisotopes for PCR-RFLP vs 100 PCR-SSO probes, radioisotopes, and adjustment of washing temperatures for PCR-SSO); (2) discrimination (a change in only one is detectable with restriction enzymes while achieving that high stringency with SSO is very time consuming); (3) cost (PCR is less costly when small samples are typed); and (4) linkage information that is provided by PCR-RFLP and not by PCR-SSO. It will be important to determine if these newest PCR-RFLP protocols work well in other laboratories and in the present context, to see whether they can discriminate the DLA alleles.

4. Polymerase Chain Reaction—Sequence-Specific Primers (PCR-SSP)

The PCR-SSP technique is based on the principle that a perfectly matched primer will be more specific in the PCR reaction than a prim-

er with one or several mismatches, especially in the first critical cycles (Olerup and Zetterquist, 1993; Olerup *et al.*, 1993). The specificity of the PCR is the discriminator, with assignment of alleles based on the mere presence or absence of the amplified product. A series of PCR amplifications is performed in parallel, each one of which contains a pair of primers that bind very specifically to only one or a very few alleles. Often the experiments are organized in two stages of increasing resolution. DR "low resolution" PCR-SSP typing employs 40 primers and is followed, if necessary, by DR4 and DR1 subtyping with a panel of additional primers. With carefully purified primers of the correct specificities, the results look very convincing (Olerup and Zetterquist, 1993). This new method seems powerful and is very fast; typical PCR-SSP typings can be performed in less than 2 hours. The disadvantage is the requirement for a large panel of very high quality primers.

IV. Models of Autoimmune Disease

Both genetic and environmental factors contribute to the initial autoimmune responses and the transformation of a benign autoimmune response into a pathological autoimmune condition (Sinha *et al.*, 1990). The reductionist dissection of the contributing factors from one another and the evaluation of the importance of each are necessary for a complete understanding of these very complex phenomena. Animal models that are especially instructive are of two classes: (1) those autoimmune reactions *induced* by injection of antigens and (2) those *spontaneous* autoimmune reactions that occur at high frequency in inbred strains (Bernard *et al.*, 1992). The best-understood model of spontaneous autoimmune disease is IDDM in the nonobese diabetic (NOD) mouse.

The NOD mouse strain was developed in the early 1980s in Japan (Makino *et al.*, 1980). Most young NOD mice spontaneously develop insulitis—lymphocytic infiltration of pancreatic islets and the destruction of many of the insulin-producing β-cells. When all β-cells are destroyed, the afflicted mice develop glycosuria and an IDDM. The full-blown disease, which is rapidly fatal without insulin treatment, is much more common in older females. Over the past decade, the NOD strain has been established in many laboratories in Japan, North America, and Europe. Individual laboratory colonies differ in the frequency of disease but all show insulitis in both sexes and a preponderance of IDDM in older females (Bernard *et al.*, 1992). Some individuals survive for over a year, and many of these older mice become

severly cachetic and jaundiced due to autoimmune hemolytic anemia (Baxter and Mandel, 1991).

Impressive progress has been made in identifying the genes that predispose NOD mice to IDDM. The initial genetic analyses suggested that there were two recessive genes on two different autosomes (Makino *et al.,* 1985). In the next year, Hattori *et al.* (1986) linked the IDDM to a MHC class II complex that was apparently unique to the NOD strain. By crossing NOD mice with the nondiabetic C3H strain, Hattori and coworkers found no diabetes in the F_1 mice, but it was present in a small fraction of the backcrosses and intercrosses. All backcross and intercross mice showing diabetes mellitus were homozygous for a 9.5-kb band on Southern blots, but some mice that were homozygous for the 9.5-kb band were disease-free. Since the 9.5-kb band was necessary but not sufficient, the investigators suggested the existence of one or more additional susceptibility genes not linked to MHC.

Molecular analysis of the responsible MHC in the NOD mice gene revealed the presence of a five-nucleotide substitution in the I-Aβ gene that alters two amino acids of the β chain from proline–aspartic acid in IA^d (wild type) to histidine-serine in the class II $I\text{-}A^{NOD}$ gene (Acha-Orbea and McDevitt, 1987). Transgenic mouse experiments support the conclusion that the $I\text{-}A^{NOD}$ gene, now designated as $I\text{-}A^{g7}$, is involved in disease development (Lund *et al.,* 1990; Miyazaki *et al.,* 1990; Slattery *et al.,* 1990). Two related sister strains of mice, ILI and CTS, which share the IA^{g7} allele but do not develop diabetes, confirm the suggestion that there are non-MHC susceptibility genes. This general pattern seems to apply for human disease as well. The HLA-DQB1*0302 allele with a homologous amino acid substitution is associated with diabetes mellitus in Caucasians (Todd *et al.,* 1987; Morel *et al.,* 1988) but not in similarly afflicted Japanese (Awata *et al.,* 1990). This striking parallel between human and mouse studies argues strongly that multiple genes, MHC and others, are involved in the development of IDDM in diverse mammalian species.

