IMMUNOPHENOTYPING OF BLOOD AND CEREBROSPINAL FLUID LEUKOCYTES IN DOGS

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by
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IMMUNOPHENOTYPING OF CEREBROSPINAL FLUID LEUKOCYTES IN DOGS

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Inflammatory neurological diseases in dogs are common, but establishing a definitive diagnosis is rarely possible. Characterization of the immune response in cerebrospinal fluid (CSF) may yield greater insight into pathogenetic mechanisms of neuroinflammation than routine diagnostic assays. Therefore, in this study, leukocyte populations of paired blood and (CSF) samples from 23 healthy dogs were characterized by flow cytometry. The results indicate that leukocyte populations in blood differ from leukocytes populations in CSF. The mean proportion of CD4+ and CD21+ cells was significantly higher in blood than in the CSF (p= 0.002 and 0.000 respectively). In contrast, the mean proportion of CD14+ and CD8+ cells were not significantly different between blood and CSF (p= 0.494, and 0.891 respectively). The greater mean proportion of CD21+ cells found in blood than in the CSF may be the consequence of a blood brain barrier (BBB) selectivity that favors movement of T cells over B cells. Immunophenotyping is a feasible and objective technique that provides additional information for CSF analysis.
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This thesis is dedicated to my parents whose sacrifices over the years have made this opportunity possible.
DECLARATION OF WORK PERFORMED

I declare that with the exception of the items below, all work reported in this thesis was performed by Maria Carolina Duque.

Complete blood counts, cerebrospinal fluid cell counts, and protein concentrations were performed by the Animal Health Laboratory at the Ontario Veterinary College, University of Guelph. The cerebrospinal fluid cytologic interpretations were performed by Dr. Dorothee Bienzle.
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Neurological diseases affecting dogs vary in severity, complexity and presentation. Clinical signs are often non-specific and it is frequently not possible to achieve a definitive diagnosis, and hence to optimally treat the dog.\(^{(1,2)}\)

Cerebrospinal fluid (CSF) analysis is performed on a regular basis as a diagnostic tool for inflammatory conditions of the central nervous system in veterinary medicine. However, the routine analysis of the fluid rarely provides conclusive information about specific etiologies for the clinical disease.

Immunophenotyping of CSF cells may reveal important information about the central nervous system (CNS) immune response. The technique provides accuracy and sensitivity when compared to manual methods used for counting and classifying cells. Immunophenotyping can be done in a relatively short period of time.

The CSF has been considered a difficult sample to immunophenotype because of its low cellularity.\(^{(3)}\) However, with some modifications in the technical approach it has been possible to analyze human and some veterinary samples.\(^{(4-7)}\) In this study, CSF and blood samples from 23 healthy dogs were collected and analyzed by immunophenotyping. The antibodies used were directed against the canine CD4, CD8, and CD21 molecules, and against the murine CD14 molecule, which is conserved across species.

The objective of the present study was to establish a reference range for CSF leukocyte populations in healthy dogs. Once such ranges are defined, it may be possible to evaluate
changes in inflammatory CSF and define the clinical importance of specific abnormalities.
1.1 HYPOTHESIS

The leukocyte subpopulations in CSF differ from the leukocyte subpopulations in blood from clinically normal dogs.

1.2 OBJECTIVES

❖ Establish a reference range for CD4+, CD8+, CD21+, and CD14+ cells in CSF from normal dogs.

❖ Compare the lymphocyte subpopulations present in the CSF and blood of normal dogs.
1.3 LITERATURE REVIEW

1.3.1 INFLAMMATORY DISEASES OF THE CENTRAL NERVOUS SYSTEM

Over the years, a large number of studies describing the clinical and pathological features of CNS diseases have been published. In a retrospective study of 220 dogs with inflammatory diseases, it was found that the signalment, history and neurological examination contributed only marginally to a specific diagnosis. For one third of the dogs in the study a definitive diagnosis could not be established. Particular neurological abnormalities were frequent but not limited to specific disorders. As an example, myoclonus was consistently present in dogs with distemper, but was also found in other meningoencephalomyelitides.

A wide variety of etiologies have been recognized in canine CNS inflammatory disorders. These include infectious pathogens such as canine distemper virus, *Ehrlichia canis*, *Neospora caninum*, rabies, tick borne viruses, and some bacterial and mycotic agents. However, a variety of meningoencephalitides such as eosinophilic meningoencephalitis, steroid-responsive meningitis/arteritis, granulomatous meningoencephalitis (GME) and other breed-related inflammatory conditions (necrotizing encephalitis in the Yorkshire terrier, necrotizing meningoencephalitis in Pug dogs) remain of unknown etiology.
The clinical signs present in neurological inflammatory diseases are diverse and non-specific and usually the condition progresses rapidly if an effective treatment is not promptly provided. Due to the close anatomical association of the meninges, brain and spinal cord, often more than one compartment is involved in the inflammatory process.\(^{(2)}\) In fact, the presence of multifocal neurological lesions is considered a hallmark of CNS inflammatory disorders.\(^{(1,8,9)}\) However, in the majority of cases at the time of neurological examination, deficits are usually indicative of a single, focal lesion. Only at post-mortem examination is the diffuse nature of the condition appreciated.

If the clinical picture of a neurological disease is suggestive of an inflammatory etiology, diagnostic tests are commonly performed. Of these, the CSF analysis is the most important allowing differentiation between inflammatory and other disease categories. However, it rarely provides a definitive diagnosis. Serology on blood samples, advanced imaging modalities, and detection of nucleic acid from specific organisms may be useful adjuncts. But, despite these diagnostic efforts to characterize causal agents, the majority of these inflammatory diseases remain undiagnosed.\(^{(1)}\) Considering that the CSF may provide valuable information related to the immune response occurring in the CNS, evaluation of the immunophenotype of cells in the fluid has recently been advocated.\(^{(4)}\) Specific types of inflammatory cells may be suggestive of specific etiological agents. In addition, changes occurring during treatment may add insight on the disease process.

The two major components of the immune response are the humoral immune system and the cell-mediated system. Humoral immunity consists of B cells and their products.
(antibodies), and cell-mediated immunity of T cells and their products (cytokines). In immunophenotyping, specific cluster of differentiation (CD) molecules found on the cell surface of leukocytes are used to identify specific populations of cells. B cells express immunoglobulins on their surface, and T cells express a T cell receptor in addition to either CD4 or CD8 molecules. Specifically designed antibodies, which recognize the CD4, CD8 and CD21 antigens, allow the quantification of each different lymphocyte population present in a specimen. A study comparing the lymphocyte subset distribution in different canine encephalitides by immunohistochemistry suggested that in viral infections, T cells were the predominant cell population in the perivascular space of the brain parenchyma. In contrast, B cells prevailed when inflammatory diseases were caused by bacterial or protozoal agents. This investigation was conducted using paraffin-embedded brain tissue. If similar changes were identified in inflammatory CSF samples, inflammatory diseases currently designated “idiopathic” may be characterized and better understood. Toward this goal, reference parameters for leukocyte subpopulations in CSF samples from healthy dogs are required.

