Pathological findings and immunohistochemical diagnosis of canine distemper in naturally infected dogs

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SUMMARY

This study describes the pathological findings and immunohistochemical distribution of viral antigen in 14 cases of canine distemper. Histopathologically, the most marked findings were non suppurative encephalomyelitis with demyelization especially in the cerebellum, and pneumonia. Lymphoid depletion was found in the spleen and lymph nodes. Eosinophil inclusion bodies were recognized in astrocytes of the cerebellum, in epithelial cells of bronchi and bronchioles and in pneumocytes, in glandular epitheliums from the stomach and in the splenic reticular cells. The avidin biotin peroxidase complex (ABC) and immunofluorescence (IF) methods were compared for the viral antigen detection. With the both tests, condensed viral immunolabelling was detected particularly in the cerebellum, lungs and stomach and in the spleen, lymph nodes, liver, kidneys and urinary bladder at a lesser extend. Despite a good agreement (94.6%) between the 2 methods, the ABC method was preferred because of a more intense staining particularly in brain samples.

Keywords: Canine distemper, immunohistochemistry, ABC method, Immunofluorescence, brain, lung, spleen, dog.

RÉSUMÉ

Données anatomopathologiques et diagnostic immunohistochimique de la maladie de Carré chez des chiens naturellement infectés

Dans cette étude, les données anatomopathologiques de la maladie de Carré et la distribution tissulaire des antigènes viraux déterminée par immunohistochimie ont été analysées sur 14 chiens infectés. Des lésions le plus souvent cérébelleuses d’encéphalomyélite non suppurative accompagnée de démyélinisation et/ou des lésions de pneumonie ont été observées à l’autopsie sur tous les animaux. La rate et les nœuds lymphatiques ont présenté une déplétion lymphocytaire. Des inclusions éosinophiles ont été mises en évidence dans les astrocytes du cervelet, dans les cellules épithéliales des bronches et bronchioles, dans les pneumocytes, dans les épithéliums glandulaires de l’estomac et dans les cellules réticulées spléniques. Deux méthodes immunohistochimiques de détection des antigènes viraux (révélation des complexes immuns formés par association d’un second anticorps biotinyle avec l’avidine couplée à une peroxydase (méthode ABC) ou utilisation directe d’un anticorps spécifique fluorescent (test IF)) ont été utilisées et comparées : des particules virales ont été identifiées par les 2 tests principalement dans le cervelet, le poumon et l’estomac, mais aussi, de façon plus ponctuelle, dans la rate, les nœuds lymphatiques, la foie, les reins et la vessie. En dépit d’un fort agrément (94.6%) entre les 2 tests, la méthode ABC permettant un marquage plus intense des structures cérébrales a été préférée.

Mots-clés : Maladie de Carré, immunohistochimie, méthode ABC, immunofluorescence, cerveau, poumon, rate, chien.

Introduction

Canine distemper (CD) is a viral infection of a wide range of carnivores including members of the Canidae, Procyonidae and Mustelidae [21]. The causative virus belongs to the genus *Morbillivirus* of the family *Paramyxoviridae*, closely related to the viruses of human measles, rinderpest, and peste des petits ruminants [18]. CD is common in young unvaccinated dogs, usually in their first year of life, but many cases are also seen in adults [21, 25, 26]. Clinically, encephalomyelitis and pneumonia, the most common manifestations of CD, are mainly responsible for the death of the affected dogs [4, 10, 15, 16]. The clinical course and neuropathological patterns of canine distemper encephalomyelitis vary with the viral strain [6, 17], and with the age of animals at the time of infection [11]. The main clinical picture of CD usually reflects a focal, multifocal or diffuse distribution of the central nervous system (CNS) lesions [22]. In addition, lung lesions complicated by bacteria is also observed [7].

The aims of the study are to describe the pathological findings, and to compare the avidin biotin peroxidase complex (ABC) and the immunofluorescence methods (IF) for detection and tissue distribution of CD viral antigens.