Several of the predisposing mouse genes have been mapped to specific chromosomes. The $I\text{-}A^{g7}$ gene, also known as *Idd-1*, is found in the murine MHC complex on chromosome 17 (Lund *et al.,* 1990; Miyazaki *et al.,* 1990; Slattery *et al.,* 1990). A second locus on chromosome 9, designated *Idd-2,* is associated with diabetes in backcross progeny (Prochazka *et al.,* 1987). Recent work by Todd and colleagues (1991) has mapped two more non-MHC genes, designated *Idd-3* and *Idd-4* to chromosomes 3 and 11, respectively, of the NOD mouse. *Idd-3* affects both the frequency of insulitis and its likelihood to progress to full diabetes.

Idd-4 was associated with diabetes in younger animals but not in those over 144 days.

Insulitis probably begins with infiltration of the pancreatic islets by macrophages (Lee *et al.*, 1988), which present β-cell antigens to T-cells. The activated T-cells predominate in the lesions (Miyazaki *et al.*, 1985). Diabetes can be prematurely induced in newborn NOD mice by transfer of T-cells from sick animals (Yagi *et al.*, 1992 and references therein). An understanding of the disease mechanisms and the steps in the process is emerging from many recent experiments using transgenic mice. For example, Katz *et al.*, (1993) produced NOD lines transgenic for TCR α and β genes from a diabetogenic T-cell clone. In these transgenic strains, the large populations of diabetogenic T-cells at birth led to "rampant" insulitis, but not immediately. Two interesting checkpoints in disease progression were revealed: 1) T-cell infiltration at 2–3 weeks, which may be related to the lag in maturation of the antigen-presenting macrophagesm, and 2) full diabetes at 4.5 months, suggesting that more than massive insulitis is required for disease appearance.

In spite of the extensive studies of IDDM, the nature of the autoantigen presented to the T-cells has remained controversial. Two important recent papers (Kaufman *et al.*, 1993; Tisch *et al.*, 1993) identify the triggering autoantigen as a secreted form of the enzyme glutamic acid decarboxylase (GAD). GAD synthesizes γ-aminobutyric acid, a neurotransmitter in the brain and a putative paracrine signal molecule in pancreatic islets. The T-cell response to the 65-kDa isoform of GAD develops at 4 weeks of age, at the same time as insulitis. The T-cell response is consistent with a presentation of GAD peptides on MHC class II molecules and involvement of T-helper cells. T-cell reactivity begins with the C-terminal regions of GAD65, and over the following weeks it spreads to other parts of the antigen, including a region with similarity to a protein of the Coxsackie virus. The sequence of reactivity argues against molecular mimicry (Sinha *et al.*, 1990; Barnett and Fujinami, 1992) between a viral antigen and GAD65 in triggering the autoimmune response. Subsequently, the T-cells gain reactivity to other β-cell antigens.

V. Autoimmune Thyroiditis in Humans and Animals

A. The Normal Thyroid

The thyroid gland is a loose aggregate of independently functioning thyroid follicles, each of which contains gelatinous colloid. The center

of each follicle is a reaction compartment, sealed from the rest of the tissues of the body by the spheroid ball of epithelial follicular cells. Within the reaction compartment are relatively high levels of iodide, hydrogen peroxide, protein substrates, and enzymatic catalysts. The critical biochemical reaction, iodination of protein-bound tyrosine, leads to thyroxine.

The follicular epithelial cells are much more than a boundary layer of the follicle. They create the reaction environment within the follicle, importing and processing precursors from the blood, passing reactants to the interior space, recovering intermediate products, performing the final steps in manufacture, and liberating the final product. At their basal surfaces, the follicular epithelial cells absorb amino acids from the blood, and within their ribosome-studded endoplasmic reticulum the amino acids are incorporated into thyroglobulin (2748 amino acids, molecular weight 660 kDa in humans) and thyroid peroxidase. Iodide, preferentially absorbed from the blood by a specific carrier molecule in the basal plasma membrane, is transported across the follicular cell, and along with hydrogen peroxide and thyroglobulin is secreted from the apical surface of the cell into the thyroid follicle.

