

## Lymphocyte Subsets in Stifle Joint Synovial Fluid of Dogs with Spontaneous Rupture of the Cranial Cruciate Ligament

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### Abstract

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Synovial fluid samples from 64 stifles of 54 dogs with spontaneous rupture of the cranial cruciate ligament were investigated for total and differential leukocyte counts and lymphocyte subsets using the methods of flow cytometry and anti-canine monoclonal antibodies. Synovial fluid samples from 19 healthy (without lameness, pain, macroscopic and roentgenological changes) joints of 12 dogs were used as a control. Total and differential leukocyte counts from joints after cranial cruciate ligament rupture did not differ from healthy joints. Less than  $5 \times 10^6$  leukocytes per millilitre were detected and neutrophils constituted less than 5% of all leukocytes. Percentages of lymphocytes expressing CD4 and CD8 were higher in synovial fluid from joints after cranial cruciate ligament rupture. On the other hand, percentages of lymphocytes expressing the CD45RA isoform were significantly lower in synovial fluid from joints after cranial cruciate ligament rupture. Results show some role of cell-mediated immunity in pathogenesis of spontaneous rupture of the cranial cruciate ligament.

*Flow cytometry, CD antigen, knee joint*

Rupture of the cranial cruciate ligament is the most common cause of stifle joint lameness in adult dogs. Moreover, osteoarthritis after cranial cruciate ligament rupture has been proposed to be an animal model for human disease of stifle (Brandt 1991ab).

Osteoarthritis caused by cranial cruciate ligament rupture ranks among non-inflammatory degenerative arthropathies (Bennett and May 1995) with a synovial fluid cytology not different from healthy joints (Halliwell and Gorman 1989). On the other hand, other authors demonstrated the presence of a synovitis and cellular infiltration of the synovial and subsynovial layers (Lipowitz et al. 1985; Brandt et al. 1991; Galloway and Lester 1995; Hewicker-Trautwein et al. 1999). Moreover, inflammatory changes have also been described in collateral stifles after experimental transection of cranial cruciate ligament (Gardner et al. 1984; Lipowitz et al. 1985).

Many features of autoimmunity have been reported in degenerative arthropathies. Niebauer and Menzel (1982) described significant C1q-binding immune complexes in 31 of 39 sera (79%) and 25 of 36 synovial fluid (69%) of dogs suffering from cranial cruciate ligament rupture. They described no antibodies to denatured collagen and only a low positivity to the rheumatoid factor. The same authors described the presence of antibodies to collagen type I and II in both sera and synovial fluid, with higher incidence in the latter compartment (Niebauer et al. 1987). Increased levels of rheumatoid factor, immune complexes, and antibodies to collagen in synovial fluid of dogs with cranial cruciate ligament rupture have been described also by other authors (Bari et al. 1989; Carter et al. 1989a; de Rooster et al. 2000). Moreover, structural alteration of IgG from synovial fluid of patients with rheumatoid arthritis and osteoarthritis leading to a higher binding affinity of rheumatoid factor has been reported (Carter et al. 1989b). Lawrence et al. (1998)

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described immunoglobulin deposition in synovial membrane biopsies from 32 dogs with spontaneous cranial cruciate ligament rupture and 7 control dogs. They found IgG deposition to be four-fold higher and IgM eight-fold higher in dogs with cranial cruciate ligament rupture.

Two papers dealing with immunohistochemical characterisation of lymphocyte subsets in synovial membranes of dogs with rheumatoid arthritis and degenerative joint disease have been published (May et al. 1992; Hewicker-Trautwein et al. 1999). Both of them described immunohistochemical detection of lymphocyte subsets in synovial membranes of dogs with rheumatoid arthritis and degenerative joint disease. May et al. (1992) used the cross reacting anti-murine IgM monoclonal antibody, anti-dog monoclonal antibody against Thy-1, and anti-dog polyclonal antibodies against IgG and IgA. They stained 10 biopsies from 7 dogs with osteoarthritis secondary to cranial cruciate ligament rupture and found only 2 biopsies to have very low numbers of cells positively stained for markers used in the study. Hewicker-Trautwein et al. (1999) extended the previous study by using different monoclonal antibodies. They stained synovial membrane specimens from 5 dogs with osteoarthritis secondary to the cranial cruciate ligament rupture. They found only a single or a few T-lymphocytes positive for CD5<sup>+</sup>, CD4<sup>+</sup> or CD8<sup>+</sup> to be present in subsynovium. Using monoclonal antibodies against gamma-delta T-cells, single or no cells were seen.