Materials and Methods

**ANIMALS AND TISSUE SAMPLING**

Fourteen dogs (8 females and 6 males) which died from naturally occurring nervous and/or respiratory infections
were chosen to investigate CD in this study. Five of animals were mongrels and nine were purebred, the ages of dogs ranged from 2 to 12 months (4.75 ± 3.4 months). Necropsy was performed in all animals within two hours after their death, and then tissue samples including brain, lungs, stomach, intestines, kidneys, liver, heart, lymph nodes, and spleen were immediately fixed in 10 % neutral buffered formalin, embedded in paraffin wax, sectioned at 5 µm and stained by routine methods with haematoxylin and eosin (HE). Replicate sections were used for immunohistochemistry.

PREPARATION OF THE HYPERIMMUN SERUM

Two rabbits, 15 month old, were inoculated intraperito-
nearly with a mixture of 1 ml distemper virus (Tissue Cell
Infection Doses [TCID]: 10^4.25 / 0.1 ml) and 1 ml of
Freund’s complete adjuvant. After four weeks, a mixture of
1ml distemper virus (TCID: 10^4.25 / 0.1 ml) and 1 ml
Freund’s incomplete adjuvant was inoculated three times at
7-day intervals. The animals were bled by cardiac puncture
15 days after the last injection, and sera were pooled. After
that, gamma globulins were initially separated using ammo-
nium sulphate precipitation then IgG was fractioned using
sephadex G-25 (Pharmacia Fine Chemicals).

IMMUNOHISTOCHEMISTRY

For immunohistochemical analysis, the avidin-biotin
peroxidase complex method (Kwik-kit; Shandon, Pittsburg,
USA) and the immunofluorescence test were used as described by
TOPLU [23] and by TOPLU and ALCIGIR [24] respectively.
Tissue sections (5 µm) were placed on poly-L-lysine-coated
glass slides. After incubation for 2h at 40°C, the sections
were dewaxed in xylene and hydrated through graded alcohols.
The tissues were digested with 0.1 % protease K for 10 min.
at 37°C and the slides were washed for 10-15 min. in
phosphate buffered saline (PBS) pH 7.3. All incubations
were performed at room temperature in a humidified chamber.

For the ABC method, endogenous peroxidase was previously
blocked with H_2O_2 3% in 70 % methanol and the non
specific staining was blocked by treatment with 2% normal
goat serum for 10 min. Then, the blocking serum was replaced
by rabbit anti-distemper serum diluted 1:32, followed by an
overnight incubation at 4°C. After washing in PBS for 10 min.,
the sections were flooded with biotinylated goat anti-
rabbit immunoglobulin for 10 min. After a further PBS wash,
the sections were covered with streptavidin-peroxidase and
incubated for 10 min. Finally, they were treated for 10-15
min. with amino ethyl carbazole (AEC) in H_2O_2 chromogen.
The sections were then counterstained with Mayer’s haema-
toxylin, washed in distilled water, and mounted with aqueous
mounting medium.

For the IF method, the sections were directly incubated
with rabbit anti-distemper virus serum diluted 1:16 for 2
hours at 37°C, and then washed for 15 min. in PBS. Sections
were covered with goat anti-rabbit gamma globulin antibody
conjugated with fluorescein isothiocyanate (Sigma, Rehorot,
Israel) for 30 min. at 37°C. Sections were then washed in
PBS for 20 min. and mounted in phosphate-buffered glyce-
rol, pH 9.0. For control purposes, replicate sections were
processed substituting the rabbit anti-distemper serum by a
normal rabbit serum. The tissue sections were examined with
fluorescence microscope (Leica, DMLB).

The percentage of the total area of the fluorescent and per-
oxidase labelling positive cells were semi-quantitatively
assessed under light and fluorescent microscopes with a 10X
ocular with grids and a 20X objective. The labelling intensity
in a given cellular compartment was assessed according the
following categories: (0+): no positively staining cells; (1+):
weak staining (1-3% positive cells); (2+): moderate staining
(4-8% positive cells); (3+): marked staining (> 8% positive
cells).