The H_2O_2 generation in the thyroid cells is controlled by thyrotropin, acting through the second messengers cAMP and the calcium-phosphatidylinositol cascade. H_2O_2 generation appears to be the limiting factor in the iodination of thyroglobulin (Corvilain *et al.*, 1991; Raspé *et al.*, 1991).

Thyroid peroxidase is an integral hemoprotein inserted into the apical membrane of the follicle cell with its catalytic domains pointing into the follicular space (Gruffat *et al.*, 1991). Human thyroid peroxidase has 933 amino acids: 61 in the putative cytoplasmic domain, 24 within the membrane, and 848 in the extracellular domain (McLachlan and Rapoport, 1992). The peroxidase oxidizes the hydroxyl groups on tyrosine residues in thyroglobulin. The activated tyrosines readily iodinate and form dimers, yielding finally two or more thyroid hormone residues per molecule of thyroglobulin. Once iodinated, the thyroglobulin is recaptured, apparently by both receptor-mediated and fluid phase processes, and enclosed within the endosomes of the epithelial cells (Rousset and Mornex, 1991). These endosomes constitute intracellular reaction compartments where iodothyroglobulin is enzymatically degraded by lysosomal enzymes to yield peptide fragments and iodinated tyrosine dimers—T_3 or T_4. It is not clear how the two hormones leave the epithelial cell, but a specific transport system, either active or facilitated, at the baso-lateral surface seems quite likely.

When passed into the blood, 99% of the T_4 and T_3 (in a ratio of *ca.* 5:1) is rapidly adsorbed to plasma proteins so that the effective unbound fraction of the hormones is low. About 0.1% of serum T_4 and 1% of serum T_3 are free (Dunn, 1989). The deiodination of T_4 to T_3 occurs readily in peripheral target tissues by a selenium-containing deiodinase (Berry and Larsen, 1993). T_3 binds more readily to the nuclear receptors for thyroid hormone and is three to five times more active physiologically than T_4. There is a fairly wide range of values for physiologically normal animals (Jeffers, 1990). Sudden fluctuations in the effective concentrations of thyroid hormones are largely offset by the high capacity of serum proteins to bind large amounts of T_3 and T_4. A consistent serious disruption of the complex equilibria between bound and free iodotyrosine derivatives and between the T_3 product and its immediate precursor T_4 would significantly affect the health of an animal, but such disruptions seem very rare.

B. The Disease Process

Autoimmune thyroid diseases are characterized by circulating antibodies to thyroid antigens, activated T-cells, and lymphocytic infiltration of the thyroid gland (Utiger, 1990; Wilkin, 1991; McGregor, 1992). The major human diseases are: (1) lymphocytic thyroiditis (Hashimoto's disease), in which there are low thyroid hormone titers and detectable circulating autoantibodies to thyroid peroxidase and thyroglobulin, and (2) Graves' disease, characterized by high levels of circulating thyroid hormones and circulating antibodies to the TSH receptor.

Primary hypothyroidism in dogs has been classified on the basis of its histopathology as either lymphocytic thyroiditis or idiopathic thyroid atrophy. At the histological level, lymphocytic thyroiditis shows many collapsed thyroid follicles, abundant macrophages, atrophy of the thyroid epithelium, infiltration of large numbers of lymphocytes, and partial or total collapse of follicles. Eventually, there is replacement of the thyroid follicles by fibrous connective tissue with a few scattered foci of inflammatory cells (Gosselin *et al.*, 1981, 1982). Idiopathic follicular atrophy, the second histopathologic class of primary hypothyroid disease, is characterized by replacement of thyroid follicular tissue by fatty tissue. Its pathogenesis is not well understood although it may simply be the result of end-stage thyroiditis (Conaway *et al.*, 1985b; Chastain and Ganjam, 1986; Dunn, 1989; Chastain, 1990; Wilkin, 1990).