1.3.2 IMMUNE SYSTEM AND FUNCTIONS

Protection of an organism results from the combination of multiple defence mechanisms that aim at curtailing or eliminating different types of deleterious microorganisms. Some microorganisms, such as bacteria, certain protozoa, and metazoa, reside predominantly in the extracellular space. Other types of microorganisms preferentially persist inside cells.
These include viruses, intracellular bacteria and certain protozoa. Defence against extracellular organisms is mainly provided by antibodies produced by the humoral immune system. In contrast, antigens from intracellular organisms are predominantly recognized by cytotoxic cells comprising cell-mediated immunity.

According to cytokine secretion patterns, two subtypes of T helper (Th) cells are defined. Th1 cells secrete predominantly interleukin (IL) 2 and γ interferon. Th1 cells can promote B cell proliferation and immunoglobulin secretion but do not stimulate specific antibody production. Th1 cells primarily enhance cell-mediated immune responses promoting resistance to intracellular organisms. In contrast, the Th2 cell subtype stimulates B cell proliferation and immunoglobulin secretion but has little effect on delayed hypersensitivity or other cell-mediated reactions. Th2 cells secrete predominantly IL 4, 5 and 6 and thus promote resistance to extracellular organisms. Interestingly, there is reciprocal suppression of cytokine production, which may account for the magnitude and intensity of cellular versus humoral immune responses elicited by a specific extracellular or intracellular antigen.

Numerous signals transmitted among cells are involved in the activation and modulation of the immune system. The initiation of humoral and cell-mediated responses is possible after the appropriate cell-to-cell interaction occurs. Through study of cell-associated molecular markers the complexity of cell surface interactions in the immune system has become apparent. Since their introduction, monoclonal antibodies have become an invaluable resource for many purposes, including characterization of specific cell
structures. By studying different cell surface molecules, it has been possible to identify and characterize functional properties of various cell types. In this study, the primary goal was to identify specific cell populations present in health through monoclonal antibody labelling to provide a background for future investigations of the canine immune response in neurological diseases.

Phagocytosed organisms are usually presented to the immune system by major histocompatibility complex (MHC) class II molecules while antigens present in the cytoplasm (as in viral infections) are presented by MHC class I molecules. In turn, foreign peptides, in conjunction with MHC, are recognized by the T cell receptor (TCR) that cooperates with accessory molecules to generate intracellular signals leading to cell proliferation, generation of cytokines, or functional development. The TCR is a surface molecule used by T cells to recognize and respond to foreign antigens in association with products of the MHC. The TCR is associated with an invariant membrane complex termed CD3. Some of the CD3 proteins have large intracytoplasmic portions which facilitate the transduction of signals to the cell interior after the TCR interacts with the antigen-MHC complex. In addition, two mutually exclusive subpopulations of T cells are recognized by expression of the CD4 or CD8 surface glycoprotein. CD4 and CD8 molecules contribute to the generation of signals through the TCR following antigen recognition. Previous studies reported that T cells expressing CD4 acted exclusively as helper or inducer cells. In contrast, T cells expressing CD8 were believed to have solely cytotoxic or suppressor activity. Exceptions to this classification have been noted, and currently the best functional correlation is with the
class of MHC recognized by the T cell.\textsuperscript{(14)} With few exceptions, CD4 cells recognize class II MHC proteins, while CD8 cells recognize class I MHC proteins. In general, the CD4 molecule stabilizes the interaction of the TCR with MHC II plus peptide on antigen-presenting cells.\textsuperscript{(14-16)} The CD4\textsuperscript{+} cell subpopulation also produces cytokines that help T-cell proliferation and that are required for antibody responses. The CD8 molecule acts as the coreceptor for MHC I in a similar fashion as CD4.\textsuperscript{(17)}

Monoclonal antibodies can be produced which bind to CD4, CD8, TCR, MHC, and CD3 surface molecules. According to expression of these surface molecules, the leukocytes can be divided in different subpopulations. Among the specific monoclonal antibodies available against dog T cells are those against the CD3, CD4, TCR and CD8 molecules.

B cells can be identified with antibodies to CD21 and surface immunoglobulins depending on their stage of maturation. B cells at early stages of ontogeny are referred to as B1 cells. Most B1 cells express CD5. They are the source of IgM antibodies that recognize different antigens. Mature B2 cells lack CD5 molecules and develop later during ontogeny. B2 cells express IgM and IgD on their cell surface before they encounter antigens. Complexes of antibodies with newly encountered antigens initiate the formation of germinal centers within secondary lymphoid organs. The germinal centers provide the appropriate environment for B2 cells to undergo immunoglobulin class switching and production of IgG, IgA or IgE. The final stages of differentiation of B2 cells into antibody-secreting plasma cells occur within the secondary lymphoid tissue.
but outside the germinal centers. CD21 is expressed on mature B cells and follicular dendritic cells of the germinal center.

1.3.3 THE BLOOD-BRAIN-BARRIER AND THE IMMUNE RESPONSE IN THE CENTRAL NERVOUS SYSTEM

The idea of a blood-brain-barrier (BBB) was initially proposed in Germany at the beginning of the 20th century, when Kraus and Lewandowsky proved that certain substances lacked central nervous activity when administered into the bloodstream, but produced dramatic responses after intracerebral and intrathecal injection. Then Goldman in 1909, after injecting trypan blue into laboratory animals, noticed that the brain and the spinal cord were remarkably white despite blue staining of most other tissues and organs. The biological purpose of the BBB is thought to be controlling the stability of the interstitial fluid to which central neurons and their synapses are exposed. Thus, neurotransmitters or neuromodulators secreted into the interstitium will not be rapidly lost to the circulation. Tight capillary junctions of endothelial cells characterize the BBB ultrastructurally. The result of this arrangement is a tight permeability barrier between blood and brain to polar molecules and ions. Passage across the BBB occurs predominantly by lipophilic transfer or carrier-mediated processes. An epithelial cell layer of choroid plexus forms another barrier between the blood compartment and the CSF known as the blood–CSF barrier. Despite the presence of the BBB and the blood-CSF barrier, the normal CNS is not isolated from the immune
processes of the body. CNS-injected antigens stimulate both humoral and cell-mediated immunity. In fact, CNS immunization can be more effective than extracerebral routes in eliciting a systemic immune response.\textsuperscript{(20)}