Results

CLINICAL AND NECROPSY FINDINGS

Clinical findings were evaluated according to anamnesis
given by practitioners. The prominent clinical findings asso-
ciated with neurological symptoms, either singly or in
various combinations, were ataxia, paresis or paralysis,
abnormal behaviour and muscular spasms. In 10 of the dogs,
respiratory symptoms associated with nasal and ocular
discharge as well as with neurological signs were also noted.
Two animals had only respiratory symptoms. Two animals
showed peracute death.

At necropsy, lungs showed marked lesions related to bron-
chopneumonia complicated by bacteria, occurred in hepa
tised and emphysematous areas. Lymph nodes and tonsils were
usually enlarged and oedematous. No remarkable lesion was
observed in other organs.

HISTOPATHOLOGICAL FINDINGS

Pathological and immunohistochemical findings are pre-
seated in Table 1. The main sites showing characteristic his-
tological lesions were the brain and lungs as well as spleen
and lymph nodes.

The 12 dogs showing neurological clinical signs also
presented inflammatory reactions in the CNS located in
different parts and in various combinations: in the cerebellum
(12/12), in cerebral hemispheres (5/12), in brain stem (6/12)
and in spinal cord (5/12). Five dogs had acute lesions
showing moderate to severe demyelization along with slight
astrocyte proliferation without perivascular mononuclear
cell infiltrates in white matter of the cerebellopontine areas.
In three dogs, there were subacute lesions consisting of
demyelization with astrocyte proliferation and perivascular
mononuclear infiltrates both in white and grey matters of the
brain. The chronic lesions characterized with demyelization
with Gitter cell proliferation, densely arranged astrocytosis
and gliosis were marked in four dogs. Intranuclear and/or
intracytoplasmic inclusion bodies were found in astrocytes
of the cerebellum in the seven dogs showing acute/subacute
CNS damage. All the dogs exhibiting acute encephalitis were
very young (2-3 months old), whereas subacute and chronic

lesions were found in older animals (more than 4 month old) (Table 1). Lytic alterative and/or atrophic changes of Purkinje’s cells appeared in different type lesions of the cerebellum. Leptomeningitis was marked in two animals. The 2 dogs which have not exhibited neurological clinical manifestations showed only demyelization in appearance “moth-eaten” as early lesions.

The lungs showed interstitial pneumonia (n = 5) or catarrhal bronchopneumonia accompanied by areas of interstitial pneumonia (n = 6). One dog exhibited only oedema and emphysema in lungs (Table 1). The interstitial pneumonia consisted of mononuclear cell infiltration in alveolar septal tissue, and pneumocyte proliferation lining alveolar walls and occasionally mild mononuclear cell infiltration of the peribronchial and bronchiolar interstitium. The epitheliums of the bronchi and bronchioles showed squamous cell metaplasia and hydropic degeneration and their lumina were filled with exudate containing desquamated epithelial cells, neutrophils and macrophages. Eosinophil intracytoplasmic and/or intranuclear inclusion bodies were seen especially in epitheliums of the bronchi and bronchioles and occasionally in pneumocytes. The alveoli showed oedema, numerous alveolar macrophages and moderate numbers of neutrophil leucocytes. Syncytial cells with intracytoplasmic inclusion bodies were also detected in the lumina of the alveoli in three animals (Figure 1).

The spleen revealed marked depletion of lymphocytes in the parafollicular areas in the white pulp, together with reticuloendothelial cell hyperplasia in 10 dogs. Sinusoids were dilated and filled with macrophages and plasma cells with a few neutrophils. In addition, reticuloendothelial cells of the spleen included intracytoplasmic and intranuclear inclusion bodies in two animals. In the mesenteric and retropharyngeal lymph nodes and tonsils, the lesions were similar to those seen in the spleen.

In the stomach, epithelial cells of the glands and crypts contained intracytoplasmic and intranuclear inclusion bodies in four animals. Inclusions were also detected in epithelium of the urinary bladder and renal pelvis of three animals.