Canine autoimmune thyroiditis is analogous to human Hashimoto's

disease (Lucke *et al.*, 1983). In the human disease, the infiltrating lymphocytes include both T-cells and B-cell, but T-cells predominate. Both helper ($CD4^+$) and cytotoxic ($CD8^+$) T-cells are present. The death of the thyroid cells occurs through antibody-dependent complement-mediated mechanisms as well as due to the action of killer T-cells. The exact sequence of events and the detailed mechanisms governing each are not well specified, but our knowledge of these stages of thyroiditis will certainly improve as there is increasing ability to establish antigen-specific T-cell clones *in vitro* (Utiger, 1991; Champion *et al.*, 1992).

An important animal model of spontaneous autoimmune thyroiditis is the Obese strain (OS) chicken, a closed flock developed at Cornell University by R. K. Cole (Cole, 1966). The young chicks become hypothyroid within a few weeks of hatching. Iodination is crucial to the development of the spontaneous autoimmune disease, since treatment with compounds that interfere with iodination *in ovo* and in the first few weeks posthatching inhibits both the onset of thyroiditis and the appearance of thyroglobulin autoantibodies (Bagchi *et al.*, 1985, 1990). According to classical genetics, there are three independent lesions: a strong autoimmune response to thyroglobulin affected by a MHC class II gene and two other genes (Bigazzi and Rose, 1985). The autoantibodies appear to be produced by the B-cells that are located in the thyroid glands and not at other sites, like the bone marrow (Maczek *et al.*, 1992). When T-cells from OS chickens were transferred into Cornell strain chickens, the disease was also transferred (Kromer *et al.*, 1985). Recent reports indicate iodine supplementation is necessary for efficient induction of thyroiditis (Brown *et al.*, 1991).

Both Graves' disease and atrophic thyroiditis involve antibodies that affect TSH stimulation of the thyroid cells (Wilkin, 1990). In Graves' disease, a circulating antibody stimulates the receptor and this creates a hyperthyroid condition. In atrophic thyroiditis, antibodies bind to the TSH receptor without stimulating it, leading to hypothyroidism. Neither of these diseases have common canine analogues.

Autoimmune thyroid disease occurs more often in women than in men (Baker, 1992) and it is especially common as a transient postpartum episode that lasts less than a year (Amino *et al.*, 1982; Jansson *et al.*, 1988). It is not yet clear whether physiological stress triggers the episodes, as careful studies have come to opposite conclusions (Gorman, 1990; Winsa *et al.*, 1991). In one study with dogs, females were reported to be more affected than males (Milne and Hayes, 1981). Alaskan husky females on racing sled dog teams occasionally become transiently hypothyroid during heavy fall training following a sum-

mer pregnancy (L. Lowry, personal communication). It has been argued that female hormones tend to be associated with the induction of autoimmune disease and that male hormones are somehow protective (Ahmed *et al.*, 1985).

C. The Thyroid Autoantigens

The four principal targets of the thyroid autoantibodies in people are: (1) thyroglobulin, which is secreted into the follicle, (2) thyroid peroxidase, an integral protein on the apical plasma membrane, (3) the receptor for TSH, facing the blood on the basal membrane, and (4) a recently described 64 kDa autoantigen. The autoantibody against TSH receptor is found in hyperthyroid Graves' patients, and will not be discussed further since autoimmune *hyper*thyroid disease is not common in dogs. The 64-kDa antigen is common to both Graves' and Hashimoto's diseases, but it has not yet been shown to be pathogenic (Dong *et al.*, 1991) and it is expressed in multiple types of cells (Ross *et al.*, 1993). The first two of these autoantigens will be discussed in more detail as they have been strongly associated with hypothyroid disease.

In dogs, the principal circulating autoantibodies are against thyroglobulin (Haines *et al.*, 1984; Beale *et al.*, 1990; Thacker *et al.*, 1992, 1994; Gaschen *et al.*, 1993). Of 1057 dogs hospitalized at the Auburn University veterinary clinic with no clinically evident endocrine disorders, 13.2% showed antithyroglobulin autoantibodies by an ELISA test (Haines *et al.*, 1984). The incidence of such antibodies is higher in the patients with thyroid disorders; in several studies, approximately half of the hypothyroid dogs had autoantibodies to thyroglobulin (Haines *et al.*, 1984; Beale *et al.*, 1990; Thacker *et al.*, 1992; Gaschen *et al.*, 1993).

Antibodies to thyroglobulin in dogs commonly react also with T_3 and to a lesser extent T_4, producing spurious immunoassay results in clinical tests. The assays may overestimate or underestimate the true hormone concentrations, depending on the particular techniques used (Young *et al.*, 1985, 1991; Rajatanavin *et al.*, 1989; Thacker *et al.*, 1992).

