ACTA UNIVERSITATIS AGRICULTURAE SUECIAE

VETERINARIA 59

Methodological Aspects on Anti-Nuclear Antibody Determination in Canine Autoimmunity and *in vitro* Studies of Antigen-Specific Cellular Responses

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Akademisk avhandling som för vinnande av veterinärmedicine doktors-examen kommer att offentligen försvaras i Ettans föreläsningssal, Klinikcentrum, Ultuna, fredagen den 22 oktober 1999 kl. 09.15.

Abstract

The presence of circulating antinuclear antibodies, ANA, is the hallmark of several systemic autoimmune diseases and has become an important diagnostic tool. Rat liver sections and the human epithelial cell line HEp-2 were compared as substrates for the detection of canine ANA using the indirect immunofluorescence (IIF) technique. The HEp-2 cell substrate was found to be superior to rat liver cryostate sections as ANA substrate for canine sera because of their low reactivity with normal sera and the ease of discernment of ANA fluorescence patterns in positive samples. Positive canine ANA sera thus could be subdivided according to the nuclear fluorescence staining patterns of non-mitotic cells and the staining of chromosomal areas in mitotic cells.

Immunoblot reactivity as well as the presence of precipitating canine antibodies, determined by Ouchterlony immunodiffusion (ID), was found to be strictly associated with one of the IIF ANA subtypes. Among the ID positive sera different antigenic reactivities were detected, represented by different ID subgroups. Only one of the ID subgroups showed identity with any of the well-defined and clinically important human ANA specificities, demonstrating anti-RNP reactivity. The response against the RNP antigen seems to be conserved between man and dog, as the canine anti-RNP reactivity was directed towards the human major antigenic region of the most prominent RNP antigen. Other prominent canine autoantigens were found not to be identical with the principal human ones, probably reflecting dog-specific subgroups of autoantigenic reactivities, which in turn may indicate different canine subgroups of systemic autoimmune disease.

In order to investigate antigen-specific cellular responses of human peripheral blood mononuclear cells (PBMC), tetanus toxoid (TT) was used as a model antigen. Tetanus toxoid conjugated to beads was found to be a more efficient stimulator of a specific CD4+ Th 1 cell response in a primary stimulation assay of human PBMC as compared with soluble TT. It is suggested that targeting of TT antigen to phagocytic antigen-presenting cells, most probably monocytes, is responsible for the enhanced stimulatory properties observed.

Key words: antinuclear antibodies, indirect immunofluorescence, immunodiffusion, immunoblot, RNP, primary stimulation assay, tetanus toxoid, cytokines

Distribution:
Swedish University of Agricultural Sciences
Department of Small Animal Clinical Sciences
S-750 07 Uppsala, Sweden

Uppsala 1999
ISSN 1401-6257,
ISBN 91-576-5439-5

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Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 1999

Acta Universitatis Agriculturae Sueciae
Veterinaria 59

ISSN 1401-6257
ISBN 91-576-5439-5

Tryck: SLU Service/Repro, Uppsala 1999

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Contents

Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Hansson, H., Trowald-Wigh, G. and Karlsson-Parra, A., 1996. Detection of antinuclear antibodies by indirect immunofluorescence in dog sera: Comparison of rat liver tissue and Human Epithelial-2 cells as antigenic substrate. J. Vet. Int. Med. **10**, 199-203.

II. Hansson, H. and Karlsson-Parra, A., 1999. Canine antinuclear antibodies: Comparison of immunofluorescence staining patterns and precipitin reactivity. Acta Vet. Scand. **40**, 205-212.

III. Welin Henriksson, E., Hansson, H., Karlsson-Parra, A. and Pettersson, I., 1998. Autoantibody profiles in canine ANA-positive sera investigated by immunoblot and ELISA. Vet. Imm. Immpath. **61**, 157-170.

IV. Hansson, H., Rönnelid, J., Grönlund, H. and Karlsson-Parra, A. Augmentation of antigen-specific human T cell responsiveness in the primary *in vitro* stimulation assay by antigen-conjugation to Sepharose beads. Submitted 1999.

Abbreviations

ACR	American College of Rheumatology
ALP	Alkaline phosphatase
ANA	Antinuclear antibodies
APC	Antigen presenting cells
BCIP	5-bromo-4-chloro-3-indolyl phosphate
B-TT	Bound tetanus toxoid (conjugated to beads)
CD	Cluster of differentiation
cpm	Counts per minute
DLA	Dog leukocyte antigen
dsDNA	Double-stranded deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ENA	Extractable nuclear antigen
FITC	Fluorescein isothiocyanate
HEp-2	Human epitheloid-2
HLA	Human leukocyte antigen
ID	Immunodiffusion
IFN	Interferon
IgG	Immunoglobulin G
IIF	Indirect immunofluorescence
IL	Interleukins
IPTG	Isopropylthiogalactosidase
LE	Lupus erythematosus
mAb	Monoclonal antibodies
MCTD	Mixed connective tissue disease
MHC	Major histocompatibility complex
OD	Optical density
PBMC	Peripheral-blood mononuclear cells
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
SFC	Spot forming cells
SLE	Systemic lupus erythematosus
Sm	Smith
S-TT	Soluble tetanus toxoid
Th1	T helper lymphocytes type 1
TNF	Tumor necrosis factor
TPO	Thyroid peroxidase
TT	Tetanus toxoid

Introduction

Autoimmune diseases

The definition of an autoimmune disease is a condition where an autoimmune reaction (an immune response mounted against normal body components) causes pathologic changes. The term autoimmune disease is, however, often used for clinical disorders presenting an autoimmune phenomenon. The evidence for a causal relationship between the autoimmune reaction and pathologic changes is often circumstantial (1).

The etiology of autoimmune diseases is mostly unknown, but it is likely to be multifactorial. Depending on the individual and the particular disease, a varying number of factors may be required to cause a clinically manifest disease. In humans, susceptibility to most autoimmune disorders shows a significant genetic component and familial associations are common (2,3). In many human autoimmune diseases a linkage to certain types of human leukocyte antigens (HLA, the polymorphic transplantation antigens expressed on the surface of all nucleated cells) is demonstrated (3). In some of the canine autoimmune disorders a breed predisposition or familial associations are noted, which also indicates a hereditary background (4-6). Moreover, one investigation has shown an association between a certain type of dog leukocyte antigen (DLA, the canine counterpart to human HLA) and autoimmune disease in German Shepherds (7). Other factors that may be crucial in the development of the diseases are infectious agents, hormonal influences, treatment with certain drugs, different inflammatory conditions and environmental factors (3,8-11).