### IMMUNOHISTOCHEMICAL FINDINGS

The results are presented in Tables I and II. The CD viral antigens with a diffuse and/or a fine granular shape were evidenced both in nucleus and in cytoplasm in infected cells with the 2 immunolabelling methods. On the 56 tissue samples (brain, lung, stomach and spleen) analysed, 44 gave positive staining with the ABC method and 43 with the IF method (Table 2). The agreement score (number of identical positive and negative results with the 2 methods) was 94.6% and disagreement was obtained in only 3 samples: 2 brain samples positive with the ABC method were considered as negative with the IF test and one lung sample negative with the ABC method was positive with the IF test. Consequently, the global relative specificity and sensibility of the IF method to the ABC method were 91.7% and 95.5% respectively. The IF relative sensibility and specificity were 100% in both the stomach and the spleen. By contrast, although no false negative results were obtained in lungs, the IF relative specificity in this organ was moderate (66.0%). The IF relative sensibility in the brain was 85.7%. On the other hand, the staining intensity observed with the 2 methods varied according to the tissues. Although this parameter was identical in 24/44 (54.5%) tissue samples, the ABC labelling was stronger in 14 samples (10 brain and 2 spleen samples) and was weaker in lungs than the fluorescent labelling.

The CD viral antigen immunolabelling was observed in nucleus, cytoplasm and cell processes of neurons especially in the cerebellum. Viral CD antigens were mainly localized in glia cells predominantly astrocytes in the substantia alba of the cerebellum (Figures 2a, 2b) and were also detected in neurons of the granular and molecular layers and in the Purkinje cells (Figure 3). In the lytic alterative Purkinje cells, viral particles were also prominent in the cell processes par-

### Table 1: Pathological findings and CD viral antigen immunolabelling by the ABC method and by the IF test in naturally CD infected dogs (n = 14).

<table>
<thead>
<tr>
<th>Lesions</th>
<th>Case number</th>
<th>Age (month) (mean ± SD)</th>
<th>CD antigen labelling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABC method</td>
</tr>
<tr>
<td>Brain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AE</td>
<td>5</td>
<td>2.5 ± 0.5</td>
<td>2+: 1 / 3+: 4</td>
</tr>
<tr>
<td>SAE</td>
<td>3</td>
<td>5.0 ± 0.0</td>
<td>2+: 1 / 3+: 2</td>
</tr>
<tr>
<td>CE/CME</td>
<td>4</td>
<td>8.8 ± 4.0</td>
<td>1+: 3 / 3+: 1</td>
</tr>
<tr>
<td>DM</td>
<td>2</td>
<td>2.0 ± 0.0</td>
<td>2+: 2</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IP</td>
<td>5</td>
<td>6.6 ± 5.0</td>
<td>0+: 1 / 1+: 2 / 3+: 3</td>
</tr>
<tr>
<td>CBP</td>
<td>6</td>
<td>3.6 ± 2.0</td>
<td>1+: 4 / 2+: 2</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td>5.0</td>
<td>1+: 1</td>
</tr>
<tr>
<td>Spleen / Lymph nodes</td>
<td>10</td>
<td>3.9 ± 1.0</td>
<td>0+: 7 / 1+: 2 / 2+: 1</td>
</tr>
<tr>
<td>Stomach / Duodenum</td>
<td>4</td>
<td>7.3 ± 4.0</td>
<td>2+: 2 / 3+: 2</td>
</tr>
<tr>
<td>Urinary tract</td>
<td>3</td>
<td>3.6 ± 3.0</td>
<td>1+: 1 / 2+: 2</td>
</tr>
</tbody>
</table>

particularly with the peroxidase method. Less frequent positive reactions were localized in neurons of the cerebral hemispheres, midbrain, medulla oblongata and medulla spinalis. Occasionally, labelling occurred in epitheliums of choroid plexus and in some meningeal cells. Moreover, the viral antigens were abundant especially in the Purkinje cells and the astrocytes of cerebellum in the 2 animals with only demyelization. The CD viral antigen staining intensity tended to be more pronounced in brain sections from acute, subacute and demyelization lesions than those from chronic lesions.

