

# Immune complexes and rheumatoid factors in canine arthritides

S D CARTER, S C BELL, A S M BARI, AND D BENNETT

*From the Arthritis Research Group, Departments of Veterinary Pathology and of Veterinary Clinical Science, University of Liverpool, Liverpool*

**SUMMARY** Thirty two domestic dogs with naturally occurring polyarthritis were investigated to determine the contribution of autoimmunity in the pathological mechanisms. Comparisons were made with canine infective arthritis (12 dogs), osteoarthritis (32), and osteoarthritis secondary to rupture of the cranial cruciate ligament (19). Rheumatoid factors, immune complexes, and complement fixation (C1q binding) were measured in sera and synovial fluids. Compared with normal dogs (32), dogs with rheumatoid arthritis (RA) had increased serum and synovial fluid immune complexes and rheumatoid factors. Increases were generally also seen in dogs with other arthropathies, however. Rheumatoid factors were higher in sera than in synovial fluids. Rheumatoid factors correlated with immune complex levels and complexed rheumatoid factor only in the group of dogs with RA. Both rheumatoid factors and immune complexes may contribute to the pathogenesis of canine RA but are considered to arise as a result of non-specific inflammatory mechanisms in the non-rheumatoid groups.

It has been suggested that a class of naturally occurring canine inflammatory joint disease is similar in many respects to human rheumatoid arthritis (RA).<sup>1-6</sup> Criteria have been defined for the diagnosis of canine RA and essentially they are as propounded by the American Rheumatism Association for use in man. Similarities between human and canine RA include the age of onset (relative to life span), the symmetrical polyarticular presentation, stiffness after rest, and the involvement of mainly peripheral joints, which show swelling and pain. The course of canine RA is progressive and may lead to severe joint destruction and deformities. Radiological findings are variable, but soft tissue swelling is always present and there is usually rarefaction and erosion of bone in one or more joints. Histopathological features include synovial membrane proliferation with villous hypertrophy and lymphocytic infiltration of the supporting layer, including aggregates of plasma cells. Gradually the articular surface becomes covered with destructive pannus.<sup>4</sup> Large numbers of polymorphonuclear neutrophils are invariably seen in the synovial fluid.<sup>3</sup>

Antiglobulins (rheumatoid factors) and immune complexes are frequently raised in the sera and synovial fluids in man and have been implicated in the pathological mechanisms of the disease.<sup>7,8</sup> There have only been limited studies in the dog; rheumatoid factors were detected in the sera of some affected dogs.<sup>9,10</sup> Immune complexes have been found in the sera in canine RA, but only six cases were tested and only three showed any increase.<sup>11</sup> To date there has been no comprehensive description of immune complexes or rheumatoid factors in dogs with RA or comparisons with other canine arthritides.

## Materials and methods

### ANIMALS

All the dogs had been referred for a second opinion to the Small Animal Hospital by veterinary general practitioners. Dogs with a wide spectrum of arthritic diseases were included in the study. Dogs with inflammatory joint disease included those with bacterial infective arthritis<sup>12</sup> and the immune based arthropathies.<sup>13</sup> The latter were classified as canine rheumatoid arthritis, having satisfied the American Rheumatism Association criteria used in man. This group included dogs with erosive and non-erosive disease.<sup>3,4</sup> Dogs with degenerative joint disease

Accepted for publication 3 March 1989.

Correspondence to Dr S D Carter, Department of Veterinary Pathology, University of Liverpool, PO Box 147, Liverpool L69 3BX.

were mostly those with secondary osteoarthritis resulting from osteochondrosis,<sup>14 15</sup> joint dysplasia and instability, or from stretching and tearing of the cranial cruciate ligament.<sup>16</sup> The last group was identified as a separate group as they formed a substantial proportion of the total cases of secondary osteoarthritis and the pathogenesis of this disease may involve immunological mechanisms.<sup>17</sup> All the other dogs with degenerative joint disease were grouped together. In addition, a normal population of dogs was sampled; these were dogs which were presented to the hospital for reasons other than locomotory disease and which were free of articular disease, as assessed clinically, radiographically, and by synovial fluid analysis. Also, beagles from a breeding colony were used as a source of serum samples and normal synovial fluids.

