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Assessment of the IgG index in dogs by indirect immunoenzimatic assays as diagnostic tool for inflammatory diseases of central nervous system ☆

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Abstract

The IgG index measures the intrathecal immunoglobulin production and it is a useful tool for diagnosis of inflammatory diseases involving the central nervous system. This index is based on the precise quantification of albumin and IgG in canine cerebrospinal fluid and serum. Here, we report the development of an indirect competitive ELISAs for the detection of both antigens.

Thirty-two dogs were included in this study, divided into three experimental groups. Group A was composed of 22 healthy animals, as determined by standard clinical examination. In group B, six animals, presented neurological pathologies associated with endogenous IgG production and, in group C four animals presented neurological diseases or symptoms not associated with intrathecal IgG production. Cerebrospinal fluid and serum samples were obtained from these animals. As expected, by using the indirect ELISAs proposed here, the IgG indexes obtained in healthy animals (A) were 0.371 ± 0.252 (SD). In B and C, the values (3.002 ± 1.897; 0.36 ± 0.306, respectively), were in agreement with the pathologic conditions of the individuals in each group. Thus, the immunometric competition ELISA methods proposed here allow the discrimination of abnormal intrathecal IgG production, in a variety of inflammatory pathologic conditions of the central nervous system.

Keywords: IgG index; Intrathecal immunoglobulin production; ELISA; Central nervous system

1. Introduction

In Clinical Neurology the examination of cerebrospinal fluid (CSF) is one of the modern tools useful not only for the diagnosis of many neurological diseases but also to evaluate the condition of the CNS. Cell number and type, as well as total protein concentration in CSF, suggest the presence of neurological pathology (Chrisman, 1992; Verbeek et al., 2005). The composition of CSF is maintained by the blood brain barrier (BBB), a selectively permeable cytological structure. The integrity of the BBB can be determined by the albumin (Alb) quotient (AQ) (Sorjonen, 1987), corresponding to the albumin concentration in CSF over its concentration in serum (S) (AQ = CSF/S) (Link and Tibbling, 1977). When the integrity of BBB is disrupted, proteins pass from the blood to the CSF (Behr et al., 2006). Some neurological diseases, with intrathecal production of IgG, are associated with chronic inflammation of the CNS (Phares et al., 2006).

The IgG index (CSF/S IgG ratio/AQ) (Link and Tibbling, 1977; McMillan et al., 1996) is used for the determination of endogen production of IgG. This index is routinely utilized for diagnosis and prognosis of some encephalic pathologies in different mammal species (human, bovine, equine, canine, feline, etc.) (Andrews et al., 1994; Chrisman, 1992; Daft et al., 2002; Dow et al., 1990; Scott,

2004; Verbeek et al., 2005). In humans this index has been used in the diagnosis and prognosis of multiple sclerosis, Alzheimer (Brandao et al., 2005; Verbeek et al., 2005; Wozniak et al., 2005; Yilmaz et al., 2006) CNS involvement associated with HIV, etc. In dogs, the diagnosis and prognosis of diseases such as chronic distemper, neoplastic encephalic pathologies, granulomatous meningoencephalitis, meningoencephalitis associated with toxoplasmosis, among other, have been oriented by the use of this index (Chrisman, 1992). A variety of techniques have been utilized for the determination of proteins present in CSF (Bichsel et al., 1984; Krakowka et al., 1981; McMillan et al., 1996; Sorjonen et al., 1989; Tipold et al., 1993). Here we propose an alternative indirect competition immunometric procedure for the quantification of IgG and albumin, present in low concentrations in CSF and serum (S).

2. Materials and methods

2.1. Animals

Thirty-two, 1–10 year old dogs were selected from the University of Chile Small Animal Veterinary Practice, after written consent from the owners. This practice operates under the direction of Licensed Veterinary Faculty Members and follows internationally accepted bioethical regulations. The animals were divided into three groups. Group A, with 22 animals were found healthy, by standard clinical examination. Group B, six animals, presented some neurological pathologies associated with endogenous IgG production (four with chronic distemper, one with atrophic degenerative encephalopathy and one with fungal meningoencephalitis) (Galie et al., 2005; Johnson et al., 1998; La Mantia et al., 1986; Nielsen et al., 2005) and group C, four animals, presented neurological diseases or symptoms not associated with intrathecal IgG production (one case of acute distemper, two cases of spinal lesion and one with hypocalcemia) (Chrisman, 1992; Vandevelde and Zurbriggen, 2005; Wuschmann et al., 2000). Cerebrospinal fluid and serum samples were obtained from these animals.

(Table 1) summarizes the general characteristics and main clinical findings in these animals.