Are most antibodies directed against epitopes in thyroglobulin that include iodinated tyrosines? Several studies suggest that iodination of thyroglobulin is required for the onset of autoimmune thyroid disease in many species (Brown *et al.*, 1991; Rayner *et al.*, 1993). Antithyroglobulin production seemed to be stimulated by the iodine supplementation given to children exposed to radiation in Chernobyl (Kinalska *et al.*, 1991). In contrast, recent evidence from a mouse model suggests that thyroglobulin epitopes need not be iodinated in this species (Carayanniotis *et al.*, 1994). Finally, nonhormogenic epitopes of thyro-

globulin must exist in dogs because some develop thyroglobulin autoantibodies without concurrent production of T_3 autoantibodies (Gaschen *et al.*, 1993). When all the conflicting evidence is considered, it remains to be demonstrated that thyroglobulin is strongly pathogenic in mammals or that the thyroglobulin autoantibodies produced by the B-cells are actually destructive to the thyroid gland; they may simply be consequences of the circulating debris produced by the attacks of the T-cells on the thyroid follicles.

The second principal autoantigen from the thyroid gland is thyroid peroxidase, which is present in the healthy thyroid as an integral protein on the basal membrane (toward the follicle). It is in fact the "microsomal antigen" that has been reported for many years in the blood of Hashimoto's patients (Baker, 1992) and is recognized by complement-fixing autoantibodies (Champion *et al.*, 1992). Injection of thyroid peroxidase can induce experimental autoimmune thyroiditis in mice. A pathogenic T-cell epitope on porcine thyroid peroxidase induces an autoimmune response in mice (Kotani *et al.*, 1992.). To date, two linear epitopes for B-cell recognition on human thyroid peroxidase autoantibodies have been reported (references in McLachalan and Rapoport, 1992). However, these are low affinity epitopes and not likely to be factors invoking the autoimmune disease. The true pathogenic epitopes are likely to be conformational and discontinuous, making their identification very difficult with current technology (McLachlan and Rapoport, 1992). The putative pathogenic role of antibodies to thyroid peroxidase remains in dispute (Bogner *et al.*, 1990).

McGregor (1992) states the antibodies to thyroid peroxidase are never significant in animals with *experimental* autoimmune thyroiditis (EAT). Furthermore, autoantibodies to thyroid peroxidase have not been demonstrated in a large group of dogs with spontaneous autoimmune thyroiditis (Thacker *et al.*, 1994).

Like most nucleated cells, thyroid epithelial cells produce MHC class I antigens, and in addition, can be induced to synthesize MHC class II antigens (Bottazzo *et al.*, 1983). Thus thyroid epithelial cells can present antigens not only to $CD4^+$ (helper) T-cells to promote differentiation of B-cells into plasma cells, but also to $CD8^+$ (cytotoxic) T-cells (Feldmann *et al.*, 1992; Weetman, 1992). The synthesis of HLA-DR class II antigens in thyroid cells is induced by the cytokine interferon-γ (IFN-γ) and enhanced by tumor necrosis factor (TNF-α) (Hanafusa *et al.*, 1983; Champion *et al.*, 1991; Asakawa *et al.*, 1992). The IFN-γ and TNF-α also induce production of cell adhesion molecules to which T-cells bind (Tolosa *et al.*, 1992). Since thyroid epithelial cells are themselves capable of synthesizing both IFN-γ and TNF-α (Zheng *et al.*,

1991, 1992), there may be autocrine regulation of adhesion of T-cells to the thyroid epithelium. Transforming growth factor-β (TGF-β) is a negative signal. When TGF-β is applied *in vitro*, it suppresses proliferation of T-cells from the thyroids of Graves' patients and inhibits class II expression by the cells of the thyroid epithelium (Widder *et al.*, 1992).

D. The Significance of MHC and TCR Polymorphism for Thyroid Disease

HLA associations with human thyroid disease have been reported by several laboratories. HLA-DRBI, -DRBII, -DQAI, and -DQB gene products may be involved in the onset or development of Hashimoto's disease (Farid, 1991, 1992), but no associations appear to be very strong. Badenhoop and coworkers (1990) used RFLP to look for the association of HLA-DQB1 alleles with Hashimoto's disease and found a positive correlation with DQw7 and some correlation also with the adjacent DQA1. However, this association was not confirmed by the later work of Roman *et al.* (1992). The HLA-DR3 loci have often been implicated in the development of Graves' disease in Caucasians, but the tight linkage between haplotypes A1, B8, DR3, DRw52, and DQw2 makes it difficult to assess the true significance of each (Farid, 1992). In spite of these reports, several authors have argued that the expression of class II antigens does not initiate the autoimmune attack on the thyroid, but rather it amplifies an ongoing process (DeGroot and Quintans, 1989; McGregor, 1992).