#### SERUM SAMPLES AND SYNOVIAL FLUIDS

Serum samples were obtained from venous blood. Synovial fluid was aspirated under aseptic conditions from a number of joints. Total and differential white cell counts were performed. Cells were removed from synovial fluid by centrifugation (1000 *g*) and the supernatant stored in aliquots at  $-23^{\circ}\text{C}$ .

#### IgM RHEUMATOID FACTOR RADIOIMMUNOASSAY

Mouse monoclonal antibody to human IgM (Oxoid, clone AF6) was used as the developing probe for canine IgM ( $\mu$  chain) by enzyme linked immunosorbent assay (ELISA) and Western blotting (Carter, Bell, Bennett, unpublished data). The IgG fraction was prepared by 40% ammonium sulphate precipitation of the ascites and labelled with  $^{125}\text{I}$  by the iodogen method.<sup>18</sup>

Canine IgG was purified from canine sera by 40% ammonium sulphate precipitation, diethylaminoethyl ion exchange chromatography, and gel filtration in ACA-34 (LKB).<sup>19</sup> Purity was shown by acetate electrophoresis and polyacrylamide gel electrophoresis<sup>20</sup> in which only  $\gamma$  chains and light chains were detected. Polyvinyl chloride microtitre plates (Linbro) were incubated with monomeric IgG at 10  $\mu\text{g}/\text{ml}$  in phosphate buffered saline (PBS) at  $37^{\circ}\text{C}$  for one hour and  $4^{\circ}\text{C}$  overnight. Unbound material was decanted and the plates incubated with 0.5% bovine serum albumin at  $37^{\circ}\text{C}$  for two hours. The plates were washed with PBS, dried, and stored in a desiccator at  $4^{\circ}\text{C}$  until use. Canine sera and synovial fluids (1/1000) and immune complex preparations (1/50) were diluted in PBS/0.5% bovine serum albumin + 0.05% Tween 20 and incubated in the wells in duplicate at  $37^{\circ}\text{C}$  for one hour and  $4^{\circ}\text{C}$  for one hour, washed with PBS/Tween, and then IgM

rheumatoid factor detected with  $^{125}\text{I}$  anti- $\mu$  at 20 ng/ml ( $37^{\circ}\text{C}$  for one hour and  $4^{\circ}\text{C}$  for one hour). The plates were washed with PBS/Tween, then dried, and the individual wells counted for  $^{125}\text{I}$  in a gamma detector (LKB). The results were expressed as the percentage uptake of  $^{125}\text{I}$  anti- $\mu$  by the wells.

#### IMMUNE COMPLEXES

Immune complexes were precipitated from sera and synovial fluids with 2.5% polyethylene glycol 6000 (BDH) at  $4^{\circ}\text{C}$  for 90 minutes.<sup>21</sup> Each precipitate was washed twice with cold 2.5% polyethylene glycol, resuspended in PBS, and the protein determined by the absorbance at 280 nm in an LKB spectrophotometer.

The immunoglobulin content of the complexes was determined by radial immunodiffusion. Rabbits were immunised with dog IgG at 1 mg/ml in Freund's adjuvant (complete and incomplete) and hyperimmune sera used in 1% agarose gels (in PBS) for radial immunodiffusion.<sup>22</sup> Five microlitre samples were added to wells and incubated at  $37^{\circ}\text{C}$  for 24 hours. Precipitin rings were measured with a vernier micrometer. Purified dog IgG was used to prepare a standard curve. Although the rabbit antisera had some reaction with light chains (by ELISA), the major reaction was to IgG and there was no precipitation with either purified dog IgA or IgM.