In the lung, intense diffuse or granular labelling was observed particularly in the alveolar lining epithelium, alveolar macrophages and syncytial cells (Figures 4a, 4b). Slight labelling was also detected in the epitheliums of the bronchi and bronchioles. Nevertheless, the CD viral antigen staining intensity was not closely related with any type of lung lesions.

Strong immunolabelling was also seen in the epithelial cells of the glands and crypts in the stomach and occasionally in the duodenum (Figures 5a, 5b). Slight positive reactions were localized in some epithelial cells of the urinary bladder, and in epitheliums of the tubules and pelvis renum in the kidneys. In the spleen, lymph nodes and tonsils, positive reactions were not strong, and restricted to sinusoidal macrophages and reticular cells. In the liver, CD immunolabelling was also evidenced in the Kupffer cells.

<table>
<thead>
<tr>
<th>ABC+</th>
<th>Overall</th>
<th>Brain</th>
<th>Lung</th>
<th>Spleen</th>
<th>Stomach</th>
</tr>
</thead>
<tbody>
<tr>
<td>44</td>
<td>14</td>
<td>11</td>
<td>7</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>IF+</td>
<td>43</td>
<td>12</td>
<td>12</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>ABC-</td>
<td>12</td>
<td>0</td>
<td>3</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>IF-</td>
<td>13</td>
<td>2</td>
<td>2</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>- (1+)</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>24</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>+ (1+)</td>
<td>14</td>
<td>12</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

| Relative SpIF/ABC | 91.7% | ND | 66% | 100% | 100% |
| Relative SeIF/ABC | 95.5% | 85.7% | 100% | 100% | 100% |
| Agreement score  | 94.6% |

TABLE 2: Comparison of the ABC method and the IF test for the detection of CD viral antigens in organs (brain, lung, spleen and stomach) from naturally CD infected dogs (n = 14).

Discussion

The most common cause of nervous symptoms and pneumonia in dogs is canine distemper infection. The clinical diagnosis of CD is particularly difficult when systemic signs, preceding neurological symptoms and/or accompanying respiratory symptoms are absent [7, 21]. Clinical findings with nervous signs and histopathological changes with acute to chronic demyelization encephalomyelitis and pneumonia are important for certifying the CD diagnosis [8, 10, 27]. However, inflammatory reaction may be absent in the CNS because of the immunosuppressive effect of CD infection especially in young puppies, and typical CD brain lesions could not always being evidenced [2, 10, 18, 20, 29]. Similarly, two cases in the present study showed only demyelization in the cerebellum. For this reason, histopathological brain analysis may not always sufficient for the CD diagnosis. In such cases, it is emphasized that the immunohistochemical CD viral antigen detection is of great diagnostic value [9, 14].

Analysis of lung lesions is also relevant for histological CD diagnosis but specific CD pulmonary lesions (i.e. interstitial pneumonia) are generally complicated by secondary bacterial pneumonia at necropsy. Intracytoplasmic and/or intranuclear inclusion bodies in epithelial cells of the bronchi and bronchioles are pathognomonic for CD, especially when coupled with the brain lesions [7]. Moreover, syncytial cells in alveolar lumina also supports CD, as described in peste des petits ruminants and human measles [7, 23]. In the present study, lung lesions were unequivocal among the cases. Interstitial to acute catarrhal pneumonia was observed while no lesion was observed in some cases. Therefore, CD viral antigen immunolabelling was essential to clarify the diagnosis. The both 2 immunohistochemical methods used in the
FIGURE 1: Lung. Syncytial cell formation with intracytoplasmic inclusions (arrows) in the alveolar lumen. HE. 40X.

FIGURE 2: Cerebellum. Viral labelling (arrows) in nucleus, cytoplasm, and cell processes of neurons in the substantia alba. A: ABC method. 20X. B: IF method. 20X.