Autoimmune diseases are usually divided into organ-specific diseases and systemic diseases. One example of organ-specific autoimmune diseases is autoimmune thyroiditis, seen in both humans and dogs (12,13). The most well-known systemic autoimmune disease is systemic lupus erythematosus, SLE, which also has been observed in both humans and dogs. In human patients it has been known as early as from antiquity. These multisystem diseases have variable clinical signs, leading to major diagnostic challenges. In 1880 the following clinical observation was made: "Anyone who has seen half a dozen examples of common lupus and of lupus erythematosus is able with ease to distinguish one from the other...but let him wait a while and see more, and he will find before long that there are examples of mixed forms of the disease which it is impossible to denote concisely without employing hybrid names" (14). This is as relevant today as it was more than 100 years ago. Human SLE is usually classified based on the American College of Rheumatology, ACR, revised Criteria for the Classification of SLE (15), which in recent years has been updated (16). The presence of four or more criteria is mandatory for the diagnosis.

Clinical signs that may be seen in human SLE are lesions involving skin and mucous membranes (e.g. malar rash, erythema over the malar eminences), non-erosive arthritis, serositis, neurologic disorders, renal abnormalities and hematologic disorders. Fatigue and fever are common complaints. The clinical course of SLE is characterized by periods of remission and chronic or acute relapses (17). Several SLE-related syndromes are also described in human patients with overlapping diagnosic features. Such diseases/syndromes may be exemplified by scleroderma, Sjögren´s syndrome and mixed connective tissue disease (MCTD) (18,19).

The diagnosis of systemic autoimmune diseases in the dog has still mainly been restricted to the diagnosis of SLE, and several efforts have been made to identify definite criteria for this disease (20-22). SLE-related syndromes, as seen in humans, are less investigated in the dog. However, canine systemic autoimmune diseases are usually characterized by musculoskeletal clinical signs, accompanied by "morning stiffness" and pain while moving. An intermittent lameness is commonly seen. The arthritis is usually multiarticular and symmetric, and may be acute or chronic. Often the signs of disease wax and wane and considerable time may elapse before the patient is presented for examination. Other clinical signs that may be involved, mainly in the diagnosis of SLE, are skin disorders (often with ulcerations and crustings), anaemia (sometimes with a positive Coomb´s test), thrombocytopenia, polymyositis, nephropathy and fever (20,21,23).

During recent years, dogs with suspected systemic autoimmune diseases have been increasingly observed. This may, at least partly, be due to increased awareness on the part of clinicians or a more ready availability of the immunological tests necessary to support the diagnosis.

In many of the autoimmune diseases in both man and dog, circulating autoantibodies are displayed. For instance, circulating autoantibodies against thyroid structures may be demonstrated in both human and canine patients suffering from autoimmune thyroiditis, while SLE patients usually present antinuclear antibodies, ANAs (22,24-30). The pathophysiologic importance of autoantibodies is mostly unknown. In a limited number of autoimmune diseases, the autoantibodies are known to exert a directly pathologic role in the disease process (31-33). However, in many of the autoimmune diseases, such a direct role in the pathogenesis cannot be demonstrated. Irrespective of the nature of their involvement in the development of the autoimmune disorders, the autoantibodies usually are of great value as diagnostic markers in organ-specific diseases as well as systemic diseases.

Antinuclear antibodies

Antinuclear antibodies are a heterogeneous population of autoantibodies directed against various nuclear antigens that normally are found in mammalian nuclei. These autoantibodies in general lack tissue or species specificity and cross react with nuclei from different sources (34).

In 1948, Hargraves et al. (35) described that a so-called lupus erythematosus (LE) cell phenomenon could be seen in SLE patients. LE cells later were demonstrated as neutrophils containing ingested nuclear material. In 1960, Kunkel et al. (36) showed that the LE cell phenomenon was correlated to the presence of ANA. Lewis et al. (37) in 1965 were the first to describe a disease in dogs that closely resembled SLE in humans, with the presence of both LE cells and autoantibodies. These early studies have been followed by many investigations identifying several different ANAs, which in turn has involved elucidation of the molecular nature of these nuclear autoantigens. Nowadays, the demonstration of LE cells has been replaced by ANA determination (38).

ANA may sometimes be present in healthy individuals but in these cases are usually found in low titres. The presence of ANA is detected more often in elderly people and in women (39,40). An increase in the ANA level may be observed following treatment with certain drugs and in relation to some inflammatory reactions. However, this increased titre is usually temporary. Some neoplastic conditions, such as lymphoma, may also be accompanied by elevated ANA, which should be considered while evaluating a positive ANA test (21,40).

In veterinary as well as in human medicine, several of the various nuclear antigens that constitute a target for the disease-associated autoantibodies have been characterized by molecular biological techniques. Most of these structures have been recognized as nucleic acids, proteins or their complexes, usually with a central biological function (27,41-43). Some of these ANA specificities have been demonstrated in man to strongly associate, alone or in certain combinations, to different distinct autoimmune diseases. This may be exemplified by antibodies against the Smith (Sm) antigen (a complex of RNA and proteins), double-stranded (ds) DNA and the proliferating cell nuclear antigen (PCNA, a cell-cycle related protein) that are all strongly associated with SLE, while reactivity against RNP (ribonucleoprotein, a complex of RNA and proteins) is associated with MCTD (27,44-46). A partial list of different human disease associated autoantigenic specificities are displayed in table 1.

Table 1. Antinuclear antibodies: Partial list of antinuclear antibodies (ANA) in human systemic autoimmune diseases. Modified from von Mühlen CA and Tan EM, 1995 (ref no. 29) and from Nakamura RM et al., 1985 (ref no. 46). - = seldom or never occurring

Specificity	IIF pattern	SLE	MCTD	Sjögren's syndrome
dsDNA	Homogeneous	40-70%	-	-
Histone	Homogeneous	50-70%	-	-
Sm	Speckled	15-30%	-	-
RNP	Speckled	30-40%	100%	-
SSB/La	Speckled	9-35%	-	71-87%
SSA/Ro	Neg/speckled	24-60%	-	88-96%

The usefulness of ANA determination in canine autoimmunity is still mainly restricted to the diagnosis of SLE (22,47). As canine SLE related syndromes have been lesser investigated, the presence of ANA in these diseases in dogs is unclear. Determination of specific antibodies to certain nuclear antigens, such as histones (DNA-associated proteins), Sm, RNP and some antigens not previously characterised in human patients, has also been performed in dogs with systemic autoimmune disease (41-43, 47-49). However, a clear-cut association between certain specificities of ANA and certain systemic autoimmune diseases as seen in humans, has so far not been identified in the dog.