#### C1q BINDING ASSAY

Human C1q (Sigma) was coated onto micro-ELISA plates (Flow Ltd) at 10  $\mu\text{g}/\text{ml}$  in PBS. Serum samples and synovial fluids, diluted 1/100 in PBS/Tween (0.05%), were added to the plates. After incubation at  $37^{\circ}\text{C}$  for one hour the plates were washed with PBS/Tween and bound immunoglobulins detected with rabbit antidog IgG (1/1000 in PBS/Tween). Alkaline phosphatase conjugated goat antirabbit IgG was used for secondary antibody measurement, followed by the substrate (*p*-nitrophenyl phosphate). Colour reaction was measured at 405 nm in a Titertek ELISA reader.

Data were analysed by Student's *t* test (paired and unpaired) and linear regression analysis.

#### Results

##### IgM RHEUMATOID FACTOR IN SERA AND SYNOVIAL FLUIDS

Figure 1 shows the results of the IgM rheumatoid factor assays. In both sera and synovial fluids dogs without joint disease had low levels of rheumatoid factor compared with the groups with arthropathy. Many of the dogs with rheumatoid disease had raised serum rheumatoid factor—that is, more than

two standard deviations above the mean of the normal dogs (31/32). Synovial fluid rheumatoid factor was lower than serum rheumatoid factor in all groups, particularly in normal dogs, in which the rheumatoid factor was usually zero. The rheumatoid factor levels were generally much higher in synovial fluids in the rheumatoid-like group than in the synovial fluids from the control group (66/68  $\geq 2SD$  of the mean). Similarly, increased levels were detected in sera and synovial fluids in dogs with infective arthritis, osteoarthritis, and cruciate disease. Table 1 shows the statistical analyses. Comparisons of the data when rheumatoid factor measurements were available for synovial fluids and sera from the same dog showed lower levels of rheumatoid factor activity in the synovial fluids.

Linear regression analysis (Table 1) showed that there was no correlation between serum and synovial fluid rheumatoid factor in the control group, presumably because the levels were low in both. Significant correlations were seen, however, in dogs with infective arthritis, osteoarthritis and, particularly, in dogs with cruciate rupture or RA.

#### IMMUNE COMPLEXES IN SERA AND SYNOVIAL FLUIDS

Measurements of complexed protein did not show increased levels in the serum samples of any of the patient groups (data not shown). In a comparison

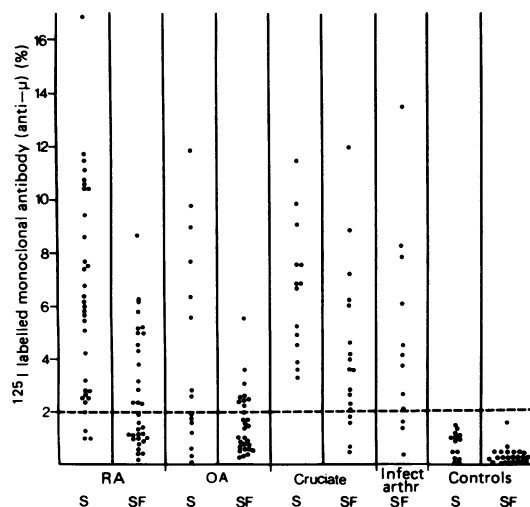


Fig. 1 IgM rheumatoid factor in sera and synovial fluids from dogs with rheumatoid arthritis (RA), osteoarthritis (OA), osteoarthritis secondary to cruciate rupture (cruciate), infective arthritis, and normal dogs (controls). Results are percentage of  $^{125}I$  monoclonal anti-IgM bound to the microplate. S=serum; SF=synovial fluid. Each point represents one dog.

Table 1 IgM rheumatoid factor in canine arthritides; statistical analyses comparing data from patient groups with those from normal dogs (Student's *t* test) and correlating sera and synovial fluid levels in same dog (linear regression analysis). Values are mean (SD)

Group	Serum RF*	Synovial fluid RF	Sera RF v SF* RF (r)
Rheumatoid arthritis	13.58 (1.17)	5.54 (0.43)	0.52
n	32	58	52
Osteoarthritis	16.23 (1.50)	7.85 (7.63)	0.43
n	28	34	30
Cruciate*	14.27 (1.78)	6.30 (0.85)	0.77
n	19	28	23
Infective arthritis	16.03 (1.81)	8.07 (1.62)	0.63
n	12	15	12
Control	3.42 (0.42)	0.43 (0.05)	NS
n	32	58	57