The highly regulated communication between the brain and the immune system is frequently compromised in CNS disorders. Due to the limited expression of MHC molecules on neurons, and the presence of few antigen-specific lymphocytes in the brain, the immune response against brain antigens cannot occur completely independently in the brain. The absence of an appropriate lymphoid drainage system and the lack of antigen-presenting dendritic cells support the concept that immune reactions in the CNS are remarkably different than those at other anatomical sites.\textsuperscript{(12)}

Flow cytometric analysis of lymphocytes is a useful tool in assessing immune responses in many sites, and may be valuable for refining the diagnostic and therapeutic approach to canine patients with inflammatory CNS diseases. Lymphocytes develop from pluripotent stem cells present in the fetal liver and bone marrow. While in mammals B cells are presumed to reach maturity within the bone marrow, T cells must travel to the thymus to complete their development. After lymphocytes reach maturity in the primary lymphoid organs (thymus and bone marrow), they migrate to secondary lymphoid organs (Peyer's patches, spleen and lymph nodes). The secondary lymphoid organs facilitate antigen trapping and provide an appropriate environment for processed antigens to be presented to antigen-sensitive cells.\textsuperscript{(11,21)} Lymphocytes have the unique ability to migrate from blood into tissue and back to blood by passing through lymph nodes and eventually
entering the thoracic duct.\(^{(22)}\) This process is known as re-circulation and is essential in immuno-surveillance of the body and the dissemination of immunological memory.\(^{(23)}\)

Many conditions other than disease, such as stress, treatment with drugs, physical activity and aging, can influence the number of blood lymphocytes.\(^{(21)}\) Concurrent determination of lymphocyte subsets in blood and CSF may provide greater utility for understanding CNS inflammatory disorders, than when each compartment is evaluated independently.

In general, the immune response involves the recognition of antigens and the generation of antibodies and effector T cells attempting to eliminate those antigens. In cases of neurological disease, specific immunogenic material from the CNS must reach the spleen or lymph nodes in order to activate a systemic immune response.\(^{(20)}\) Cerebral interstitial fluid drains with cerebrospinal fluid from the brain to blood, across the arachnoid villi, or to lymph, along certain cranial nerves (primarily olfactory) and spinal nerve root ganglia. This connection - from the brain to the blood and regional lymphatics - provides possibility for the continuous flow of immune cells and antigens from the central nervous system to extracerebral immune tissues.\(^{(20)}\) The mechanisms responsible for migration of immune cells into the CNS are not completely understood. Apparently, cerebrovascular endothelial cells (EC), part of the BBB, are involved in the migration and interaction of lymphocytes.\(^{(24)}\) Similarly to the blood-CSF barrier for proteins, lymphocyte selection appears to take place at the endothelial BBB of the brain microvasculature and, presumptively, at the epithelial blood-CSF barrier of the choroid plexus.\(^{(5)}\) The process of homing and transmigration of lymphocytes is dictated by a variety of adhesion molecules including selectins, integrins and molecules of the immunoglobulin family reciprocally
expressed on endothelial cells and leukocytes. These adhesion molecules play an essential role in mediating cell to cell contact between the vascular endothelium and blood leukocytes.

In previous studies it was reported that T cells have to be activated in order to cross the BBB. In contrast, more recent studies have shown that under normal conditions lymphocytes do not need to be activated to enter the CSF but appear to be part of the recirculating pool of lymphocytes. Naive B cells are rarely encountered in the CSF mainly because of their restricted passage from the peripheral blood. The presence of activated IgG secreting cells in the CSF could be due to the normal transfer of activated but pre-secretory B cells through the BBB, or due to the presence in the CNS of factors which promote B cell activation, differentiation and secretion.

Immunoglobulin-secreting cells play an important role in CNS inflammatory reactions. Although these cells are useful markers of inflammation, they provide no information as to the exact nature of the infectious agent, unless specificity of their antibodies can be defined. Within the last few years determination of titers against specific infectious agents (Neospora caninum, Ehrlichia canis, distemper virus, tick borne encephalitis virus and Toxoplasma gondii) has become possible. However, the validity of such antibody titers in CSF is controversial. Even though a B cell may secrete about 2000 antibody molecules per second, there still may be insufficient numbers of such cells during certain stages of a disease to produce a detectable antibody titer. Moreover, Ig levels in CSF
are influenced by many factors such as integrity of the BBB, catabolism of Ig’s, and local protein production, that at times make titers difficult to interpret.\textsuperscript{(29)}

In summary, the etiology and mechanism of many neurological disorders affecting companion animals remain undiagnosed. Greater definition of the immune response associated with characteristic leukocyte responses, as well as attempts to directly identify specific etiologic agents, may be helpful.

1.3.4 FLOW CYTOMETRY

1.3.4.1 HISTORY

After several years of contributions from many disciplines, the flow cytometer, a sophisticated analytic tool, evolved. Flow cytometry allows quantification and characterization of cells in a fluid medium. The first attempt to count cells flowing through a capillary tube was made by Moldavan in 1934.\textsuperscript{(30)} Then, Crosland Taylor designed a chamber for optical counting of red blood cells. Considering that frequent obstructions of the channels by large cells was an important limitation of his method, Taylor decided to inject cell suspensions into a faster flowing stream of fluid.\textsuperscript{(30)} This became the basis of the hydrodynamic focusing principle. Twenty years later, Van Dilla Fulwyler developed the first fluorescence detection cytometer that used an argon ion laser for excitation and a 90 degrees optical configuration. In 1975, the production of monoclonal antibodies was described which nicely complemented flow cytometric
analysis. Now that it was possible to analyze markers expressed on specific cells by fluorescence measurements, efforts were directed toward improving and refining flow cytometry instruments. Commercial machines began to appear on the market by the mid 1970’s.\(^{31}\) As science generally, and computer systems specifically, advanced, sophisticated flow cytometers became more widely available. Researchers and clinicians are now increasingly aware of the multiple applications this laser-based technology provides.

In flow cytometry, multiple properties of individual particles flowing in a stream of fluid are simultaneously assessed. Cells from a variety of sources such as solid tissues, cerebrospinal fluid, urine, whole blood and bone marrow can be analyzed in suspension. The characteristics determined include cell size, cytoplasmic complexity, nucleic acid content and a wide range of membrane-bound and intracellular proteins.\(^{32}\) The underlying principle of flow cytometry is that light is scattered and fluorescence is emitted as each cell is exposed to an excitation source with a specific wavelength that usually is a laser beam. The immunological markers that bind to molecules on the cell surface allow cell identification. The cell surface molecules act as antigens reacting with specific fluorochrome-tagged antibodies.\(^{33}\)

**1.3.4.2 THE FOUR COMPONENTS OF A FLOW CYTOMETER**

A combination of physical, electronic, chemical and optical principles are applied in flow cytometry technology. The unique advantage of flow cytometers relative to other
detection instruments is that they provide a collection of individual measurements from a large number of cells in seconds. This is an important feature if the time required to analyze hundreds of cells with a microscope is considered. In flow cytometry, each cell is denominated as an event, and usually sample evaluation includes at least 10,000 events of interest.