Clinical ANA test methods

The indirect immunofluorescence (IIF) technique is by far the most widely used technique for ANA routine screening in man as well as in animals (22,50). The IIF ANA test is evaluated by fluorescence microscopy. In positive samples, different nuclear fluorescence patterns (fig. 1) are seen, thus allowing further subdivision into different groups according to the kind of staining pattern shown (50,51). When conducting the IIF ANA test, the importance of substrate has become apparent. In the case of nuclear antigens present in abundance, such as the antigens dsDNA, RNP and SSB/La, cells from differentiated animal tissue, such as rat liver, appear to be adequate for the demonstration of the respective antibodies. Some cellular antigens, such as the SSA/Ro antigen and certain nucleolar antigens, however, may be poorly represented in animal tissue. Other antigens, such as the PCNA and the centromeric antigen (proteins bound to centromeric DNA), may also be absent due to the highly differentiated state of certain organ tissues (27).

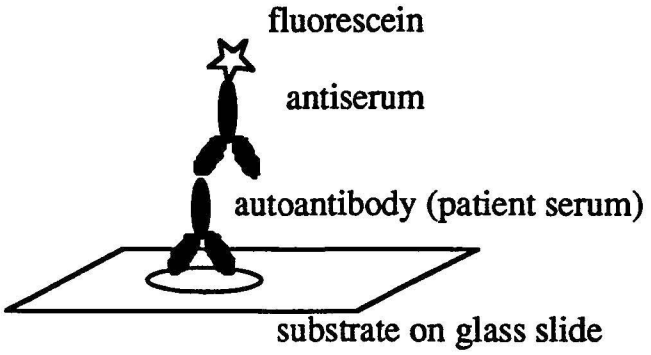

Figure 1. The indirect immunofluorescence (IIF) antinuclear antibody (ANA) test. First, the substrate is incubated with patient serum for 30 minutes, followed by a second incubation with for another 30 minutes with FITC-labelled anti-canine IgG. The slides are examined by fluorescence microscopy.

Other difficulties in interpreting the IIF ANA test result using rodent tissue sections involve pattern discernment, which may give clues to the specific ANA reactivity. Fluorescence pattern detection may sometimes be difficult due to relatively small nuclear size, oblique sectioning of nuclei and the frequent occurrence of extraneous staining due to the presence of extracellular protein matrix substances (50). Considering the aforementioned limitations of IIF ANA testing on sectioned animal tissue substrates, many laboratories conducting human testing now use monolayers of cultured human cells (e.g. the monolayer of human epithelial-2, HEp-2, cells) (52-54). In human patients a clear correlation between different nuclear fluorescence patterns and ANA specificity is shown, which in turn is valuable in the further management of the individual patient (50). In most reports of IIF ANA screening in dogs, cryopreserved sections of rodent organs or mouse blood smears have been the main substrate used (5,22,55,56).

Since a number of specific ANAs appear to be of diagnostic and/or prognostic value in the evaluation of different systemic rheumatic diseases in humans, further specificity determination of IIF ANA positive sera is frequently employed to display the ANA reactivity. A number of assays are available for such determination. The most commonly used methods, due to the relative ease of application, are the Ouchterlony immunodiffusion (ID) test (57) and enzyme-linked immunosorbent assay (ELISA). Other methods that may be used are counter immuno electrophoresis, agglutination techniques and immunoblotting (46,58-60).

Antigen-specific cellular responses

Different cellular interactions have proven important in autoimmune disorders. In these interactions, soluble substances, cytokines, are secreted and act as important mediators between the cells. Examples of different cytokines are interleukins (IL), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). The demonstration of T cell responses and cytokine production may provide further information of certain aspects of autoimmune diseases as compared with the demonstration of autoantibodies. The demonstration of autoantibodies may, e.g., have a more limited value in the monitoring of disease activity or in monitoring the effects of antigen-specific immunotherapy (61,62). In organ-specific autoimmunity such as insulin-dependent diabetes mellitus, multiple sclerosis, and rheumatoid arthritis, there is now strong evidence that T helper type 1 (Th1) cells are important for the initiation and perpetuation of the disease process (63,64). Th1 cells are a subset of CD4+ (expressing the surface molecule cluster of differentiation 4) T cells secreting, e.g., IFN-γ and TNF-α.

The *in vitro* determination of antigen-specific cellular responses, such as lymphocyte proliferation and cytokine production, has emerged as

possible indicators in order to evaluate immunological effects of specific immune interventions (65-67).

A commonly used method for measuring antigen-specific cellular responses is the primary stimulatory test (also called the lymphocyte proliferation test), which most simply reflects measurement of the proliferation of T cells after addition of antigens to a cell culture. Adequate antigen presentation, performed by antigen-presenting cells (APC), is essential to evoke proliferative responses of T cells. In the primary stimulatory test with peripheral-blood mononuclear cells (PBMC), monocytes, B cells and dendritic cells constitute the APC population (68,69). However, since several autoantigens reveal only a low responsiveness in the primary stimulatory tests using PBMC, such monitoring has often been performed by using laborious and time-consuming assays including establishment of short-term T cell lines (66,67,70). Furthermore, several investigations have tried to enhance the stimulatory capacity by conjugating antigen to different kinds of matrix (71-76). Antigen conjugated to microspheres (as compared with soluble antigen) has been shown to drastically enhance the response of established CD4+ T cell lines/clones against macrophage-mediated antigen presentation (71,72). However, the effect of such antigen-conjugation on T cell responsiveness during a single primary stimulation of human PBMC has, to our knowledge, not been evaluated.

Aims of the study

The aims of the present studies were:

* to compare cryopreserved rat liver sections with tissue culture monolayers of human epithelial-2 (HEp-2) cells as antigenic substrate when conducting the IIF ANA test using canine sera (paper I)

* to study the correlation between IIF ANA staining patterns and precipitating canine antibodies using immunodiffusion technique and to identify possible precipitin lines of identity with any of the well-defined and clinically important human ANA specificities (paper II)

* to investigate the canine response against human nuclear antigens using immunoblot technique with HeLa cell nuclear extract (paper III)

* to evaluate the effect of antigen conjugation to beads on lymphocyte proliferation and cytokine production using PBMC from antigen-primed human subjects (paper IV).

Materials and methods

The materials and methods employed during the course of these studies are described below. For further details, see papers I-IV.