\*RF=rheumatoid factor; SF=synovial fluid; Cruciate=osteoarthritis secondary to cruciate rupture.

with normal dogs, however, dogs with arthritic changes had statistically increased levels of synovial fluid complexes, as measured by protein content. The increases were seen in all four groups (Table 2). Statistically, there was no difference between the levels in the different patient groups tested. Measurement of the immunoglobulin content of the synovial fluid precipitates paralleled the total protein measurement, and a correlation was found in normal dogs and all of the four patient groups ( $r \geq 0.874$ ;  $p < 0.01-0.001$ ). Complexed immunoglobulin was found in both the sera and synovial fluids in normal dogs, and increased amounts were

Table 2 Synovial fluid immune complexes in canine arthropathies; protein measurements (mean (SD)) in 2.5% polyethylene glycol (PEG) precipitates (Student's *t* test) and correlation of total IgM rheumatoid factor (RF) with precipitated complexes and with IgM RF in PEG precipitates

Group	Complexed protein ( $\mu\text{g/ml}$ )	RF v complexes (r)	RF v complexed RF (r)
Rheumatoid arthritis	362 (67)	0.724	0.984
n	26	17	13
Osteoarthritis	201 (79)	NS	NS
n	28	16	16
Cruciate*	149 (264)	NS	NS
n	29	14	18
Infective arthritis	895 (344)	NS	NS
n	8	6	6
Control	16 (3)	NS	NS
n	60	34	37

\*Cruciate=osteoarthritis secondary to cruciate rupture.

measured in most of the dogs in the patient groups (Fig. 2). The high concentrations recorded in the control dogs were often more than the total protein, as calculated by absorbance at 280 nm, but this is at best a crude estimate. The only group with a significant increase were the dogs with RA, which showed an increase in both sera and synovial fluids ( $p < 0.05-0.001$ ).

#### C1q BINDING MATERIAL

The solid phase C1q binding assay showed increased complement fixing complexes in the serum samples of some dogs in all the arthritides groups (Fig. 3). In comparison with the control group, the increases were significant in all the disease groups: rheumatoid arthritis  $p < 0.01$ , osteoarthritis  $p < 0.001$ , cruciate rupture  $p < 0.01$ , infective arthritis  $p < 0.001$ .

#### IgM RHEUMATOID FACTOR IN SYNOVIAL FLUID IMMUNE COMPLEXES

High levels of rheumatoid factor were found in the

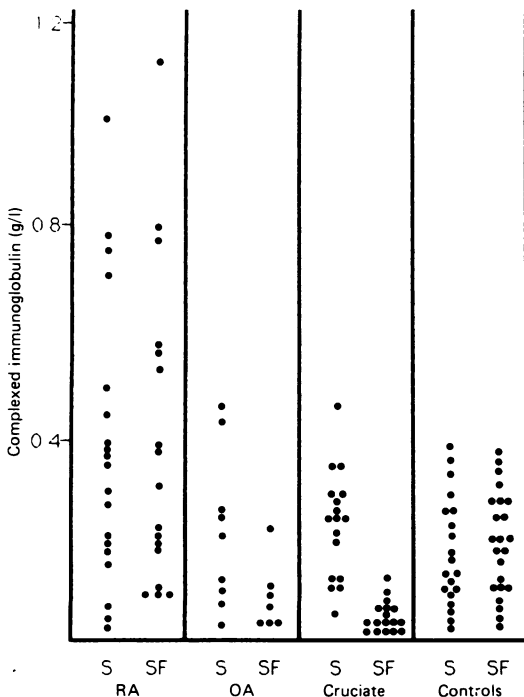


Fig. 2 Complexed immunoglobulins in polyethylene glycol precipitates from sera and synovial fluids in canine arthritides: rheumatoid arthritis (RA); osteoarthritis (OA); osteoarthritis secondary to cruciate rupture (cruciate); and normal dogs (controls). S=serum; SF=synovial fluid. Each point represents one dog.