Specimens to be analyzed are prepared as single-cell suspensions in phosphate-buffered saline (PBS). The PBS is used as a sheath fluid to exert pressure against the suspended particles in order to align them in a single-file fashion. This process is called hydrodynamic focusing and allows each cell to be analyzed individually while traveling within the sheath fluid stream. After the fluidic system prepares the sample to flow adequately in a laminar fashion, the illumination system provides the laser excitation source. The illumination system focuses the laser emission to ensure that the particle will receive the appropriate stimulus despite any minor positional variation within the sample stream. When the laser beam strikes the individual cell, light is scattered and fluorescence is emitted.\textsuperscript{31} Fluorescence occurs when a molecule excited by light of one wavelength returns to the unexcited (ground) state by emitting light of a longer wavelength.\textsuperscript{34}

Fluorescence is detected using mirrors, optics and beam splitters that direct the light toward highly specific optical filters. The filtered light is then measured by a special type of detector, the photomultiplier tube (PMT).\textsuperscript{31} In current cytometers usually three channels are available allowing the examination of three different types of fluorescent
emission. Each channel is designed to detect a narrow range of wavelengths. Fluorescence obtained from the green fluorochrome fluorescein isothiocyanate (FITC) is typically detected in a band of wavelength that is designated as the FL-1 parameter. FITC is the most commonly used fluorochrome. R-phycoerythrin (PE) generates orange-red light detected in a wavelength range designated as the FL-2 parameter. Red fluorescence is detected as a third FL-3 parameter.(31)

In addition to the fluorescence emission, two other important parameters are recorded when cells are analyzed by flow cytometry. The forward angle light scatter (FSC), which reveals physical properties of the cells such as size, and the right angle side light scatter (SSC) that represents the internal complexity of the cell. All five parameters (FSC, SSC and the three fluorescent emissions) are collected quantified and converted to electrical signals by the optical and electronic systems. The generated signals are stored by a computer system as a list-mode file. A list-mode file contains unprocessed data of all the measured parameters for each cell. When the data is acquired in this way, retrospective analysis of all gathered information can be easily conducted, according to the specific experimental objectives. Finally, specific software is used to analyze data collected. The software provides basic statistical information of the cell populations studied and a visual display (histograms, dot plots or contour plots) of the final results.
1.3.4.3 PRACTICAL PROBLEMS AND IMPORTANT CONSIDERATIONS IN FLOW CYTOMETRY

When acquiring flow cytometry data, all sources of error must be identified and controlled to obtain meaningful information. Recent technological advances have resulted in more user-friendly flow cytometers. However, obtaining accurate and precise results necessitates attention during sample preparation and careful monitoring of the performance of the flow cytometer. At the time of sample preparation, different dilutions of antibodies must be tested until the ideal antibody concentration is defined. Increasing the amount of antibody can result in non-specific reactions that interfere with specific staining. Non-specific binding of the Fc region of antibody to Fc receptors may further result in increased background staining. The presence of surface immunoglobulins is used to identify B cells in some immunophenotyping assays. However, in many previous studies it was not recognized that B cells, some T cells, monocytes, neutrophils, basophils and mast cells have surface receptors with binding affinities for the Fc portion of Ig. For a long time, this problem resulted in an overestimation of B-lymphocyte numbers. Recently, the use of F(ab)’2 fragments instead of the intact immunoglobulin was implemented to eliminate non-specific Fc receptor binding by non-B cells. \(^{(33)}\) Alternatively, the Fc region can be blocked by pre-incubating cells with serum containing Ig to reduce nonspecific binding.
A set of standard operating conditions must be applied each time a sample is evaluated by flow cytometry. Controversy exists regarding the best negative controls for immunophenotyping. At least, appropriate unstained and isotypic antibody controls must be analyzed concurrently with each sample. All cells have some autofluorescence as a result of their type, size and normal cellular components such as flavins and cytochromes. By analyzing a control of unstained cells, the researcher can recognize how autofluorescence influences the results. Resting lymphocytes have relatively little autofluorescence, activated lymphocytes typically have more, and granulocytes and monocytes have the most.\(^{35}\)

Every time that a monoclonal antibody is conjugated to a fluorochrome a new complex is created which can change the charge, hydrophobicity and aggregation state of the antibody.\(^{35}\) Therefore, it is important to analyze the effects of the fluorochrome and the antibody even on parameters such as forward and side scatter. Flow cytometry only measures relative fluorescence intensity and does not distinguish between specific and non-specific sources. Increasing the amount of dye on or in a cell may not result in a proportional increase in the specific fluorescence signal. The final fluorescence emission depends on the length of the incubation time, the concentration of antibody added, and on the number of cells being stained.\(^{35}\) The most critical factor is the concentration of the antibody. Usually there is a significant excess of antibody over antigen so the number of cells becomes a less relevant factor.\(^{36}\)
Intersample variation is another factor that has to be addressed. It is recommended that duplicate samples be tested whenever possible. This is frequently difficult to accomplish in samples of limited supply such as with the CSF. In these cases, all technical precautions must be taken before the sample is analyzed. Ideally, a stained cytospin preparation of the sample processed by flow cytometry should be analyzed in parallel with light microscopy to ensure concordance of observations.\(^{(33)}\)

Other factors important for ensuring accurate cytometric analysis include careful preparation of the sample and appropriate mixing of the cell suspension before the sample is introduced in the flow cytometer. Assay calibration is an important component of internal quality control and constitutes the evaluation (and adjustment) of the test system to provide a known relationship between the measured response and the value of the substance being measured by the test procedure.\(^{(36)}\) Assay calibration makes use of reference material with a known value or characteristic. It is of critical importance for the reference material to be stable over time (at least several weeks, preferably months) and to have interassay properties comparable with those of the samples analyzed.