Subjects

The patients and controls included in the different studies were as follows:

In study I-III, a total of 112 canine serum samples that had previously been analysed for IIF ANA due to suspected autoimmune disease, were selected on the basis that they all were positive for ANA, using cryopreserved sections of rat liver as substrate, at a dilution of 1/20. 43 different breeds were included among these dogs. All serum samples were stored in aliquots at -20°C (study I). The 62 canine patient sera included in studies II and III, were all among the autoimmune patients in study I. These sera were ANA positive by IIF at the dilution ≥ 1/100, using HEp-2 cells as substrate. These dogs comprised 24 different breeds and the age distribution was one to 11 years, with a median age of four years. Serum samples from 100 apparently healthy family dogs (examined at the Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences) were stored at -20°C until analysed. Thirty-two different breeds were included among these dogs, with an age distribution between four months and 13 years (median age four years).

In study IV, peripheral venous human blood samples were obtained with informed consent from tetanus toxoid (TT) primed healthy adult volunteers.

Indirect immunofluorescence (studies I-III)

In order to evaluate this assay for the canine patients, the IIF ANA test was performed using cryopreserved sections of rat liver (study I) or monolayers of human epitheloid, HEp-2, cells (study I-III) as substrate. First, the substrate was incubated with serum for 30 minutes, followed by a second incubation for another 30 minutes with FITC labelled anti-canine IgG. The incubations were carried out at room temperature in a humid atmosphere and followed by washings in PBS. The slides were mounted in PBS-glycerol and examined by fluorescence microscopy.

Immunodiffusion (studies II-III)

The sera were analysed with the commercially available Auto I.D. Autoantibody Test System (Immuno Concepts, Sacramento, CA) using lyophilized mammalian nuclear antigen. The nuclear antigen substrate was added to the central well and each canine serum sample added to one of six peripheral wells in the agar plate. After incubation for 24-48 h at room temperature, nuclear reactivity was identified as a visible precipitation line between the canine serum sample added to one of six peripheral wells in the

agar plate and the nuclear antigen substrate added to the central well. Specific human antisera reactive to SSA/Ro, SSB/La, Sm, RNP, Jo1 and Scl 70 were used in order to determine the specificity of precipitating dog sera.

Immunoblotting (study III)

As only a few canine sera showed reactivity identical to specific human antisera, as determined by immunodiffusion, the immunoblotting technique was used to obtain an overview of canine response against nuclear antigens. Thus, immunoblotting of HeLa cell nuclear extract or the nuclear extract used in the immunodiffusion assay, was performed as previously described (77,78). The nuclear proteins were separated on a 10% SDS-PAGE gel and transferred electrophoretically to a nitrocellulose membrane. Alkaline phosphatase conjugated anti-human IgG antibodies and anti-dog IgG antibodies were used with the substrate nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Human reference sera from several previously identified ANA-positive patients were used to identify the different nuclear antigens.

ELISA measurement of antinuclear antibodies (study III)

A limited number of canine sera displayed immunoblot reactivity similar to common and disease specific human ANA specificities. By using the enzyme-linked immunosorbent assay (ELISA) technique with recombinant proteins as antigens, we were able to investigate in greater detail the specific properties of the antinuclear antibodies in these dogs. Soluble recombinant fusion proteins were produced with, for the U1-70K protein, the protein containing a fragment corresponding to the most frequently recognized human domain (amino acids 63-195) (78,79). The SSB/La (80,81) and the SSA/Ro (82) fusion proteins encoded the full length proteins. The recombinant fusion proteins were transformed into *Escherichia coli* cells and expression was induced by addition of isopropylthiogalactosidase (IPTG). The recombinant proteins were purified using an amylose column and the protein concentration was determined. Recombinant proteins were used to coat medium binding ELISA plates overnight at +4°C. Plates were incubated 2 h with primary serum, whereafter affinity purified alkaline phosphatase (ALP) conjugated anti-dog IgG antibodies or anti-human IgG specific antibodies was added. The final steps included addition of p-nitrophenyl phosphate, stopping the reaction with 1M NaOH and reading optical density (OD) at 405 nm.

Lymphocyte proliferation test (study IV)

Human PBMC were separated from heparinized blood by Ficoll-Hypaque density centrifugation. Soluble tetanus toxoid was obtained from

SBL Vaccine, Stockholm, Sweden. After purification of the TT antigen by size fractionation gel filtration, the toxoid was conjugated to 2 μm Sepharose beads (performed by Pharmacia & Upjohn Diagnostics, Uppsala, Sweden). The PBMC were cultured in 96-well round-bottom microtiter plates at 37°C in a humidified atmosphere of 5% CO_2 for 7 days. The cells were stimulated with soluble TT (S-TT) or TT conjugated to Sepharose beads (B-TT) with different final concentrations. PBMC not stimulated at all or stimulated with unconjugated Sepharose beads were used as controls. During the final 16 h of the 7-day incubation period, the cells were pulsed with [^{3}H]thymidine and incorporated label counted by liquid scintillation. Results were expressed as mean counts per minute (cpm) of [^{3}H]thymidine incorporation for triplicate cultures.

For inhibition of MHC (major histocompatibility complex) class II-dependent reactions, a pool of three different monoclonal antibodies (mAb) reacting against human MHC class II (anti-HLA DR, DP and DQ) was used at a total final concentration of 3 μg/ml as earlier described (83). As control antibodies, an isotype matched mixture of two monoclonals directed against keyhole limpet hemocyanin were used (83).

ELISA measurement of cytokines (study IV)

PBMC were cultured and TT stimulated for 6 days as described above, whereafter supernatants were collected. Analysis of IFN-γ, TNF-α, IL-6 and IL-10 concentrations were performed by ELISA. Briefly, ELISA plates were coated with primary antibodies overnight at +4°C. The plates were blocked with PBS-1% bovine serum albumin and then incubated with culture supernatants at room temperature for 4 h. Biotin labelled secondary antibodies were added and incubated overnight. Finally, avidin-alkaline phosphatase was added and incubated for 60 min whereafter p-nitrophenyl phosphatase substrate in dietanolamine buffer was added. A_{405} was measured and correlated to standard curves established with recombinant cytokines.

Detection of IFN-γ producing cells by ELISPOT (study IV)

The method was used as earlier described (84) with the addition of a pre-incubation step. In short, the primary monoclonal antibody was diluted in PBS and adsorbed to plastic ELISA plates at 4°C overnight. PBMC were separated and cultured for 48 hours while stimulated with antigens (as described above). A cell suspension was then added to the coated plastic plates and plates were incubated overnight. The cell suspension was flicked off, plates washed, diluted secondary antibody added to each well and the plates left overnight at 4°C. Finally, avidin-alkaline phosphatase was added followed by a BCIP phosphatase substrate solution and the plates were developed at room temperature for 5 h. Spot-forming cells (SFC) were counted using an inverted microscope.