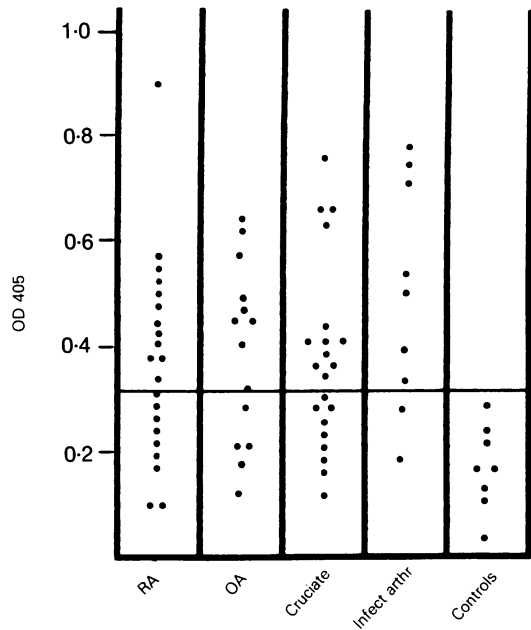


Fig. 3 C1q binding assay in dog sera. Enzyme linked immunosorbent assay (ELISA) data for each dog sample. RA=rheumatoid arthritis; OA=osteoarthritis; cruciate=osteoarthritis secondary to cruciate rupture.

synovial fluid immune complexes from some dogs with joint disease. Linear regression analysis of the data showed correlation of complexed rheumatoid factor with total synovial fluid rheumatoid factor only in those dogs with rheumatoid arthritis (Table 2); this correlation was highly significant ( $p < 0.001$ ).

#### RHEUMATOID FACTOR CORRELATION WITH AGE AND BREED

Dogs of known ages were assayed, but linear regression analysis showed no correlation of rheumatoid factor with age in the rheumatoid group (22 dogs), the osteoarthritis group (33 dogs), or the cruciate group (21 dogs). No correlation was seen when rheumatoid factor presence or levels were compared with the breed of dog; no breed was overrepresented in the positive results or in those with high levels of rheumatoid factor. In all the groups the sexes were represented equally, though the group numbers are not really large enough for adequate statistical analysis. In previous work we have not seen an imbalance of the canine arthritides in the sexes.<sup>3 4</sup>

## Discussion

This study showed that dogs suffering from various arthritides have, in common with man, a number of autoimmunological features which may be important in disease pathogenesis. The finding of rheumatoid factors in canine RA sera is not a new one, though previous studies have only used agglutination methods.<sup>9-10</sup> Synovial fluid rheumatoid factor levels have not been extensively studied; the one available report describes results from only 12 dogs.<sup>17</sup> Some workers have measured serum immune complexes previously in dogs with connective tissue disorders by using a heterologous system of binding to human C1q, but only in those with cruciate disease.<sup>11</sup> No reports have been found of analyses of immune complexes from dog synovial fluids.

Normal dogs had lower levels of rheumatoid factor than dogs with joint diseases. As in man, however, the use of a rheumatoid factor assay in the dog does not appear to be able, alone, to distinguish rheumatoid arthritis from other joint diseases; increased amounts of rheumatoid factor were also found in some dogs with osteoarthritis (including those with cruciate disease) and infective arthritis. In contrast with man, very high levels of rheumatoid factor were not found in any of the dogs with RA,<sup>23</sup> a finding previously reported in the canine disease.<sup>9-10</sup> Assessment of clinical disease activity in the dog is difficult and only generally possible by radiography.<sup>2</sup> Both erosive and non-erosive forms of canine RA had raised rheumatoid factor and immune complexes; indeed there were no apparent differences between the two groups in any of the laboratory measurements, suggesting that the same pathological mechanisms are at work in both. Probably, they are different presentations (or degree of progression) of the same disease. The radioimmunoassay used in this study is analogous to the assay previously developed for use in man; indeed it uses the same monoclonal antibody to human IgM.<sup>25</sup> The use of a Rose-Waaler assay or a latex agglutination system in dogs has been described and shown to be relatively insensitive,<sup>10</sup> although we have found that the sera with the highest titre against dog or rabbit IgG coated sheep red blood cells did have the highest results in the radioimmunoassay. The cleaner system of the radioimmunoassay removes the problems of other factors which influence sheep red cell agglutination and produces more quantifiable results.