FIGURE 3: Cerebellum. Viral CD antigens (arrows) in nucleus, cytoplasm and cell processes of Purkinje cells. ABC method. 20X.

FIGURE 4: Lung. CD viral antigen (arrows) immunolabelling within alveolar epithelial cells and macrophages. A: ABC method. 40X. B: IF method. 40X.

FIGURE 5: Stomach. Positive reactions (arrows) within epithelial cells of the glands. A: ABC method. 40X. B: IF method. 20X.
present study clearly revealed the occurrence of CD viral antigens especially in alveolar lining cells, alveolar macrophages, and bronchiolar epitheliums. Moreover, it was important that immunohistochemical methods were found to be highly sensitive in alveolar epitheliums of lungs and in brain sections which could present no histological lesion.

The ABC method would be more sensitive than the IF test, since the enzyme fixed on antibodies can have a continuous action on the substrate leading to the massive production of the reaction product and to the consequent amplification of the signal linked to the formation of immune complexes with CD antigens. By contrast, in the IF test, the observed fluorescence is directly proportional to the amount of CD antigens available in the specimen for binding labelled antibodies [14, 24]. In the present study, the 2 immunohistochemical methods were equally sensitive and specific for detection of CD viral antigens in spleen and stomach. In brain tissue, relative specificity and sensibility of the ABC method were over than the IF method. Furthermore, the ABC especially in chronic encephalitis was more useful than the IF. Although the relative specificity in the lungs was being to the fluorescent method’s advantage, the ABC method appeared to be more advantageous for CD diagnosis since this method required the utilisation of more simplified microscopes and allowed the storage of stained slides for a long time.

Lymphoid depletion or necrosis or both are recognized as features of Morbillivirus infections [1, 7]. In the present study, viral antigens were occasionally found particularly in reticuloid cells and sinusoidal macrophages and were less evident in cells of the lymphocytic series, in accordance with previous observations on rinderpest and peste des petits ruminants [5, 23]. TAJIMA and USHIJIMA [19] hypothesized that reticuloid cells are much less susceptible than lymphocytes to destruction by rinderpest virus. Similar situation has been also noted in canine distemper and PPR [7, 23]. It seems probable that the slight immunoreactivity in lymphoid tissues in the present study was the result of severe destruction of the lymphoid tissues.

Natural infection of dogs results in a high incidence of brain lesions despite the absence of evident neurological signs [12, 28]. The occurrence and development of the different types of CD encephalitis depend on the age of the animals at the time of infection, of the virus strain, and of the efficiency of the immune response [11, 17]. Histologically, brain lesions have been classified as early lesions, acute, subacute and chronic lesions [11]. In this study, 12 dogs showed acute to chronic demyelization encephalomyelitis and two dogs had only demyelization as early lesion. Immunohistochemical methods revealed that CD viral antigen immunolabelling was found especially in the brain in accordance with the histological lesions. The CD viral antigens were abundant in cerebellopontine areas with disseminated demyelization and reactive gliosis. Positive labelling of neurons and astrocytes was much stronger in acute encephalomyelitis with demyelization lesions. However, it gradually decreases or disappears in areas with progression of the intense inflammation, neuronal degeneration and necrotic changes especially in chronic lesions, in agreement with other studies [2, 3]. TOPLU and ALCIGIR [24] have also emphasized that intense inflammatory reactions have caused the weakness of viral antigens in the CNS during avian encephalomyelitis (genus hepatitis, family Picornaviridae). It is interpreted that pronounced CD viral antigen weakness in such cases of CD is due to circulating antibodies or restricted protein synthesis and/or rapid degradation [3, 13]. On the other hand, the increased positive staining cells for acute CD encephalitis could be due to strong viral replication in the early stage of infection.

Immunohistochemistry could not perfectly reveal the immunolabelling of CD viral antigen in cases with chronic encephalitis. Therefore, it is suggested that molecular biological methods would be useful especially in brain sections with chronic lesions for certifying the CD diagnosis.

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References