T-cells are clearly involved in the pathogenesis of autoimmune thyroid disease (Davies *et al.*, 1991, 1992), and unusual lymphocyte subsets, including $CD4^+CD8^+$ and $CD4^-$ $CD8^-$ cells, are found in thyroids of patients with autoimmune thyroid disease (Iwatani *et al.*, 1993). Martin and Davies (1992) have reviewed the characteristics of the intrathyroid T-cells and find that the epitopes recognized by T-cells can be quite specific. After injection of thyroglobulin into mice, an EAT results and cytotoxic T-cells attack the thyroid follicle cells (Weigle, 1980). Champion *et al.* (1987) isolated two murine T-cell clones that recognize iodinated thyroglobulin but not thyroxine-deficient thyroglobulin. In a synthetic nonomer peptide, these T-cell clones fail to recognize thyroglobulin when the residue at site 2553 is tyrosine (or any other of the standard amino acids) but recognize 2553 when the tyrosine has been derivatized to thyroxine. The MHC class II molecules from the thyroids of these mice bind the nonomer and activate such T-cell clones *in vitro*. When transferred to naive recipient mice, the

activated T-cells induced thyroiditis in their new host (Hutchings *et al.*, 1992). This is the first case of a precisely defined pathogenic epitope in thyroglobulin that activates T-cells; it is especially interesting that the residues in thyroglobulin must be iodinated.

The associations of particular TCR allelic subfamilies with multiple sclerosis in humans (Beall *et al.*, 1989; Seboun *et al.*, 1989) suggested a similar relationship for autoimmune thyroid disease. By PCR amplification of the TCR gene transcripts for the α-chain and the β-chain, Davies and his colleagues concluded that of 18 possible families of α-chains for T-cell receptors, an average of only 4 were present in thyroid glands suffering autoimmune attack. The β-chain usage did not show such bias (mean 14.1 out of 19 families) (Davies *et al.*, 1991, 1992). However, another careful study failed to find any restriction in the TCR α-chains of intrathyroidal T-cells for patients with Graves' disease (McIntosh *et al.*, 1993). The question remains unresolved. However, an intriguing recent report implicates γδ T-cells in thyroid autoimmunity (Iwatani *et al.*, 1992) .

E. Environmental Factors

Iodine deficiency is classically linked to thyroid dysfunction. This is even more true of dogs than people since dietary iodine requirements are greater for dogs than for people. Dogs are less effective at conserving iodine and excrete it at a higher rate in the feces as well as being less efficient in its utilization in the thyroid gland (Belshaw *et al.*, 1975). Dietary iodine is important not only as a constituent of thyroid hormones but also because it modulates the autoimmune response. In Europe, regional and seasonal increases in dietary iodine are associated with increased autoantibodies to thyroid antigens and increased lymphocytic infiltration of the human thyroid gland (McGregor, 1992). Animal models likewise demonstrate the important contribution of iodine to thyroid autoantibodies and disease [Bagchi *et al.*, 1985 (chickens); Allen *et al.*, 1987 (rats); Cohen and Weetman, 1988 (rats); Braverman, 1990].

Selenium deficiencies affect thyroid hormone concentration at several levels. The most obvious need for selenium is as a component of the liver deiodinase which converts T_4 to T_3 (Beckett *et al.*, 1993; Berry and Larsen, 1993). In addition, selenium is required for intracellular glutathione peroxidase activity; thus low levels of selenium mean increased peroxide supply and perhaps greater hormone synthesis (Corvilain *et al.*, 1993).

One source of thyroid damage due to high iodine could be the excess

production of free radicals (Mahmoud *et al.*, 1986; Hall and Lazarus, 1987). If free radicals play a role in the early stages of thyroid disease, their reduction might afford protection. When the antioxidants ethoxyquin and butylated hydroxyanisole were included in the diet of the OS chicken, infiltration of the thyroid by lymphocytes, increases in autoantibodies to thyroglobulin, and the onset of the spontaneous thyroiditis were delayed. Weaker antioxidants like β-carotene afforded no protection (Bagchi *et al.*, 1990).