The finding of increased rheumatoid factors in both erosive and non-erosive RA, infective arthritis, and osteoarthritis might suggest that rheumatoid factors are a non-specific effect (or mediator) of joint inflammation or that all the arthritides in the

dog are immune mediated. It was clear, however, that the levels of synovial fluid rheumatoid factors and the levels of rheumatoid factors in synovial fluid immune complexes only correlated in synovial fluids in canine RA, and this correlation was very strong. These findings indicate an association between immune complexes and rheumatoid factors in canine RA, suggesting that the complexes are, at least partially, formed by rheumatoid factor complexing with native IgG. Alternatively, rheumatoid factor may be produced as a result of stimulation by immune complexes, either articular or within extra-articular lymphoid tissue. This rheumatoid factor could then bind to produce a larger, potentially more inflammatory, immune complex. Hence in canine RA the role of rheumatoid factor in creating immune complexes is still tenable and would be in agreement with the antiglobulin/immune complex hypothesis of Zvaifler.<sup>24</sup> The finding of lower levels of rheumatoid factors in synovial fluids compared with sera, particularly in canine RA, might be explained by complexing and possibly clearance in the joint space and hence non-detection in our assay system. The ability of complexes to fix C1q is indicative of a further pathological role by activation of the complement cascade, possibly leading to inflammation. The C1q binding material found in the non-rheumatoid groups needs to be explained; it may not necessarily be bound by immunoglobulins nor result in C3 activation.

The relevance of rheumatoid factor in canine osteoarthritis is unclear but does not seem to be related to immune complex formation within the diseased joint. The sensitivity of the radioimmunoassay may explain these findings and supports the contention that immune responses to altered IgG may occur in osteoarthritis in man.<sup>25</sup> Linear regression data suggest that although both rheumatoid factors and immune complexes are present in the joints in osteoarthritis, they are unrelated events—that is, they do not coincide in the same dog. Furthermore, rheumatoid factors are generally not found within immune complexes in osteoarthritis. The immune complexes found in osteoarthritis resulting from cruciate disease were also found by Niebauer and Menzel<sup>17</sup> with a C1q binding assay. In an attempt to define an antigen inducing immune complex formation these authors looked for evidence of autoimmunity to collagen (types I and II) in osteoarthritis. No link was found, but in a later study Niebauer *et al* showed serum and synovial fluid antibodies, in cruciate disease, to collagen I and II.<sup>26</sup> We are now investigating the involvement of anticollagen antibodies and other antigen-antibody systems, which have been implicated in man, in canine RA.

This work was supported by a project grant from the Wellcome Trust.