Flow cytometry analysis, contrary to immunohistochemistry, allows less invasive sample collection. Since this method has not been used in canine medicine, we previously used flow cytometry for characterisation of lymphocyte populations in synovial fluid from healthy joints of dogs (Faldyna et al. 2004). We found a relatively high variance of results which rules out the possibility to use this method for improvement of individual diagnosis but does not exclude the use of flow cytometry for studies based on inter-group comparison. Therefore, in the present study, we used flow cytometry for characterisation of lymphocyte populations in synovial fluid from stifles after spontaneous rupture of the cranial cruciate ligament.

## Materials and Methods

### Animals

A total 64 of synovial fluid samples from stifles of 54 dogs with spontaneous rupture (i.e. not after surgical transection) of cranial cruciate ligament were included in the study. Forty-four and ten of them suffered from unilateral and bilateral rupture, respectively. The dogs were referred to the Surgery Department of the Small Animal Clinics of the University of Veterinary and Pharmaceutical Sciences Brno. The diagnosis was based on clinical and radiological evaluation and confirmed by arthrotomy or arthroscopy. Synovial fluid samples were taken immediately before the surgery procedure.

The dogs were of 25 breeds; the most numerous were: Pitbull Terrier (6 cases), Brazilian Fila (6), Boxer (5), Labrador Retriever (5) and Golden Retriever (4). Mean age was  $4.2 \pm 2.8$  years, with range from 9 months to 12 years.

Samples of synovial fluid from 19 healthy (without lameness, pain, macroscopic and roentgenological changes) joints of 12 dogs were collected as control (Faldyna et al. 2004).

### Cytological evaluation

Total leukocyte counts were determined using the Coulter Counter® (Coulter Electronics Ltd., Harpenden Herts, England). Differential leukocyte counts were enumerated from smears stained with May-Grünwald and Giemsa-Romanowski.

### Immunostaining and flow cytometry

The synovial fluid samples for flow cytometry were processed within two hours after collection. Samples were centrifuged and the supernatants were then gathered and stored in refrigerator for further study. The remaining sediments were subsequently stained.

Indirect immunofluorescence was used for single colour flow cytometry as described elsewhere (Faldyna et al. 2001). The cells were incubated with mouse anti-dog monoclonal antibodies (Table 1) and Fc-receptor-mediated binding was blocked by adding heat-inactivated non-immune swine serum. After a 15 min incubation, the cell suspension was washed in a lysing solution (8.3 g NH<sub>4</sub>Cl, 1 g KHCO<sub>4</sub> and 1 mM EDTA per liter of distilled water). Binding of primary immunoreagents was visualized with fluorescein isothiocyanate-labelled swine anti-mouse

immunoglobulin (Sevapharma Prague, Czech Rep.; diluted 1:360). Negative control samples were labelled with the secondary antibody only. After a 20 min incubation with the secondary antibody, cells were washed in a washing buffer (PBS containing 0.1% sodium azide and 0.05 mM EDTA, all reagents from Sigma). Propidium iodide was used for staining DNA in dead and damaged cells and exclusion of these events from analysis. Samples of peripheral blood were stained using the whole-blood lysis technique (Faldyna et al. 2001).

Detection of CD45 and SWC3 antigen was used for assessment of “gate purity” as described elsewhere (Faldyna et al. 2001). The antigen CD45 is expressed on all leukocytes and SWC3 is not expressed on lymphocytes. Therefore, only cells positive for CD45 and negative for SWC3 should belong to lymphocytes.