Since CSF specimens have significant cell deterioration and altered immunofluorescent properties, stabilization or immediate analysis is necessary. Reference laboratories are available to verify research lymphocyte immunophenotyping.\(^{(37)}\)

Careful attention to details prior to analysis is paramount with such sensitive techniques as flow cytometry. The computer and the software also play an essential role. These are
not only used to analyze data, but also to calibrate all parameters for optimal data acquisition. Errors in setting the compensation values can significantly affect results and yield erroneous conclusions. It is important to recognize the spectral properties of the fluorochromes used in a particular experiment. In general it is recommended to use the brighter fluorochrome on the less abundant antigen. When determining the appropriate compensation for positive samples, it is prudent to re-evaluate fluorescence of unstained cells in order to identify staining problems from a particular subset of cells.\(^{(35)}\)

The hypocellularity characteristic of CSF is a limiting factor for analysis by flow cytometry. Many investigators have nevertheless been able to use the technique successfully under carefully controlled conditions.\(^{(5,38-41)}\) Cell loss during centrifugation is a common problem with CSF samples. Up to 90% of CSF cells do not survive more than 120 minutes after the sample is collected.\(^{(3)}\) Since monocytes and granulocytes decay more rapidly than lymphocytes, skewing of relative subpopulations may occur. In one experiment, a mean cell reduction of 16.6% was found with each centrifugation step.\(^{(3)}\) Therefore, the use of antibodies directly conjugated to fluorochromes is preferred to decrease the number of centrifugation steps required for cell staining. Even when only one centrifugation step is performed, one investigator reported cell losses for lymphocytes between 29% and 33%, and loss of monocytes between 60% and 80%.\(^{(5)}\)
1.3.4.4 GENERAL APPLICATIONS OF FLOW CYTOMETRY

Different biochemical, morphologic and metabolic states can be defined by studying the scatter and emission properties of cells. By correlating FSC and SSC light signals, it is generally possible to differentiate viable, necrotic and dead cells. Useful information is obtained and specific cell populations are classified based on flow cytometry results.

Due to the increasing availability of monoclonal antibodies, clinical applications of flow cytometry are increasing steadily in veterinary and human medicine. Although different lymphocytes are very similar in their morphological appearance, important functional differences exist. With flow cytometry, these can be correlated with expression of specific markers. This process is known as immunophenotyping and has become a powerful technique for identifying and monitoring different diseases. As an example, this method is used to evaluate the status of HIV-infected patients.\(^{(32,37,42)}\) Progressive HIV disease is characterized by the loss of CD4\(^+\) cells and the absolute CD4\(^+\) cell count is a good indicator for the stage of HIV disease.\(^{(32)}\)

Other applications of immunophenotyping include assessment of the structural and functional properties of erythrocytes, leukocytes and platelets in different immunological and neoplastic disorders.\(^{(43-46)}\)

As in human medicine, the use of flow cytometry in veterinary medicine has increased substantially over the past years. In the last five years cells from numerous specimens
including sperm, skin biopsies, bone marrow and peripheral blood have been analyzed by immunophenotyping.\(^{(47-53)}\) The results support that flow cytometry can be a useful diagnostic adjunct in veterinary medicine. A better understanding of the immunological processes associated with specific diseases may lead to an improvement in the diagnosis and therapy of diseases in animals.

### 1.3.4.5 FLOW CYTOMETRY AND NEUROLOGICAL DISEASES

Although CSF has been considered difficult to analyze by flow cytometry because of its low cell count, knowledge of the immune characteristics of CSF cellular constituents would be a very useful tool in neurology. According to Tipold, a high degree of individual variation was present in CSF lymphocyte immunophenotyping of normal dogs.\(^{(4)}\) Paired CSF-blood sample analysis may overcome some of this variability, and has been recognized as essential for immunophenotyping human CSF samples.\(^{(40,41,54)}\) In one study of inflammatory CNS diseases of humans, the distribution of lymphocyte subsets in CSF differed markedly from the distribution in peripheral blood. A significantly larger proportion of CD\(^{3+}\) cells and CD\(^{4+}\) cells were present in the CSF of patients with multiple sclerosis and other inflammatory conditions compared to blood.\(^{(6)}\)

In a study conducted by Vrethem, the CD\(^{4+}/CD^{8+}\) cell ratio was increased in CSF in comparison to peripheral blood in all individuals assessed.\(^{(55)}\) It has been shown that infectious and neoplastic diseases can cause an inversion of the CD\(^{4+}/CD^{8+}\) cell ratio in peripheral blood of humans and dogs.\(^{(33)}\) In general, T-cell ratios should be interpreted in
association with absolute lymphocyte counts. An inverted (decreased) CD4+/CD8+ cell ratio might result either from CD8+ cell elevation or from a CD4+ cell reduction.\(^{(47)}\)

According to various investigations, the proportion of CD8+ cells in human CSF is lower than in the peripheral blood.\(^{(55,56,57)}\) In contrast, dog CSF was reported to have lower CD3+ and CD4+ cell fractions compared to peripheral blood.\(^{(4)}\) Important time-dependent changes in CSF lymphocyte subsets during the course of infection have been recognized.\(^{(54)}\) The properties of the BBB and the CSF-blood barrier change in acute and subacute inflammatory processes of the CNS.\(^{(19)}\) Apparently, the modified barrier favors the transfer of T cells from blood into CSF depending on the stage of the disease. In several studies of human inflammatory diseases, the percentage of B cells in the CSF appeared lower than in the peripheral blood.\(^{(54,57,58)}\) Similar results have been reported for normal human CSF, indicating that B cells do not have the capability to re-circulate between the CNS, lymphoid tissue and peripheral blood to the same extent as T lymphocytes.\(^{(54,59)}\)

Reference ranges for blood lymphocyte subsets in dogs have been reported in different publications. In 1992, Gebhard and Carter described a set of monoclonal antibodies that identified T lymphocyte subsets expressing CD4 and CD8. The median percentages of peripheral blood lymphocytes expressing CD4 was 37.8% and CD8 was 17.7%.\(^{(60)}\) Similar results were reported by Cobold who found 41% of peripheral blood lymphocytes expressed CD4 and 20% expressed CD8.\(^{(17)}\)
In 1992, Moore and co-workers demonstrated the expression of CD4 by canine neutrophils, which has not been recognized in any other mammalian species studied so far. In the same work, the authors indicated that 46.4% of peripheral blood lymphocytes expressed CD4, and 16.4% CD8. In 1994 at the first international canine leukocyte antigen workshop (CLAW), information on the reactivity of 127 monoclonal antibodies with canine cells was compiled. At this workshop, the canine equivalents of human CD4, CD8, CD5 and Thy-1 were studied. Other monoclonal antibodies defined included the canine homologues of CD11/CD18, CD44, CD45/CD45R, as well as antibodies binding to platelets, granulocytes, MHC class II and immunoglobulins (as a marker for B cells).

In summary: Collective information to date suggests that surface antigen molecule expression on canine leukocytes can be constructively used for the assessment of multiple inflammatory diseases in dogs, including those of the CNS.
1.4 REFERENCES


2.0 INTRODUCTION

Inflammatory and infectious conditions of the central nervous system (CNS) are common among dogs presenting with neurological abnormalities. Available diagnostic modalities are limited, and despite therapy these diseases frequently result in death of the patient. Despite substantial advances in clinical neurology, establishing a definitive diagnosis for many such conditions continues to be a challenge. Studies are needed to better characterize the nature of the inflammatory or immune response, the organisms thought to be involved, and the efficacy of therapy.