Infectious agents affect the onset of autoimmune disease. Parasitic infections have been linked to the breaking of T-cell tolerance to self-antigens (Röcken *et al.*, 1992; Röcken and Shevach, 1993). In animal EAT, sterilization of the gut protects against the development of the disease while restoration of the gut microorganisms increased the incidence (Penhale and Young, 1988). Normal gut pathogens have surface proteins that can bind TSH (Ingbar *et al.*, 1987). There is potential for molecular mimicry in which the immune response to that pathogen might trigger an autoimmune disease (Sinha *et al.*, 1990). A more direct association between infection and thyroiditis has been suggested by Belfiore *et al.*, (1991) who report that a viral infection triggered the local elaboration of IFN-γ, which induced HLA-DR expression on the surface of thyroid epithelial cells, rendering them susceptible to immune attack. Endogenous retroviruses are potential etiologic agents in autoimmunity (Krieg *et al.*, 1992). The role of retroviruses, which can infect and transform rat thyroid cell lines, is intriguing (Weetman and Borysiewicz, 1990; Wick *et al.*, 1993). Retrovirus-like sequences have also been demonstrated in Southern blots of DNA from the thyroid glands of humans with Graves' disease (Ciampoillo *et al.*, 1989).

VI. Future Research Applications

A. Research Directions for Future Work

1. DLA Histotyping

It would be very advantageous to have reliable and convenient methods for DLA typing. The potential is not only for studies of immune function but also for characterizing the various breeds and populations and for deducing the relationships among breeds and lines (Bull and Gerlach, 1992). The recent advances in HLA typing with molecular techniques will need to be applied to DLA typing. The initial base sequence for a DLA-DRB allele provided by Sarmiento and Storb

(1990) has been followed by sequences for the variable regions of more alleles of this gene as well as the sequences of other DLAs (Sarmiento *et al.,* 1990, 1992, 1993); these base sequences allow one to precisely define the primers for PCR-based typing techniques of DLA genes. The PCR-RFLP approach offers great promise for distinguishing among the DLA alleles. Development of the techniques is now in progress in our laboratory.

2. DLA Types and Canine Autoimmunity

The evidence from humans and mice convincingly links specific MHC histotypes with predisposition to spontaneous autoimmune reactions and autoimmune disease. As Day and Penhale (1987) argued, it is almost certain that a significant portion of the genetic predisposition toward autoimmune diseases in dogs lies in the genes of the immune system. It is important to identify the genes that are putatively responsible. Spontaneous canine thyroiditis offers promising opportunities to look for correlations between disease incidence and the DLA types. We are attempting such correlations by using PCR-RFLP to compare the DLA in euthyroid and hypothyroid dogs within the same family.

3. Improving Diagnostic Criteria

Diagnosis of thyroiditis is problematical in dogs because (1) only about one-half of the affected animals demonstrate thyroglobulin antibodies, (2) clinical assays for these canine antibodies are not currently available, and (3) only a few affected dogs have circulating anti-T_4 and/or anti T_3 antibodies (Beale *et al.,* 1990; Thacker *et al.,* 1992; Gaschen *et al.,* 1993). Once the diagnosis is clear, the clinical management of thyroiditis is comparatively simple; in veterinary medicine as in human medicine, supplementation with levothyroxine usually ameliorates the symptoms. It could be argued that since there are likely to be several genes that contribute to predisposition, the specification of each and the unraveling of their respective roles will be largely of academic interest and irrelevant to clinical management of the disease. However, when one considers the present ambiguity in the clinic, additional genetic criteria should add an important dimension for more accurate diagnosis and realistic prognosis.

4. Genetic Improvement of Breeds

Once the connections between particular alleles and the predisposition to autoimmune diseases are discovered, one could screen for carriers of deleterious genes in dogs that are considered for breeding. Most autoimmune diseases do not become patent until middle age, and even

then their deleterious impact often is expressed only when particular sets of environmental factors, including infection and dietary factors, coincide. When the breeding dog is young or when the impact of environmental factors is absent or delayed, "silent" carriers of potential defects are bred. The unfortunate consequences appear in the descendants. An effective genetic screen would allow many such breedings to be avoided. As more information on inheritance patterns is collected and correlated with the molecular biological information (Smith, 1994), selection to minimize genetic disease should become increasingly efficient.