2.2. CSF and serum samples

CSF and S samples were obtained, under anesthetic premedication (atropin, 0.04 mg/kg; acepromazin, 0.04–0.2 mg/kg), anesthesia induction (2% sodium thiopental, at 8–10 mg/ kg) and intubation. CSF samples (1.5–2 ml) were obtained from the Magnum Cistern, with spinal needles (Chrisman, 1992; Bagley and Higgns, 1985; Takasugi et al., 2005; Takizawa et al., 1986; Wilsson-Rahmberg et al., 1998). Blood samples (5–10 cc) were obtained from saphena and/or cephalic veins. Serum was obtained by standard procedures. After standard centrifugation, CSF and S samples were stored in Eppendorf tubes at −20° C.

2.3. Canine IgG purification

Canine IgG was affinity purified from normal serum in a Sepharose–Protein G column (Amersham Pharmacia Biotech AB, Uppsala, Sweden), by using standard procedures.

2.4. Immunization procedures

For immunization purposes, two rabbits (female, New Zealand White, 3–5 month old, 2–3.5 kg), were used. Affinity-purified dog IgG was used to immunize one rabbit. A second rabbit was immunized with pure commercial dog serum albumin (DSA). After a pre-immune bleeding, the animals were immunized three times at weekly intervals (in the first injection complete Freund's adjuvant was used, while the incomplete version was used in the remaining ones). A week after the last immunization the animals were bled three times, also at weekly intervals. Given its high titer, the first two immunizations were subcutaneous and, the last two intraperitoneal after titration of all bleedings, the third one was chosen for all assays.

Table 1
Clinical histories of dogs used in the IgG index determinations

Patient ID	Age (years)	Clinical signs	Diagnosis
Group A[a]	1–8		
1–22		Normal	Normal
Group B[b]	2–10		
24, 25, 29, 30		Ataxia and incoordinated, hypermetria	Chronic distemper
27		Ataxia, voluntary trembling	Atrophic encephalic degeneration
31		Seizures, blindness, ataxia	Meningoencephalitis *C. neoformans*
Group C[c]	1–4		
23			Spinal lesion L4–L5
26		Bronchopneumonia	Acute distemper
28			Spinal lesion L–S
32		Seizures, ataxia	Hypocalcemia

[a] Healthy animals.
[b] Animals with neurological pathologies associated with endogenous IgG production.
[c] Animals with neurological diseases or symptoms not associated with intrathecal IgG production.

2.5. Competition ELISA for the detection of IgG

In order to build a standard inhibition calibration curve, microtitration polyvinylchloride (PVC) plates were sensitized with 100 µl of a 1/5000 dilution of normal dog S (in 0.1 M, pH 9.6, carbonate buffer), as an IgG source. The plates were then washed with PBS–Tween 20, 0.05% v/v, and the remaining active sites were blocked with 200 µl PBS containing 0.5% w/v soybean proteins (Aguillon et al., 1992). Then, 50 µl of a rabbit serum anti-canine IgG, diluted 1/160 000 (in pilot direct ELISA experiments, this Ag concentration generated about 70% of the maximum signal) were added in the presence of equal volumes of different concentrations of pure canine IgG (0.52/100 to 0.52/2000 mg/ml). Rabbit IgG, bound to solid phase canine IgG, was detected with the corresponding immune probe (affinity-purified and peroxidase conjugated goat Igs, diluted 1/5000), anti-rabbit IgG. As peroxidase substrates, 3-dimethylaminobenzoic acid (DMAB, 80 mM) and monohydrate, 3-methyl-2-benzotiazolinone hydrazone hydrochloride (MBTH, 1.6 mM) was used. The reaction was stopped with 50 µl, 2 M, H_2SO_4, per well. The plates were read at 600 nm. The IgG concentrations in different CSF or S samples, tested at different dilutions (CSF: 1/0.33 to 1/10) (S: 1/1000 to 1/10 000), were determined by interpolating the inhibitory signal to the standard curve.

2.6. Competition ELISA for albumin quantification

In order to build a standard inhibition calibration curve, PVC plates were sensitized with 100 µl of 20 µg/ml commercial DSA (Fraction V, Sigma, USA) (in pilot direct ELISA experiments, this Ag concentration generated about 70% of the maximum signal). The plates were then washed and blocked. Then, 50 µl of a rabbit serum anti-DSA, diluted 1/80 000, were added to the wells, in the presence of equal volumes of different DSA concentrations (1.38×10^{-2}–3.45×10^{-4} mg/ml). Rabbit IgG, bound to solid phase DSA, was detected with the corresponding immune probe. Peroxidase substrates, stopping reagents, reading and interpolation procedures for the signals obtained with CSF and S (1/50 to 1/250 and 1/20 000 to 1/80 000 dilutions, respectively), were those described in Section 2.5.