The CNS tissue is inaccessible for many diagnostic procedures commonly applied to other organs. Biopsy is an invasive and expensive procedure with the added risk of permanent neurological damage. As a consequence, the cerebrospinal fluid (CSF) is used as an indirect measure of diseases affecting predominantly the CNS parenchyma. The CSF is relatively accessible and because of its close proximity to the inflammatory CNS lesions, may reflects the changes present in parenchymal diseases. Currently, analysis of CSF consists of determination of the total cell count, the differential count, and the total protein concentration. While these indices have proven useful in the assessment of neurological diseases, they yield little information beyond indicating a general inflammatory response. Furthermore, controversy persists regarding the identity of specific mononuclear cell subpopulations that appear lymphoid with older sedimentation techniques, but are more commonly classified indeterminate as “mononuclear cells” with the cytocentrifugation techniques currently applied in most diagnostic laboratories.\(^{(1,2)}\)

Findings in human neurology suggest that specific etiologic agents or conditions induce
characteristic changes in lymphocyte or monocyte/macrophage populations in the CSF. Similar associations are suspected in veterinary medicine, but as yet are uncharacterized. Recent development of reagents specific for canine cell surface antigens has facilitated better dissection of the immune response in a variety of diseases of dogs. Recent development of reagents specific for canine cell surface antigens has facilitated better dissection of the immune response in a variety of diseases of dogs. These monoclonal antibodies may also be of value in the investigation of canine neurological disease. In order to assess characteristic changes associated with specific diseases or infectious agents, a reference framework for health is required. Furthermore, leukocyte populations in blood commonly differ from those in CSF in most species, necessitating separate examination of both fluids. Thus, in this study, monoclonal antibodies against canine leukocyte antigens were carefully titrated, and specific binding to leukocytes in blood and CSF samples from clinically healthy dogs was assessed by flow cytometry. A range for each subpopulation present in health was determined. The hypothesis that leukocyte subpopulations in blood differ from those in CSF was tested.
2.1 MATERIALS AND METHODS

Experimental animals and samples

Twenty-three random source, pure or mixed breed dogs of medium to large size were available for this study. The dogs were euthanized for teaching purposes subsequent to sample collection. Each dog was determined to be healthy based on physical examination, complete blood count (CBC) findings, and routine CSF analysis. From each dog sedated with ketamine\(^a\) (5mg/kg) and xylazine\(^b\) (1mg/kg) 4 ml of blood were collected into EDTA anticoagulated vacutainer tubes\(^c\) by jugular venipuncture. The dogs were then euthanized with an overdose of pentobarbital\(^d\), and 6 ml of CSF were immediately collected by gravity flow into serum vacutainer tubes from the cerebello-medullary cistern. Blood and CSF samples were processed for routine and immunophenotypic analysis in less than 1 hour after collection to maximize cell preservation. This project was reviewed by the University of Guelph animal care committee and was performed in accordance with the guidelines set by the Canadian Council on Animal Care and the Animals for Research Act (Ontario 1980).

Antibodies and reagents

Monoclonal antibodies against the following antigens were used: anti CD14-PE\(^e\), anti-canine CD4-FITC\(^f\), anti-canine CD8-PE\(^f\) and anti-canine CD21-PE\(^f\). For the analysis of blood leukocytes, unstained cells and an isotype antibody were the negative controls. For
CSF leukocytes analysis unstained cells served as the negative control. All the antibodies used in this study were from the IgG isotype.

A wash solution consisting of phosphate buffered saline (PBS) with 10% fetal calf serum, 0.1% sodium azide, and 0.1M EDTA was prepared. The stock red blood cell lysis buffer consisted of a solution of 1.68 M NH₄Cl, 90 mM KHCO₃ and 1 mM EDTA. Commercial flow buffer solution was used during analysis.

**Cell preparation**

The blood samples were incubated with a 1:14 dilution of lysis buffer for 5 minutes at room temperature, and then centrifuged at 600x g for 5 minutes. After discarding the supernatant, the cell pellets were re-suspended in 5 ml of wash buffer and divided into five 12 x 75 mm polystyrene tubes. Four tubes with 1000 ul to 1250 ul of CSF from each dog were prepared. The tubes were centrifuged at 450x g for 10 minutes. Discarding the required volume, all CSF tubes were brought to 250 ul. Five ul of the respective antibody was added and incubation followed at 4°C in the dark for 15 minutes. For CD4-FITC and CD8-PE double staining, both fluorochrome-labelled antibodies were added at the same time. After a final wash, 0.05 ml of flow buffer was added, and samples were immediately analyzed.

**Flow cytometric analysis**

Samples were analyzed on a FACScan flow cytometer. Particular attention was given to minimizing particulate matter in the sheath fluid that would confound results from low-cellularity samples. Samples were carefully but thoroughly vortexed immediately prior to
analysis to yield evenly concentrated, single cell suspensions. Leukocyte populations in blood samples were identified based on forward and side scatter characteristics of unstained samples and samples stained with isotypic control antibodies, and gates were identified for lymphocyte and monocytes-macrophage populations. Identical settings for all gates were applied for all blood and CSF samples. All events in each of the CSF samples and a minimum of 10,000 events in the blood samples were collected and the fluorescence emission of cells analyzed with Cell Quest software.

CBC and CSF analysis

Routine hematological analysis was performed with an automated hematology analyzer, and manual differential counting. The CSF cell count was determined in a hemocytometer chamber, and cytospin smears were prepared from 200 ul aliquots of CSF. The total protein concentration was determined spectrophotometrically following Ponceau Red staining. Differential counts of CSF leukocytes were performed according to established criteria.

Statistical Analysis

The proportion of CD4+, CD8+, and CD21+ cells in the lymphocyte gate, and of CD14+ cells within the monocytes-macrophage gate, were determined in each blood and CSF sample. A paired t test was used to compare the proportion of each cell type in blood and CSF samples. Because the assumption of normality was not met for the CD8+ and CD14+ cells, their percentages were compared using the Wilcoxon Signed Rank test for
non-parametric distributions. A p value of <0.05 was considered significant. All the statistical analysis were performed using the Minitab software.
2.2 RESULTS

The total hemocytometer CSF leukocyte count ranged from 0-0.005 × 10^9/L cells (0-5/ul). Lymphocytes and monocytes were the most frequent cell types morphologically identified in CSF smears. Macrophages and segmented leukocytes were occasionally noted. The protein concentrations ranged from 15 to 38 mg/dl (Table 1). All CBC numerical values for leukocytes, erythrocytes, and platelets, as well as the white blood cell differential counts, were within reference ranges. Therefore the 23 dogs included in this study were considered healthy animals free of clinical neurologic disease.