Results and discussion

ANA determination of canine sera by indirect immunofluorescence and immunodiffusion techniques (studies I-II)

In study I, rat liver sections and HEp-2 cells were compared as substrate for IIF ANA determination. The major finding was that HEp-2 cells were superior to rat liver cryostate sections as ANA substrate due to their low reactivity with normal sera and the ease of discernment of ANA fluorescence patterns in positive samples. Thus, the use of HEp-2 cells allowed the possibility of further subdividing the IIF ANA-positive canine sera into different groups, according to the kind of staining pattern shown. The predominant fluorescence pattern was the speckled subtype, without concomitant chromosomal staining in mitotic cells, which was displayed by 83% of the ANA-positive dogs. The remaining 17% were considered as homogeneous with a concomitant chromosomal staining of mitotic cells (fig. 2). The speckled sera without chromosomal reactivity could furthermore be divided into subtypes of nuclear staining patterns. The majority of these serum samples exhibited a fine speckled nuclear fluorescence pattern with an almost homogeneous appearance in non-mitotic cells. A second subgroup showed a grainy speckled nuclear pattern and a third subgroup exhibited a combination of fine and grainy speckles, a mixed speckled staining pattern.

None of the IIF ANA chromosomal positive, homogeneous sera displayed any anti-dsDNA reactivity using *Crithidia luciliae* as substrate. This finding is in concordance with earlier studies, where a DNA-binding protein was demonstrated in dog sera and thus made canine anti-DNA antibodies difficult to detect (48,85). More recently, the majority of canine sera reacting with chromosomal antigens have been shown to present anti-histone antibodies (41,42,86).

After the completion of study I-III, 34 additional dogs with suspected autoimmune disease, examined at the Department of Small Animal Clinical Sciences, Uppsala, Sweden, have been found to demonstrate IIF ANA positivity. When adding these dogs to the ones in study I-III, 21% among a total of almost 100 IIF ANA-positive canine serum samples displayed a homogeneous, chromosomal positive, staining pattern (unpublished data).

The finding of different subgroups of fluorescence staining patterns prompted us to study whether such subgroups also represented different specific ANAs as determined by the commercially available ID technique (study II). The presence of precipitating antibodies was found to be strictly associated with a positive IIF ANA exhibiting a speckled staining pattern without any chromosomal reactivity. All the IIF ANA chromosomal positive, homogeneous sera were found negative by immunodiffusion (fig. 3).

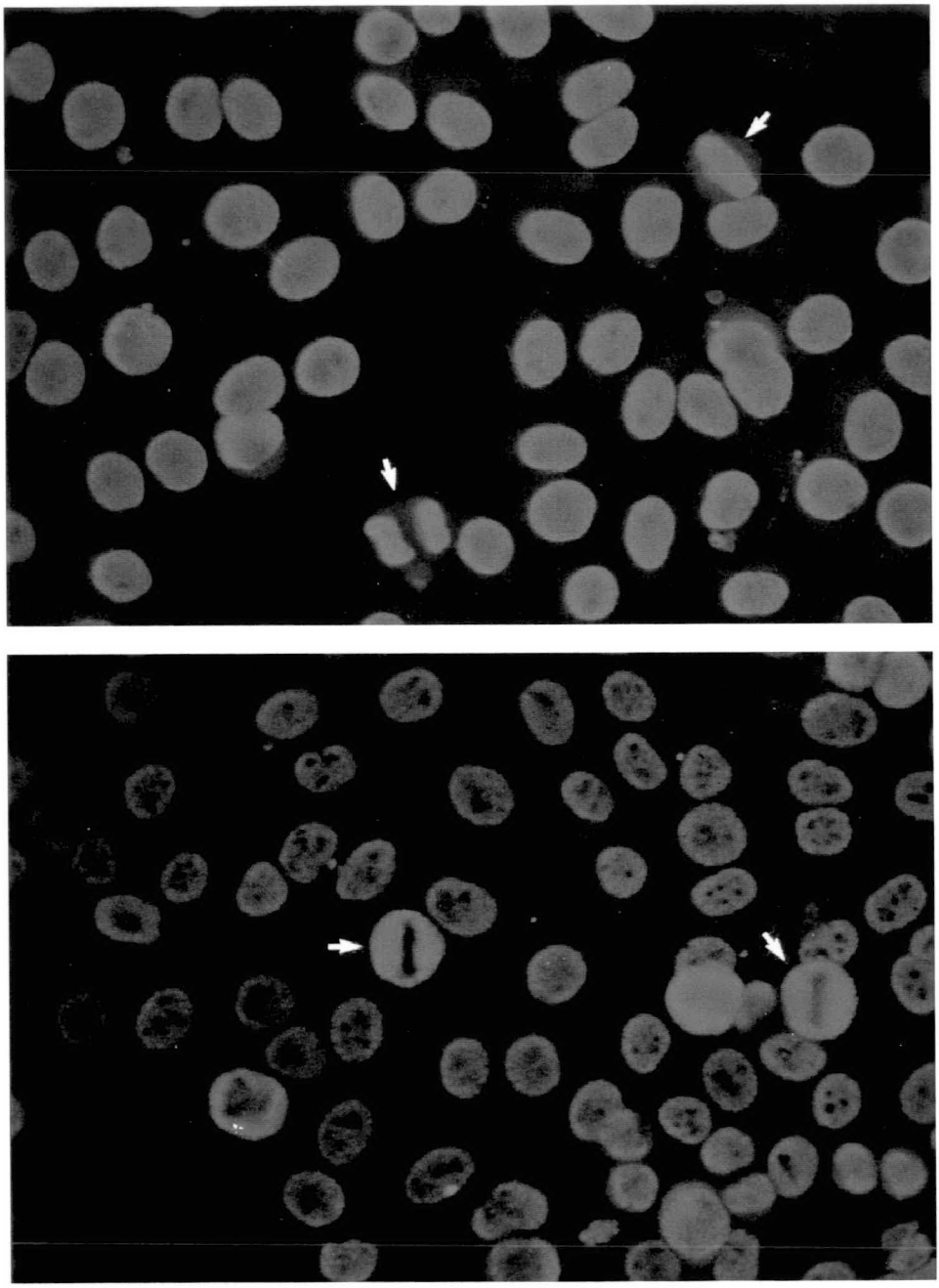

Figure 2. IIF ANA staining patterns on HEp-2 cells. (A) A homogeneously stained fluorescence pattern with a concomitant chromosomal staining of mitotic cells (arrow). (B) A speckled staining pattern without concomitant chromosomal staining of mitotic cells (arrow).