2.7. IgG index

An IgG index was established according to the expression ([CSF IgG]/[S IgG] × [S Alb]/[CSF Alb] × correction factor (CF)). It is accepted that, in clinically healthy individuals, the index should be below 0.9 (Bichsel et al., 1984).

2.8. Calculation and statistical analysis

For each group (A; B; C) the means and standard deviations (SD) were obtained. Subsequently, all the groups (A v/s B; A v/s C; B v/s C) were compared by using one-tailed student t tests.

3. Results

3.1. Purification of dog IgG

Dog IgG was purified from heat-inactivated, filtered, whole normal serum by affinity chromatography in a Sepharose–Protein G column. In 10% Coomasie Blue-stained SDS–PAGE, under reducing conditions, both the heavy (H) and light (L) IgG chains are observed. More than 98% purity was obtained, as assessed by SDS–PAGE and immunowestern blotting (IWB). No other contaminating serum proteins were detected, as shown in Fig. 1.

3.2. Specificity of rabbit anti-dog IgG

Pure dog IgG was analyzed by immune western blotting against our rabbit anti-dog IgG antiserum. As shown in Fig. 2, both H and L chains were specifically detected by this reagent.

3.3. Specificity of rabbit anti-DSA IgG

The whole rabbit anti-dog albumin antiserum is highly reactive and specific, as shown in the ELISA, in dilutions over 1/8000 (Fig. 3).

3.4. IgG indexes

In agreement with the literature, the IgG indexes obtained in normal individuals (group A, Table 2) were lower than those obtained in individuals from B, a group displaying clinical conditions consistent with endogenous IgG production ($p = 0.009$; Table 2). As expected, no differences ($p = 0.475$) were obtained when group A was compared with C (representing pathologies not associated with endogenous IgG production). Likewise, group B produced more endogenous IgG than C, a group displaying a behavior similar to group A (Table 2; $p = 0.009$).

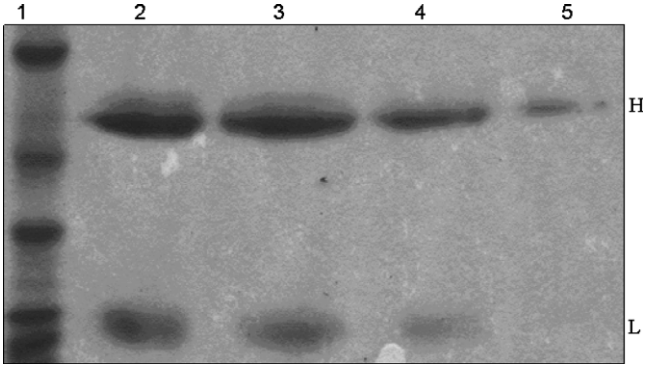

Fig. 1. Reducing 10% SDS–PAGE of affinity purified dog IgG. Track 2–5: Sepharose Protein-G elution (5.25; 2.63; 1.3; 0.66 µg) profile of canine IgG. Pure H (53–54 kDa) and L (28–29 kDa) chains are observed. Track 1: molecular weight markers (66; 45; 36; 29; 24 kDa, from top to bottom).

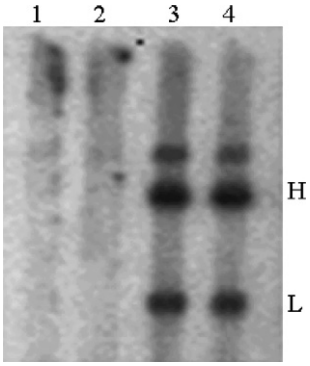

Fig. 2. Specificity of a rabbit anti-canine IgG. Affinity purified canine IgG was analyzed in 10% reducing SDS–PAGE (Fig. 1) and transferred to a nitrocellulose sheet. After blocking, the presence of H and L chains was detected with a rabbit anti-dog IgG (track 3–4), generated as described in Section 2. Tracks 1–2, developed with preimmune rabbit serum.

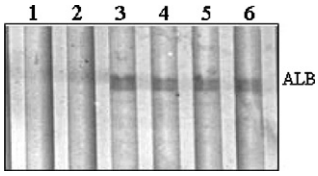

Fig. 3. Specificity of a rabbit anti-canine albumin antiserum. Whole canine serum was analyzed in 10% reducing SDS–PAGE and transferred to a nitrocellulose sheet. After blocking, the presence of canine albumin was detected with a rabbit anti-dog serum albumin, diluted 1/8000 (tracks 3–4) and 1/16000 (tracks 5–6), generated as described in Section 2. Tracks 1–2 were developed with preimmune rabbit serum.