The volume of CSF obtainable by free flow from dogs in this study ranged from 5 to 6 ml. The number of CSF cells evaluated by flow cytometry per antibody combination ranged from 480 to 4280. Lymphocytes and monocytes were gated according to their forward and side light scatter characteristics (Fig. 1, 2, 3). Lymphocytes formed defined populations while monocytes varied in their distribution among different samples. Lymphocyte and monocyte gates identified in blood samples were directly applicable to cells in the CSF (Fig. 4). CD4 expression by canine neutrophils was prominent, and granulocytes were therefore carefully excluded from analysis.

The mean proportion of CD4^+ cells in CSF lymphocyte gate (28.55% ± 12.41%) was significantly lower than in the blood lymphocyte gate (40.07% ± 11.37%) (p = 0.02) (Table 2,3). The mean proportion of CD21^+ cells was also significantly lower in CSF lymphocyte gate (7.11% ± 2.04%) compared to blood lymphocyte gate (18.73% ± 9.11%) (p = 0.000). In contrast, there was no significant difference between blood and CSF in the mean proportions of CD14^+ cells in the monocyte-macrophage gate and CD8^+
cells in the lymphocyte gate (Table 2.3). Blood and CSF CD14^+ cells constituted a mean proportion of 75.46% ± 15.46% and 75.16% ± 16.50% of cells in the monocyte gate, respectively. The mean proportion of CD8^+ cells was 19.51% ± 7.89% in blood, and 22.04% ± 13.62% in CSF samples. P-values of 0.494 and 0.891 were derived from comparison of the proportion of CD14^+ cells and CD8^+ cells, respectively, in blood and CSF using the Wilcoxon signed rank test.
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Mean ± SD*  40.07 ± 11.37  28.55 ± 12.41  19.51 ± 7.89  22.04 ± 13.62  75.46 ± 15.46  75.16 ± 16.50  18.76 ± 9.11  7.11 ± 2.04

The percentages of positive cells were derived according to the lymphocyte and monocyte gates

* SD: Standard deviation
**Table 3. Total events of CD4+, CD8+, CD21+ and CD14+ cells in blood and CSF according to lymphocyte and monocyte-macrophage established gates.**

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*Total events in the CSF double stained with CD4-FITC and CD8-PE
**FIG 1.** A. Blood cytogram from dog 5. Forward and side light scatter characteristics; monocytes-macrophage gate. B. Cytogram of CD14-PE staining cells in the gated population in A. A total of 848 events were analyzed in the monocyte-macrophage gate. The CD14-PE⁺ population (upper left quadrant) represented 84.43% of the gated events.
Fig 2. A. CSF cytogram from dog 3. Forward and side light scatter characteristics; lymphocyte gate. B. Cytogram of CD4-FITC and CD8-PE staining cells in the gated population in A. A total of 660 events were analyzed in the lymphocyte gate. The CD4-FITC$^+$ population (lower right quadrant) represented a 23.18% of the gated events. The CD8-PE$^+$ population (upper left quadrant) represented 36.67% of the gated events.
Fig 3. A. Blood cytogram from dog 7. Forward and side light scatter characteristics; lymphocyte gate. B. Cytogram of CD4-FITC and CD8-PE staining cells in the gated population in A. A total of 972 events were analyzed in the lymphocyte gate. The CD4-FITC$^+$ population (lower right quadrant) represented 56.58% of the gated events. The CD8-PE$^+$ population (upper quadrant) represented 14.20% of the gated events.
Fig 4. A. Blood cytogram from dog 23. Forward and side light scatter characteristics; lymphocyte gate. B. Cytogram of CD4-FITC and CD8-PE staining cells in the gated population in A. A total of 2801 events were analyzed in the lymphocyte gate. The CD4-FITC\textsuperscript{+} population (lower right quadrant) represented 41.16\% of the gated events. The CD8-PE\textsuperscript{+} population (upper left quadrant) represented 11.53\% of the gated events.
C. CSF cytogram from dog 23. Forward and side light scatter characteristics; lymphocyte gate. D. Cytogram of CD4-FITC cells and CD8-PE staining cells in the gated population in C. A total of 880 events were analyzed in the lymphocyte gate. The CD4-FITC+ population (lower right quadrant) represented a 35.86% of the gated events. The CD8-PE+ population (upper left quadrant) represented 16.90% of the gated events.
Inflammatory CNS diseases are frequently encountered in dogs. Severe neurological impairment often results from CNS inflammation, and may cause death of the patient despite treatment. The current approach to canine neurological diseases consists of the clinical examination, routine laboratory assessment, imaging studies, and CSF analysis. Of these, the CSF analysis is the most specific for inflammatory CNS disease\(^{(13)}\); however, the results are rarely suggestive of a specific organism or process.

Classification of cells based on expression of specific antigens by immunophenotyping is a potentially useful and less subjective adjunct in the interpretation of CSF samples.

Experience in other species suggested that obtaining sufficient CSF for analysis from dogs might be difficult, and that CSF leukocyte populations might differ substantially from those in blood.\(^{(3,4,11)}\) Therefore, the goals of this study were firstly to assess the feasibility of flow cytometric evaluation of canine CSF cells, and secondly to establish the immunophenotype of leukocytes in the CSF relative to those in the blood of healthy dogs.

Collection of a volume of CSF sufficient for flow cytometric evaluation using 4 specific markers was feasible in dogs in this study. This volume of CSF is difficult to obtain from most clinical cases; however, the increased cellularity generally present in inflammatory CSF samples should yield meaningful results even if a smaller volume of fluid is available for immunophenotypic analysis. Marked individual variation was found in the proportion of CSF cell types among dogs in this study, suggesting that the reference
range is very broad. This was previously reported in the assessment of pooled canine CSF samples.\textsuperscript{(14)} Thus, CSF flow cytometric assessment is a technique applicable for investigation of select canine neurologic diseases, but the study of paired CSF and blood samples is always recommended.

In agreement with a previous report, the proportion of CD4\textsuperscript{+} cells in CSF was significantly lower than in blood.\textsuperscript{(14)} According to reports on human subjects\textsuperscript{(3,11)} healthy individuals exhibited a higher proportion of CD4\textsuperscript{+} cells in the CSF than in blood, which differs from findings of this study. Considering that a lower proportion of CD4\textsuperscript{+} cells are present in normal canine CSF, and that immune-mediated neurological diseases in other species may be associated with increases in this population it is of interest to assess canine diseases suspected to be of immune-mediated origin.\textsuperscript{(15)}

CD8\textsuperscript{+} cells appeared in a higher proportion in CSF than in blood, as previously reported by others.\textsuperscript{(14)} This observation was not statistically significant in this study, possibly due to a sample size limited to 23 animals. Nevertheless, the greater proportion of CD8\textsuperscript{+} cells in the CSF suggests that cell-mediated immune responses may be of greater importance in the CNS than in other sites.