Data were acquired on the standard FACSCalibur™ flow cytometer (Becton Dickinson, Mountain View, CA) operated by the CELLQuest software. In each sample, at least 5 000 cells were measured and the data were saved in the list mode. The WinMDI software was used for data analyses.

Table 1  
Mouse monoclonal antibodies used in the study

mAb	Specificity	Isotype	Distribution	Source
CA13.1E4	caCD4	IgG1	Helper T-cell subset	P. F. Moore <sup>1</sup>
CA9.JD3	caCD8	IgG2a	Subsets of T and NK cells	P. F. Moore
CA2.1D6	caCD21	IgG1	B- cell lineage	P. F. Moore
CA20.8H1	caTCR $\gamma\delta$	IgG2a	$\gamma\delta$ T-cells	P. F. Moore
CA12.10C12	caCD45	IgG1	All leukocytes	P. F. Moore
CA4.1D3	caCD45RA	IgG1	B cells, T cell subset	P. F. Moore
DH59B*	SWC3	IgG1	Phagocytes, progenitors	VMRD, Inc. <sup>2</sup>

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\* Monoclonal antibody DH59B raised against other species reacted with canine homologues of surface molecules; SWC3 (Swine Workshop Cluster 3), is a member of Signal Immunoregulatory Protein-like family

#### Statistical evaluation

Means and standard deviations were calculated for characterisation of synovial fluid lymphocyte compartments. Inter-compartment differences were evaluated using Mann-Whitney *U*-test for non-parametric comparisons. All calculations were performed with Prizma® (Graph Pad Software, Inc.) software.

## Results

### Total and differential leukocyte counts in synovial fluid

Total leukocyte counts in synovial fluid samples from stifles after cranial cruciate ligament rupture similarly to healthy joints did not exceed  $5 \times 10^6$  cells per millilitre in all but 3 samples in which total counts were  $5.6$ ,  $5.9$ , and  $7.1 \times 10^6$ . Mean leukocyte count was  $2.7 \pm 1.6 \times 10^6$  cells per millilitre. Neutrophils constituted less than 5% of all leukocytes as determined by light microscopy.

### Lymphocyte subsets in synovial fluid

Similarly to healthy synovial fluid, about 90% of cells in the lympho-gate were positive for the common leukocytic antigen CD45. Less than 3% of cells in this region were positive for SWC3 antigen, which is expressed on the surface of myeloid lineage.

Distribution of lymphocyte subsets in synovial fluid from stifles after spontaneous cranial cruciate ligament rupture and comparison with those from healthy synovial fluid is shown in Table 2. Synovial fluid from affected joints contained a higher proportion of lymphocytes positive for both CD4 and CD8 antigens. Since the increase of proportion of CD4+ lymphocytes was more intensive, this change led to significantly higher CD4/CD8 ratio when compared with healthy joints. Proportions of lymphocytes positive for  $\gamma\delta$ -TCR and CD21 were comparable. Contrary to healthy synovial fluid, we found a significantly lower percentage of lymphocytes expressing CD45RA isoform.

Table 2  
Lymphocyte subsets in synovial fluid from normal joints and from joints with osteoarthritis secondary to cranial cruciate ligament rupture (CCL)

Parameter	Normal (n = 19)	CCL (n = 64)	Significance
CD45RA+ %	69.4 ± 16.7	39.5 ± 11.5	<i>P</i> < 0.01
CD4+ %	13.6 ± 9.5	31.6 ± 12.0	<i>P</i> < 0.01
CD8+ %	16.5 ± 7.5	21.4 ± 9.5	<i>P</i> < 0.05
CD4/CD8 ratio	0.8 ± 0.6	1.9 ± 1.5	<i>P</i> < 0.01
γδ -TCR+ %	1.9 ± 2.1	2.7 ± 2.4	n.s.
CD21+ %	2.2 ± 3.1	4.0 ± 3.5	n.s.