Nearly 50% of the IIF ANA-positive speckled sera, without chromosomal staining, were positive by ID. Thus, a number of the canine sera with a speckled staining pattern did not present precipitating antibodies detectable with the ID technique. Experience of human patients indicates a similar condition, in which certain autoantibody specificities do not present precipitin lines. Among the ID-positive sera different antigenic reactivities were detected, represented by different ID subgroups. The serum samples in each subgroup thus presented ID reactivity identical to the other sera in the same ID subgroup. An identical reactivity was defined as a continuous precipitation line between the adjacent serum sample wells and the central well containing the nuclear extract (fig. 4). Interestingly, there were only four different ID subgroups demonstrated, which indicates that the precipitating sera were restricted as to specificity. However, no clear-cut correlation was obtained between different subgroups of speckled ANA fluorescence patterns and certain subgroups of precipitating antibodies.

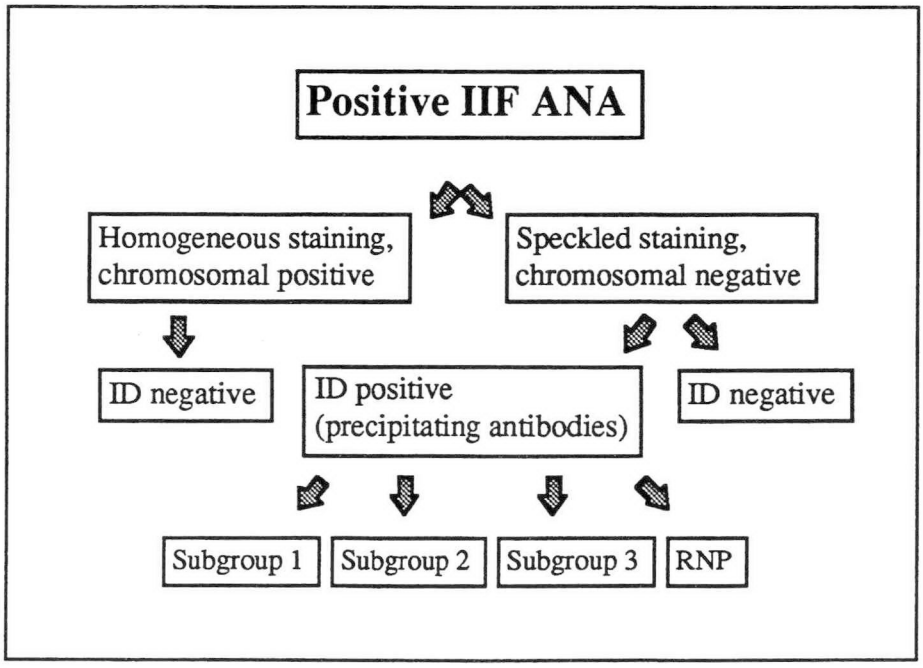

Figure 3. IIF ANA positive canine sera subgrouped according to fluorescence staining patterns and to precipitin reactivity obtained by immunodiffusion (ID). The presence of precipitating antibodies was strictly associated with a positive IIF ANA exhibiting a speckled staining pattern without any chromosomal reactivity.

Only one of the four ID subgroups showed identity with any of the well-defined and clinically important human ANA specificities. This subgroup, consisting of six canine sera, all demonstrated anti-RNP reactivity. One serum sample concomitantly exhibited precipitating antibodies against the Sm antigen. The majority of the canine sera, however, did not react against common disease-associated autoantigens in the human system (fig. 3). This may reflect difficulties in interactions of precipitating antibodies between the two different species. However, since six of the canine sera produced precipitin lines of identity with one of the human specificities, the differences in precipitin line formation probably reflect dog-specific subgroups of autoantigenic reactivities. This may further indicate that the different subgroups reflect different canine subgroups of systemic autoimmune disease.

The German Shepherd (fig. 5) was the clearly dominating breed among IIF ANA-positive dogs, although this is a common breed in the Swedish dog population in general (9-10% of the total registered dog population in Sweden during the years comprised in studies I-III).

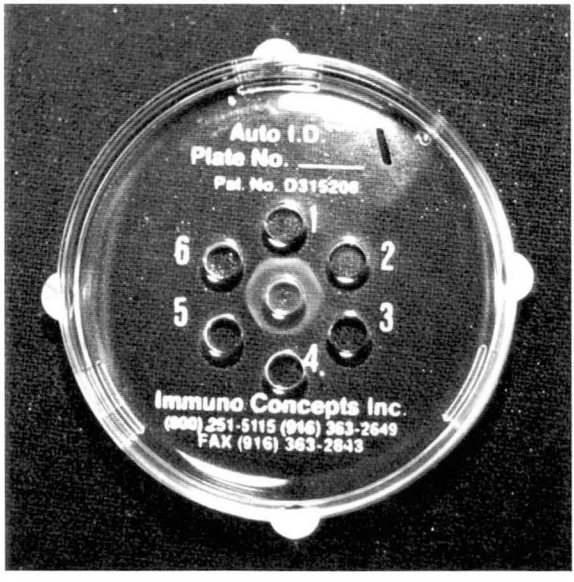

Figure 4. Immunodiffusion (ID). Six canine sera demonstrating identical ID reactivity. A nuclear antigen substrate is added to the central well and each canine serum sample added to one of six peripheral wells in the agar plate. After incubation, nuclear reactivity is identified as a visible precipitation line between the canine serum sample added to one of six peripheral wells in the agar plate and the nuclear antigen substrate added to the central well. An identical reactivity is defined as a continuous precipitation line between the adjacent serum sample wells and the central well containing the nuclear extract.

48% of the IIF ANA-positive dogs investigated in studies I-III and 42% of the total number of IIF ANA-positive dogs (when adding the 34 ANA-positive dogs found in recent years as mentioned above, unpublished data) consisted of German Shepherds. All but one of the German Shepherds´ sera demonstrated a speckled ANA fluorescence pattern without chromosomal reactivity. An interesting finding was that 14 out of 15 precipitin-positive German Shepherds´ sera also exhibited an identical but unidentified specificity as confirmed by ID. Moreover, this breed completely dominated one of the four ID subgroups. These findings may indicate a breed-specific disorder with a common autoantigenic reactivity. If so, it might reflect a hereditary susceptibility among German Shepherds to achieve a certain subtype of systemic autoimmune disease. One study has also proven such a hereditary susceptibility in this breed, by demonstrating an association between a certain DLA antigen and SLE (7). This finding is in concordance with observations in humans, where autoimmune diseases often are associated with certain types of HLA (3). However, the genetic relationship between the German Shepherds in our investigations was not studied, and it is not known if these dogs reflect a certain related line of dogs or a disorder more generally distributed throughout the breed.