A significantly greater proportion of CD21\textsuperscript{+} cells was found in the blood than in the CSF. This is in agreement with previous studies in human immunology and in contrast with the findings reported by Tipold and coworkers.\textsuperscript{(4,5,14,16)} An apparent BBB selectivity favors movement of T cells over B cells potentially explaining the relatively higher proportion of CD21\textsuperscript{+} cells in the blood. The exact mechanism of how lymphocytes cross the BBB
remains unclear. It appears likely that adhesion molecules such as specific selectins, integrins and their ligands mediate this process.\(^{17}\) Lymphocytes found in the CSF are part of the blood lymphocyte recirculating pool, however, with an apparent bias toward T cells.\(^{17}\)

In a previous study analyzing paraffin-embedded brain tissue T cells were the predominant cell population in perivascular cuffs in confirmed cases of distemper and tick borne encephalitis. In contrast, in the same study it was reported that in bacterial and protozoal meningoencephalitis, the percentage of B cells was higher than that of T cells.\(^{18}\) Valuable information may be gained if such changes can be detected antemortem in CSF samples. If T cells are indeed the predominant cell population in brain tissue of animals with distemper and tick borne encephalitis, it may be feasible to obtain characteristic results from the analysis of CSF samples. Moreover, repeated analysis may allow assessment of response to therapy to greater detail than numerical and microscopic CSF analysis.

Because CSF is of exceptionally low cellularity and protein content, morphological assessment necessitates cell concentration and rapid processing. The most widespread method for CSF cell concentration is cytocentrifugation. With this technique slides of good quality are obtained in short time.\(^{19,20}\) However, the technique is prone to morphological artifacts from cell distortion. The cells overall appear larger, lymphocytes may develop nuclear “holes”, eccentrically placed nuclei or perinuclear clear areas.\(^{12}\) Monocyte/macrophage cell morphology may also be altered through appearance of cytoplasmic vacuoles, eccentrically placed nuclei, and cytoplasmic pseudopods.\(^{12}\) Many of the morphological changes induced by cytocentrifugation are believed to be due
to mechanical distortion.(1,21) Sedimentation is a less commonly used method for CSF preparation, is relatively simple and provides good cell recovery. However, drying artifact is prominent and may cause severe cell disruption.(12) In addition, large mononuclear cells upon contact with the glass slide during the sedimentation process can “transform” into large vacuolated mononuclear cells identified as macrophages.(22) Both techniques induce morphological changes that are more pronounced in certain types of cells. Granulocytes and small lymphocytes appear similar in blood and CSF preparations. In contrast, CSF mononuclear cells other than small lymphocytes appear to vary greatly in shape and size. Although these mononuclear cells comprise the majority of cells in canine CSF, their identification criteria are subjective and variable. Thus, there are inconsistencies in the classification of mononuclear cells in CSF based solely on microscopic appearance. Immunophenotyping is an alternative that may yield more objective cell classification than microscopic analysis.

When light scatter properties were analyzed for CSF samples lymphocytes, formed discrete populations. In contrast, monocytes and macrophages exhibited variable sizes and consequently variable light scatter properties, making the definition of a mononuclear-macrophage gate more difficult. However, in general, a preponderance of lymphocytes over cells of the monocyte-macrophage lineage was found in this study. Due to CSF hypocellularity, modified protocols are required when performing immunophenotyping. The additional centrifugation steps required for unconjugated antibodies preclude their usefulness in CSF immunophenotyping.
Six of the 23 samples included in this study contained low number of erythrocytes. In humans, CSF with greater than 0.030 x 10⁹/L (30/ul) erythrocytes is considered to be blood contaminated. Similar findings were reported in canine CSF. Only CSF samples containing erythrocytes > 0.030 x 10⁹/L (30/ul) had cytological examinations significantly different from CSF samples that were free of blood. In the present study, only samples with fewer than 30 RBC’s/ul were included.

The upper reference limit for CSF nucleated cells in dogs is 0.003 x 10⁹/L (0-3/ul). In the present study, three animals (dogs 4, 16 and 20) with cell counts of 0.004, 0.004 and 0.005 x 10⁹/L, respectively, were included. These animals were included since both the microscopic assessment and the protein concentration were within reference parameters. Cell recovery on cytocentrifuged preparations and during flow cytometric analysis was similar to samples with lower cell counts, and thus the mild elevation in total cell count was considered to be possibly due to counting error in the hemacytometer chamber. Nevertheless, ideal assessment of the health status of dogs in this study would have included a full necropsy and histologic examination of the CNS. This was not possible; thus, underlying mild CNS disease cannot be entirely ruled out in these 3 cases.
2.4 CONCLUSIONS

It was concluded that the leukocyte subpopulations in CSF differ from the leukocyte subpopulations in blood from clinically normal dogs. Marked individual variation was found making it difficult to define a reference range for the proportion of leukocyte populations studied. Therefore, the analysis of repeated CSF-blood samples is recommended when performing CSF immunophenotyping to study the course of neurological diseases.

Immunophenotyping is a unique technology that provides additional information for CSF analysis. The technique is feasible, rapid, and may yield more objective cell classification than microscopic analysis. Attention to minimizing cell loss during processing and reducing background fluorescence detection during analysis is essential.

Footnotes


bRompun. Bayer Inc. 77 Belfield Road, Toronto, Ontario, M9W1G6.

Beckton Dickinson and company. 1886 Franklin Lakes, New Jersey, 07417.

eEuthansol. Schering plough. 200 Galloping Hill Road, Kenilworth, New Jersey, 7733-0530.

DAKO Diagnostics Canada, Inc. 12 Falconer Drive, Unit 4, Mississauga, Ontario, L5N3L9.

SEROTEC Inc. 717 Main Campus Drive, Suite 2450, Raleigh, North Carolina, 27606.

Beckton Dickinson Canada Inc. 2464 South Sheridan Way, Mississauga, Ontario, L5J2M8.
h6 series Allegra centrifuge. Beckman Coulter Canada Inc. 6755 Missisauga Road, Suite 600, Missisauga, Ontario, L5N7Y2.

Cytospin 2 cytocentrifuge. Johns scientific Inc. 175 Hanson Street, Toronto, Ontario, M4C1A7.


Minitab software. Pearson Higher Education. 1900 East Lake Avenue, Glenview, Illinois, 60025.
2.5 REFERENCES