### Discussion

In the present study, we characterized lymphocyte subsets in synovial fluid from dog stifles after spontaneous cranial cruciate ligament rupture. Breed and age distribution is in agreement with the fact that cranial cruciate ligament rupture preferentially affected middle-aged dogs of larger breeds (Nečas et al. 2000; Whitehair et al. 1993). Also the fact that 10 of 54 dogs had bilateral rupture is consistent with previous studies (Bennett et al. 1988; Duval et al. 1999; Pond and Campbell 1972).

Synovial fluid cytology of joints after cranial cruciate ligament rupture did not differ markedly from healthy joints. Neutrophils in a degenerative or traumatic joint disease do not generally exceed 10% of the total cell counts (Griffin and Vasseur 1992; Halliwell and Gorman 1989; Werner 1979; Lewis et al. 1987).

Based on the increase of percentage of CD4-positive lymphocytes associated with the increase of CD4/CD8 ratio, this cell type seems to play the key role in immune reaction in this type of joint disease. The CD4-positive lymphocytes, known as helpers, produce cytokines that can control the mode of immune reaction. Cytokines are also involved in pathogenesis of osteoarthritis (Fernandes et al. 2002) and chondrocytes from osteoarthritis cartilage, but not from healthy one, produce many of these cytokines (Moos et al. 1999). Cytokines can be divided by the effect on cartilage development into two groups - with catabolic and anabolic effects, the latter includes also interferon-gamma. Now, there is no doubt that rheumatoid arthritis and osteoarthritis are diseases associated with elevated level of interferon-gamma in synovial fluid (Al-Janadi et al. 1993; Keystone et al. 1991). Description of changes in presence of different lymphocyte subsets in a canine model of joint diseases should be followed by studies of cytokine production. Studies of this type are limited by the lack of tools for cytokine detection and quantification. Recently, cross-reactivity of monoclonal antibodies against interleukin-4 and interferon-gamma, originally raised against bovine cytokines, has been found and described (Pedersen et al. 2002).

Contrary to healthy synovial fluid, we found a significantly lower percentage of lymphocytes expressing the CD45RA isoform. Since the CD45RA expression is restricted to B-lymphocytes and to virgin T-lymphocytes, we concluded that increased numbers of lymphocytes in synovial fluid from stifles after spontaneous cranial cruciate ligament rupture were primed by antigen. It is not known which antigen is involved in that lymphocyte activation, but it is another proof of participation of the cellular part of the immune system in pathogenesis of osteoarthritis after spontaneous cranial cruciate ligament rupture.

Changes in lymphocyte subsets described in our work, are in agreement with other authors (May et al. 1992; Hewicker-Trautwein et al. 1999) and indicate that not only humoral but also cellular parts of immune system are involved in the pathogenesis of canine osteoarthritis after cranial cruciate ligament rupture.

### Subpopulace lymfocytů v kolenní synoviální tekutině psů se spontánní rupturou předního zkříženého vazů

Celkový a diferenciální počet leukocytů a subpopulace lymfocytů byly detekovány ve vzorcích synoviální tekutiny z 64 kolenních kloubů z 54 psů se spontánní rupturou předního zkříženého vazů. Synoviální tekutina z 19 normálních (bez kulhání, bolesti, makroskopických a rentgenologických změn) byly použity jako kontrola. Celkový a diferenciální počet leukocytů v synoviální tekutině z postižených kloubů nelišil od kloubů normálních. V mililitru bylo méně než  $5 \times 10^6$  leukocytů a neutrofilů představovaly méně než 5% všech leukocytů. V postižených kloubech bylo větší procentuální zastoupení lymfocytů charakterizovaných přítomností antigenů CD4 a CD8 než v kloubech normálních. Naopak, přítomnost CD45RA pozitivních lymfocytů bylo signifikantně nižší v kloubech postižených. Tyto výsledky svědčí o podílu buněčné složky imunitního systému v patogenezi spontánní ruptury předního zkříženého vazů.

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