#### References

- 1 Bennett D. Inflammatory joint disease of the dog. Faculty of Veterinary Medicine, University of Glasgow, 1980. (PhD thesis.)
- 2 Bennett D. Immune-based non-erosive inflammatory joint disease of the dog. 3. Canine idiopathic polyarthritis. *Journal of Small Animal Practise* 1987; **28**: 909–28.
- 3 Bennett D. Immune-based erosive inflammatory joint disease of the dog. Canine rheumatoid arthritis. 1. Clinical, radiological and laboratory investigations. *Journal of Small Animal Practise* 1987; **28**: 779–97.
- 4 Bennett D. Immune-based erosive inflammatory joint disease of the dog. Canine rheumatoid arthritis. 2. Pathological investigations. *Journal of Small Animal Practise* 1987; **28**: 799–819.
- 5 Newton C D, Lipowitz A J, Halliwell R E, Allen H L, Biery D N, Schumacher R. Rheumatoid arthritis in dogs. *J Am Vet Med Assoc* 1976; **168**: 221–7.
- 6 Pedersen N C, Pool R R, Castles J J, Weisner K. Non-infectious canine arthritis: rheumatoid arthritis. *J Am Vet Med Assoc* 1976; **169**: 295–303.
- 7 Elson C J, Carter S D, Cottrell B J, Scott D G I, Bacon P A, Wallington T B. Complement activating properties of complexes containing rheumatoid factor in synovial fluids and sera from patients with rheumatoid arthritis. *Clin Exp Immunol* 1985; **59**: 285–92.
- 8 Carter S D, Bourne J T, Elson C J, Hutton C W, Czudek R, Dieppe P A. Mononuclear phagocytes in rheumatoid arthritis: Fc-receptor expression by peripheral blood monocytes. *Ann Rheum Dis* 1984; **43**: 424–9.
- 9 Wood D D, Hurwitz A I, Schultz R D. A latex test for canine rheumatoid factor. *Vet Immunol Immunopathol* 1980; **1**: 103–11.
- 10 Bennett D, Kirkham D. The laboratory identification of serum rheumatoid factor in the dog. *J Comp Pathol* 1987; **97**: 541–50.
- 11 Terman D S, Moore D, Collin J, *et al.* Detection of immune complexes in sera of dogs with rheumatic and neoplastic diseases by <sup>125</sup>I-C1q binding test. *J Comp Pathol* 1976; **89**: 221–7.
- 12 Bennett D, Taylor D J. Bacterial infective arthritis in the dog. *Journal of Small Animal Practise* 1987; **28**: 207–30.
- 13 Bennett D, Kelly D F. Immune-based non-erosive inflammatory joint disease of the dog. 2. Canine polyarthritis/polymyositis complex. *Journal of Small Animal Practise* 1987; **28**: 891–908.
- 14 Bennett D. Joint disease. In: Chandler E A, Sutton J B, Thompson D J, eds. *Canine medicine and therapeutics*. 2nd ed. Oxford: Blackwell Scientific, 1984: 166–205.
- 15 Bennett D, Duff S R I, Kene R O, Lee R. Osteochondritis desiccans and fragmentation of the coronoid process in the elbow joint of the dog. *Vet Rec* 1981; **109**: 329–36.
- 16 Bennett D, Tennant B, Baughan J, May C, Lewis D G, Carter S D. Cruciate disease of the dog. A reappraisal. *Journal of Small Animal Practise* 1987; **29**: 275–97.
- 17 Niebauer G W, Menzel E J. Immunological changes in canine cruciate ligament rupture. *Res Vet Sci* 1982; **32**: 235–41.
- 18 Fraker P J, Speck J C. Protein and cell membrane iodinations with a sparingly soluble chloroamide 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochem Biophys Res Commun* 1978; **80**: 849–57.
- 19 Carter S D, Leslie R G Q, Reeves W G. Human monocyte binding of homologous monomer and complexed IgG. *Immunology* 1982; **46**: 793–800.
- 20 Laemmli U K. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 1970; **227**: 680–5.
- 21 Carter S D, Brennan F M, Grace S A, Elson C J. Clearance and organ localisation of particles and soluble complexes in mice with circulating complexes. *Immunology* 1984; **52**: 761–9.
- 22 Mancini G, Carbonara A O, Heremans J F. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry* 1965; **2**: 235–54.
- 23 Gioud-Paquet M, Auvinet M, Raffin T, *et al.* IgM rheumatoid factor (RF), IgA RF, IgE RF, and IgG RF detected by ELISA in rheumatoid arthritis. *Ann Rheum Dis* 1987; **46**: 65–71.
- 24 Zvaifler N J. The immunopathology of joint inflammation in rheumatoid arthritis. *Adv Immunol* 1973; **16**: 265–336.
- 25 Carter S D, Makh S R, Ponsford F M, Elson C J. Rheumatoid factor has increased reactivity with IgG from synovial fluids of patients with rheumatoid arthritis and osteoarthritis. *Br J Rheumatol* 1989; **28**: 233–8.
- 26 Niebauer G W, Wolf B, Bashey R I, Newton C D. Antibodies to canine collagen type I and II in dogs with spontaneous cruciate ligament rupture and osteoarthritis. *Arthritis Rheum* 1987; **30**: 319–27.