Figure 5. The clearly dominating breed among dogs presenting antinuclear antibodies (ANAs) in our studies was the German Shepherd. This ANA positive German Shepherd initially presented skin lesions, later followed by stiffness and pain while moving.

The clearly dominating clinical signs among the IIF ANA positive dogs were musculoskeletal problems mainly accompanied by stiffness and pain while moving or intermittent lameness involving multiple limbs. Musculoskeletal signs were distributed among the dogs in all the different IIF ANA and ID subgroups. Thus, in general it was not possible to relate certain clinical signs to certain subgroups of dogs. However, one interesting difference was found when comparing dogs in the two major IIF ANA subgroups: A greater proportion of the dogs displaying a homogeneous, chromosomal-positive staining pattern displayed other clinical signs as dominating symptoms, e.g. anaemia. The dominating clinical signs of the dogs with a speckled ANA fluorescence pattern were almost always musculoskeletal disorders. This finding was even more evident when looking at the larger group of IIF ANA positive dogs, i.e. when the 34 dogs more recently demonstrated were included. As SLE usually is more associated with clinical signs from different organ systems, it is possible that the dogs with a homogeneous, chromosomal positive staining in our studies might suffer from a "true" SLE disorder. A homogeneous, chromosomal positive staining pattern may reflect the presence of antihistone and/or anti-dsDNA antibodies. As mentioned above, it has been shown that canine sera reacting with chromosomal antigens usually present anti-histone antibodies. In humans, SLE is associated with many different autoantibody specificities, although autoantibodies associated with chromosomal reactivity are a common finding (table 1) (28).

ANA determination of canine sera by immunoblot and ELISA (study III)

In human patients, as described above, some of the ANA specificities have been demonstrated to strongly associate, alone or in certain combinations, with different well-defined autoimmune diseases. As only a few canine sera showed reactions with immunological identity to specific human antisera when conducted by immunodiffusion, further characterization of the canine ANA specificities was needed. The same IIF ANA positive canine sera that were investigated by immunodiffusion were therefore also characterized using HeLa nuclear extract immunoblot.

Immunoblot reactivity was found to be strictly associated with a positive IIF ANA exhibiting a speckled staining pattern without any chromosomal reactivity. On HeLa cell nuclear extract immunoblot, a common observation among the dog sera was a reaction against a nuclear protein corresponding to approximately 43 kD. This reactivity was not correlated to the presence of precipitating antibodies, which probably is explained by the finding that this prominent canine autoantigen was missing in the commercially available extract designed for immunodiffusion testing of human sera. A 43 kD glycoprotein, hnRNP G, has previously been identified as the target antigen in sera from dogs developing SLE

or clinically related autoimmune disorders (49,87,88). Even though the immunoblot reactions indicate that the hnRNP G protein is present in human HeLa cell nuclear extract, this antigen does not seem to be autoantigenic for human patients (unpublished observations).

In several canine sera we could observe bands of varying strengths around the 48 kD level. Prominent known human autoantigens in this molecular weight range are the SSB/La-48 kD protein and the SSA/Ro 52-kD protein (28). The canine sera presenting bands in this range were assayed on ELISA with recombinant SSA/Ro and SSB/La proteins, although none of the sera exhibited any significant reactivity with these antigens. This finding was in concordance with the outcome of the immunodiffusion results described above, as none of the serum samples displayed any precipitin reactivity against the SSA/Ro or SSB/La antigen.

All six dog sera demonstrating anti-RNP antibodies by ID also displayed a classical human RNP pattern on immunoblot. The one serum sample with a concomitant Sm reactivity also exhibited a classical human Sm immunoblot pattern (59,89,90). None of the RNP positive sera reacted with the 43 kD protein on immunoblot.

We further investigated the canine anti-RNP reaction against the human principal RNP antigen, the U1-70K protein. We could establish that anti-RNP sera from dogs with systemic autoimmune disease recognise the major antigenic region of the U1-70K protein, which is in concordance with reactivity in human anti-RNP sera (78). Thus, the response against the RNP antigen seems to be conserved between man and dog.

In humans, such an anti-RNP reactivity may reflect the syndrome MCTD. Clinical signs that may be seen in human MCTD are, e.g., fatigue, fever, myositis, mucocutaneous changes, joint pain and stiffness. Joint involvement in MCTD is more common and more severe than in classic SLE (18). All but one of the dogs with the same anti-RNP reactivity showed severe musculoskeletal clinical signs, with a pronounced stiffness and pain while moving. This might indicate a disease syndrome similar to the human MCTD. The dog not showing musculoskeletal signs instead suffered from regurgitation and esophageal dysfunction. Interestingly, this is also a common finding in human MCTD.

Further studies of canine ANA specificity may provide additional information on whether similar associations exist between specific ANA and certain autoimmune diseases as seen in human patients. This may give us a possibility to offer a more individual approach for the management of the ANA-positive canine patient.

In a longer perspective, both human and canine patients may profit from a more thorough analysis of the differences and similarities between human and canine systemic autoimmune diseases. Dogs develop "spontaneous" autoimmune disease, probably due to a combination of inheritance and environmental factors. This induction may be similar to

that seen in human patients. Characterization of spontaneously occurring systemic autoimmune disease in dogs may thus provide insights not possible to obtain from genetically or chemically induced man-made models.

PBMC proliferation and cytokine response to soluble antigen and antigen conjugated to beads (study IV)

The effect of antigen conjugation to beads in antigen-specific cellular responses was investigated by using tetanus toxoid (TT) as a model antigen and PBMC from primed normal human subjects. TT conjugated to beads (B-TT) was found to be a highly efficient stimulator of the PBMC proliferative response. Ten to 100-fold lower concentrations of B-TT was usually needed in order to obtain a substantial proliferative response, as compared with soluble TT (S-TT). The levels of IFN-γ, and to some extent TNF-α, in culture supernatants at day 6 were found to be increased by B-TT as compared with identical concentrations of S-TT as measured by ELISA. A markedly increased number of IFN-γ producing cells, observed with ELISPOT technique, was also demonstrated after stimulation with B-TT. These data indicate that antigen conjugated to beads is a more efficient stimulator of a specific Th 1 cell response in a primary stimulation assay of human PBMC as compared with soluble antigen. The secretion of IL-6 or IL-10 (which would indicate a Th2 cell response) was either very low or undetectable after stimulation of PBMC by B-TT or S-TT.