5. DLA Diversity and Endangered Species

In humans, comparison between races or well-defined ethnic groups often blurs the association between a particular HLA allele and thyroid disease. The population structure of the domestic dog is profoundly different from human populations, yet some evidence indicates that the genetic differences between dog breeds is rather like that between human races (Jordana *et al.*, 1992). Canine thyroiditis is apparently widespread (Dodds, 1988), found in many registered breeds and in outbred Alaskan huskies. Comparative studies using simultaneously both the well-defined breeds and lines and outbred mongrel dogs will permit the evaluation of the importance of a particular allele or haplotype in gene pools of large or small diversity. The impact of a particular allele may be more substantial in small structured populations, such as are characteristic of many endangered species. Research on autoimmune predisposition in structured and unstructured dog populations should provide useful guidelines for assessing the risks and planning management strategies for preservation of particular dog breeds as well as endangered wild mammals, birds, and other vertebrates.

6. Therapeutic Immunosuppression

In experimental models, autoimmune disease can be prevented by tolerization to the initiating target antigen. For example, a single injection of GAD into the veins (Kaufman *et al.*, 1993) or the thymus (Tisch *et al.*, 1993) of 3-week-old NOD mice, just before the onset of the spontaneous IDDM, prevented both insulitis and diabetes. Similarly, in EAT of high responder mice, preinjection of soluble thyroglobulin tolerizes against subsequent thyroglobulin administration (Lewis *et al.*, 1992; D. C. Rayner cited in Champion *et al.*, 1992). Such a procedure might protect young dogs known to be genetically predisposed from developing the symptoms in later life. There are many other very

promising strategies for selective immunosuppression that are beyond the scope of this chapter (Champion *et al.,* 1992; Adorini *et al.,* 1993; Zhang and Raus, 1993, Matsumoto *et al.,* 1994).

7. Correction of Genetic Defects

One great promise of molecular medicine is the potential for gene replacement therapy. Such treatments have been successful for hematopoietic cell function in dogs (Karlsson, 1991). Even more dramatic is the recent partial correction of canine hemophilia B following insertion of a cloned, functional copy of the gene for canine factor IX via a retrovirus vector (Kay *et al.,* 1993). It has recently been shown that the canine thyroid follicular cell is a particularly convenient target for retroviral gene delivery (O'Malley *et al.,* 1993). With retroviral constructs, the regulation of the thyroid and the succession of events during the development of autoimmune disease could be probed in many new ways. Regimens for rescue from congenital thyroid disease might be developed (O'Malley *et al.,* 1993). The complexity of this autoimmune disease makes complete rescue unlikely in the near future, but partial rescue might provide new basic information on thyroid physiology and might permit interesting new therapies.

8. General Mechanisms of Autoimmune Pathogenesis

A broader argument can be made for the study of thyroiditis as a model autoimmune disease, for both canines and humans (McGregor, 1992). The autoantigens and their important epitopes involved in thyroiditis are being defined with increasing precision; the target organ is discrete and accessible, the pathogenic processes in the gland are well described, and there are excellent groups working on the human autoimmune disease as well as animal models. In addition, thyroiditis in genetically predisposed individuals (human or canine) may be triggered as a normal response to a foreign antigen, perhaps one sharing antigenic determinants with the self-antigen. Part of the interest in studying thyroiditis in people stems from its convenience as a model to understand the factors that precipitate general autoimmune response and to see what tips the balance between benign response and disease (McGregor, 1992). Thyroiditis is an excellent model for canine research for the same reason.

9. The Importance of the Genetic Context in Disease Development

Canis familiaris is a species that offers unique advantages as an experimental model to study the role of immunogenetics in the onset of autoimmune disease. One problem that plagues attempts to evalu-

ate combinations of human MHC and TCR alleles as candidates for production of autommune disease is the very noisy genetic context (e.g., Hibberd *et al.*, 1992). The diverse genetic contexts available for dogs present opportunities to reduce the noise. The profound morphological and physiological differentiation among the hundreds of dog breeds reveals the significant genetic heterogeneity present in the species. Each breed comprises a subset of the total gene pool and is in effect a closed population. Within many dog breeds, the relationships of lines and the breeding histories are well documented. A candidate gene or a combination of candidate genes can be examined in unique genetic contexts—lines and breeds that are already established and readily available. The same candidate genes can be assessed in outbred mongrels. From such comparative studies, one could evaluate individual alleles, combinations of genes, or even inbreeding as factors that predispose toward autoimmune reactions or protect against them. The lessons learned from dogs might apply to other mammals, including humans.

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