Table 2
IgG indexes in normal (A) and neuropathic (B and C) dogs

Group A	Group B	Group C
$n = 22$	$n = 6$	$n = 4$
(1)[a] to (22)	(24) 2.706	(23) 0.102
	(25) 1.415	(26) *0.097*
	(27) *5.370*	(28) *0.688*
	(29) *1.081*	(32) 0.553
	(30) 5.312	
	(31) 2.128	
$\overline{X}_A \pm SD$	$\overline{X}_B \pm SD$	$\overline{X}_C \pm SD$
0.371 ± 0.252	3.002 ± 1.897	0.36 ± 0.306
Range: 0.030–0.856	Range: 1.081–5.370	Range: 0.097–0.688

[a] Case number in parenthesis. Indexes were $\leqslant 0.9$ in group A and C and >0.9 in group B. *P* values: A vs. B (0.009); A vs. C (0.475); B vs. C (0.009).

4. Discussion

The main contribution of this study is of methodological nature, since it proposes an indirect competition ELISA for the determination of IgG indexes.

As expected, in reducing SDS-PAGE, affinity purified dog IgG showed high purity, as shown by the exclusive presence of H and L chains (Fig. 1). As shown in Fig. 2, in IWB of affinity purified dog IgG, besides the H and L chains, a 75 kDa band is also observed. This could represent H–L dimers (since, the reducing agent β-2 mercaptoethanol is a non charged alcohol, it remains in the loading well, thus making possible the generation of dimers in the running gel) (Coligan et al., 2001a). Alternatively, since the nitrocellulose membrane was sensitized with whole normal dog serum, previously separated in reducing SDS–PAGE, recognition of the dog IgM H chain (approximately 70 kDa) by the polyclonal rabbit serum, is possible (Coligan et al., 2001b). In such a case, given the high antisera dilutions used in this competitive assay and considering the low IgM concentration in dog serum and CSF (Krakowka et al., 1981; Sorjonen, 1981), no interference in the assay is expected.

The IgG indexes shown in Table 2 indicate that the competition ELISA proposed here allows the quantification of both IgG and albumin. The average IgG index (Table 2), obtained for normal animals (group A) in the competitive ELISA proposed here, was close to that described by Bichsel et al. (1984).

Of relevance to this proposal is the fact that the IgG indexes obtained are similar, regardless of the assay used. Moreover, the average concentrations of relevant proteins, obtained by us, are in agreement with those described in the literature (Bichsel et al., 1984). Possible factors involved in the generation of some interassay differences are the reduced number of clinically healthy animals used in this study, as well as the large variations in the genetic background and environmental origin conditions of all participating animals.

Given the methodological nature of this report, it was of interest to determine the IgG index in healthy dogs, in order to compare our results with those from the literature. The animals in groups B and C were randomly chosen from clinical cases where owner's consent was necessary, thus explaining their reduced number. In these cases, our main interest was to explore the index variations in some clinically available pathologies.

A closer clinical evaluation of groups B and C (Table 2), provided additional useful information: In agreement with previous findings (Sorjonen et al., 1989), in group B, the most frequent pathology of the CNS, with endogenous production of IgG, is canine distemper encephalitis (Tables 1 and 2). Chronic distemper has been proposed as an attractive biological model for human demyelinating diseases (Alldingers et al., 2006). An interesting clinical case number 31, was fungal meningoencephalitis, in a dog (Tables 1 and 2) with symptoms of deteriorated CNS. *Cryptococcus neoformans* was isolated from CSF (Lavely and Lipsitz, 2005; Munana, 1996) and the IgG index in this animal indicated pathology (Tables 1 and 2). Case 27 (Tables 1 and 2) presented symptoms consistent with cerebellar lesions (voluntary trembling) (Mariani et al., 2001) with brain compromise (Bagley, 1996; Hazlett et al., 2005; Li et al., 2006). The IgG index (case 27) obtained is consistent with an intrathecal IgG synthesis (Galie et al., 2005; Schelhaas et al., 1997). Thus, in all those cases where

CNS chronic diseases were diagnosed, altered IgG indexes were detected.

Differently from B, group C animals (Table 1), suffering from neurological diseases or displaying neurological symptoms not associated to intrathecal IgG production, did not show significant alterations in their IgG indexes (Table 2). These results substantiate the accuracy of the ELISA technique for IgG index measurement proposed here. Moreover, the acute distemper case was interesting, since the IgG index was normal, a result compatible with the literature (Vandevelde and Zurbriggen, 2005; Wuschmann et al., 2000).

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