Addition of monoclonal antibodies directed towards human major histocompatibility complex (MHC) class II antigens (anti-HLA DR, DP and DQ) to the culture at the start of the proliferative assay markedly reduced the proliferative response to S-TT and B-TT, indicating a HLA class II restricted response by CD4 + T cells.

We assume that the TT-conjugated Sepharose beads are targeted mainly to phagocytic monocytes, since B-cells are known to be unable, or far less effective, to ingest and present antigen covalently linked to microspheres (71,73,74). Since earlier studies have shown that macrophages are optimal as APC for Th1 cells while B-cells are optimal for Th2 cells (91-94), the expected targeting of TT to monocytes may by part explain the superiority of B-TT in eliciting a Th1 response. Possible differences in expression of certain monocyte surface molecules that are crucial for MHC class II dependent antigen presentation was furthermore investigated. Thus, the expression of MHC class II (HLA- DR and HLA-DQ) and B7 (CD80 and CD86) was compared using PBMC monocytes stimulated with S-TT and monocytes stimulated with B-TT. The expression of these structures on CD14+ monocytes/macrophages, however, did not differ between the two systems after 24 or 48 hours of incubation, which is in concordance with an earlier study (75).

Thus, a more efficient upregulation of these crucial molecules in MHC class II-dependent antigen presentation does not seem to be the cause of the enhanced B-TT response demonstrated in study IV.

Phagocytosis of inert polystyrene beads has, moreover, been shown to induce IL-12 p40 mRNA expression in macrophages (95). Since several studies have demonstrated a requirement of IL-12 production by APC for optimal Th1 proliferation (96,97), we have conducted preliminary investigations adding anti IL-12 to the cultures. The addition of anti IL-12 antibodies showed that Th1 cytokine production, but not proliferation, was markedly inhibited (data not shown). The Th1 cytokine production induced by B-TT and S-TT was however equally downregulated by anti IL-12, indicating that enhanced IL-12 production is not responsible for the effective stimulatory response obtained with B-TT as described above.

Taken together, our data indicate that antigen conjugated to Sepharose beads is a more efficient stimulator of a specific Th 1 response in a primary stimulation assay of human PBMC as compared with soluble antigen. Antigen targeting to monocytes/macrophages is probably of crucial importance for this efficiency.

The efficient antigen presentation by beads may be of interest when monitoring the Th1 response in organ specific autoimmunity, since several autoantigens reveal only a low responsiveness in primary stimulatory cultures. Experiments to study if similar cellular responses are present in an organ specific autoimmune situation are in progress. Preliminary data indicate that thyroid peroxidase (TPO) conjugated to beads has enhancing properties on PBMC stimulation in Hashimoto patients (displaying autoantibodies towards the autoantigen TPO). Further investigations have also recently been started in order to evaluate the effect of matrix conjugation of autoantigens on proliferation and cytokine production in human patients with other autoimmune diseases. Preliminary investigations have also been started using the lymphocyte proliferation test in dogs with thyroid autoimmunity.

Conclusions

Monolayers of HEp-2 cells are superior to rat liver cryostate sections as substrate for ANA determination in canine sera because of their low reactivity with normal sera and the ease of discernment of fluorescence patterns in IIF ANA positive sera.

The presence of canine precipitating antibodies (ID positivity) and immunoblot reactivity appears to be strictly associated with a positive IIF ANA exhibiting a speckled staining pattern of non-mitotic cells, without a concomitant chromosomal reactivity in mitotic cells. However, there was no clear-cut correlation between different subpatterns of speckled IIF ANA and specificity as determined by ID or immunoblot.

One ID subgroup showed identity with one of the well-defined and clinically important human ANA specificities, demonstrating anti-RNP reactivity. These canine anti-RNP sera also exhibited classical human RNP patterns on immunoblot. The human major antigenic region of the most prominent RNP antigen, the U1-70K protein, was also targeted by canine anti-RNP autoantibodies as determined by ELISA technique. Thus, the response against the RNP antigen seems to be conserved between man and dog.

Tetanus toxoid (TT) conjugated to beads seems to be a more efficient stimulator of a specific CD4+ Th 1 cell response in a primary stimulation assay of human PBMC as compared with soluble TT. It is suggested that targeting of TT antigen to phagocytic antigen-presenting cells, most probably monocytes, is responsible for the enhanced stimulatory properties observed.

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Acknowledgements

The present study was carried out at the Department of Small Animal Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala and the Department of Clinical Immunology and Transfusion Medicine, University Hospital, Uppsala.

To all my friends both off and on work - I thank you all for your encouragement and all the good times and laughter shared during these years!

In particular, I wish to express my sincere gratitude to:

Alex Karlsson-Parra, my associate scientific supervisor, for enthusiastically introducing me to the fascinating world of autoantibodies, for skilful and patient guidance throughout the work with this thesis and for always being kind and encouraging.

Gunilla Trowald-Wigh, my associate scientific supervisor, for genuine friendship, full-hearted support concerning all sorts of things, for fruitful, inspiring discussions and for always being there during my ups and downs during the years.

Birgitta Essén-Gustavsson, my scientific supervisor, for constant support in scientific as well as administrative matters and for always being cheerful.

Ann-Sofie Lagerstedt and Bitten Andrén for their sincere interest in this work and for always doing their utmost to make clinical work and research plans fit together.

Johan Rönnelid for always being interested and taking time for my questions, for constructive criticism and for generously sharing his knowledge, his blood and his antibodies.

Hans Grönlund for fruitful collaboration and for generously providing knowledge and antigen preparations from Pharmacia & Upjohn Diagnostics, Uppsala.

The staff at the Department of Clinical Immunology and Transfusion Medicine at the University Hospital for kindly teaching me different laboratory methods - and advice when it didn't work - and for offering blood and many happy sessions.

My friends (staff and colleagues) at the Department of Small Animal Clinical Sciences for skilful help and happy laughter during clinical work and research projects as well as during our different "performances".

Nigel Rollison for prompt linguistic revision of this thesis.

The owners of the dogs suffering from autoimmune diseases for their cooperation and for fruitful discussions.

All my choir-friends (in MeloDisa and FriTillAria) and my musical tutors for all the merry gatherings and the many really great moments we have experienced together, offering other aspects of existence.

Inger Lilliehöök & Pan, Anette Glöde & Silas, Ingrid Boström & Tango and Eva Lundell & Korri & Raija for many dog-specific (re)activities.

Karolina Törneke for being a very special friend - both for me and for Nanja.

My family for general support and for always being there, with an extra big hug to Jonas who always happily and generously provides expert emergency car and computor help.

Nanja

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ISSN 1401-6257
ISBN 91-576-5439-5