Histologic and Immunocytochemical Characterization of Canine Distemper-associated Metaphyseal Bone Lesions in Young Dogs Following Experimental Infection

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Abstract. The proximal metaphyses of the humerus of weanling gnotobiotic dogs experimentally infected with canine distemper virus (CDV) were investigated histologically and immunocytochemically between 4 and 41 days after infection. Viral antigen was demonstrated in hematopoietic marrow and bone cells at postinfection day (PID) 5 and PID 7, respectively. Between PID 8 and 27, CDV antigen was abundantly present in marrow cells, osteoclasts, and osteoblasts and less frequently in osteocytes. Immunopositive cells in both osseous tissues and bone marrow declined between PID 29 and PID 36 and were absent by PID 41. Chondrocytes of the growth plate were negative for viral antigen throughout the observation period. In bone, viral antigen was more frequently observed in bone cells of the primary spongiosa than in the secondary spongiosa. There was a strong correlation between occurrence of CDV antigen and osseous changes. Associated metaphyseal bone lesions were mild and most prominent between PID 8 and PID 32. Lesions consisted of necrosis of osteoclasts, which was associated with subsequent persistence of the primary spongiosa (growth retardation lattice). Atrophy and necrosis of osteoblasts and marrow cells were also noted. Infection of metaphyseal bone cells appears to be common in young dogs with experimental systemic distemper. Bone cell infection is preceded by infection of marrow cells, and infected bone cells may experience degeneration and necrosis. This subtle viral effect may result in defects in bone modeling in CDV-infected dogs.

Key words: Bone; canine distemper virus; dogs; metaphysis; osteoclasts; Paget's disease; paramyxovirus.

Canine distemper virus (CDV), closely related to measles virus (MV), belongs to the genus *Morbillivirus* of the family *Paramyxoviridae*. The host spectrum of CDV comprises dogs, many species of carnivores, marine mammals, and monkeys. Distemper in dogs has been associated with clusters of multiple sclerosis; however, this observation has not been confirmed. Epidemiologic studies showed a possible link between dog ownership and patients with Paget's disease of bone. Recently, CDV RNA was demonstrated in Paget's disease bone tissues, suggesting that CDV or a closely related morbillivirus may play an important etiologic role in this disease. However, other studies have failed to support this hypothesis.

In the dog, CDV infection may result in a completely subclinical disease or may produce several different distinct clinical and pathologic manifestations, depending on the main organ(s) involved. In general, lesions are most prominent in the respiratory and alimentary tracts or in the central nervous system (CNS). Enamel hypoplasia has been observed in adult dogs who experienced CDV infection during permanent tooth formation.

The pantropic nature of CDV is demonstrated by the presence of viral antigens in many organs and tissues and in many different cells. Few studies have mentioned the presence of CDV antigen or RNA in bones and associated lesions of dogs with distemper.

Human Paget's disease of bone (= osteitis deformans) is a focal or multifocal chronic illness of the skeleton associated with an increase in bone resorption and formation, which leads to progressive destruction and remodeling. In most cases, this bone disorder remains unnoticed; when patients do have clinical problems, symptoms arise from nerve impingement, pain, pathologic fractures, bone deformity, and tumorlike proliferations. Several techniques, including electron microscopy, immunohistology, in situ hybridization, and the polymerase chain reaction, have been used to identify viral structures, antigens, or RNA in Pagetic bone lesions. Viruses thus far associated with the Paget's disease bone lesions include MV, respira-
Table 1. Microscopic lesions in dogs experimentally infected with canine distemper virus.

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<th>Dog No.</th>
<th>PID*</th>
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* Postinoculation day.
† Age at death.
‡ - = none; + = minimal; ++ = mild; +++ = moderate; ++++ = severe.

...tory syncytial virus, simian virus 5, human parainfluenza virus 3, and CDV. Similarly, MV antigen has been demonstrated in human active otosclerosis.

Little is known about morbillivirus infection of bone cells and the resultant osseous lesions. Moreover, the pathogenesis of virus-induced bone lesions and mechanisms that result in new bone formation or destruction of existing osseous tissue are still undetermined. The purpose of the present study was to investigate sequentially the cellular tropism and the distribution of viral antigen in the metaphyses of young dogs experimentally infected with CDV and to correlate these findings with microscopic lesions.

Materials and Methods

Experimental protocol and tissue sampling

Thirty-eight outbred, colostrum-deprived gnotobiotic Beagle dogs were inoculated intraperitoneally between 19 and 75 days of age with 0.2 ml freshly thawed 20% spleen-thymus homogenate in RPMI 1640 medium containing 10^4.5 TCID_50/ml of the R252 CDV strain as previously described. CDV nucleoprotein-specific monoclonal antibodies were unsuitable for demonstrating viral antigen in glutaraldehyde-fixed tissues (W. Baumgärtner, personal observation). Therefore, a polyclonal polyspecific or a nucleoprotein-specific...
(rabbit No. 162, provided by C. Orvell, Karolinska Institute, Stockholm, Sweden) rabbit anti-CDV antibody was used as the primary label. Both were employed at a dilution of 1:1,000, and to reduce nonspecific staining they were adsorbed with 10% (w/v) liver powder prior to use. Immunocytochemical staining of CDV antigen was performed as described previously.\textsuperscript{2,13} Tissues were deparaffinized in xylene and hydrated through graded alcohols, and endogenous peroxidase was quenched with 0.5% H\textsubscript{2}O\textsubscript{2}, diluted in methanol. After washing in Tris-buffered saline (TBS, 0.1 M Tris-base, 0.9% NaCl, pH 7.6), sections were incubated with undiluted normal swine serum for 10 minutes at room temperature in a moist chamber to block nonspecific binding sites. Excess serum was removed without rinsing, and sections were incubated with the primary antibody overnight at 4°C, followed by biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) as link-antibody and the avidin–biotin–peroxidase complex (ABC, Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. All antibodies were diluted in TBS. Between each incubation step, sections were washed three times for 5 minutes each with TBS. Positive antigen–antibody reaction was visualized by incubation of the slides with 0.2% 3,3'–diaminobenzidine–tetrahydrochloride in 0.1 M imidazole, pH 7.1, containing 0.01% H\textsubscript{2}O\textsubscript{2}, for 10 minutes. Sections were slightly counterstained with hematoxylin and mounted with Corbit-Balsam (Hecht, Kiel-Hasse, Germany). CDV-specific immunoreaction was scored based on the number of CDV antigen-positive cells in each section: – = none; (+) = single cells; + = few; ++ = moderate; +++; = numerous. The data points in the histogram represent the mean values obtained after evaluation of all animals at that time interval: – = 0; (+) = 0.5; + = 1; ++ = 2; +++ = 3. Controls for immunocytochemistry included 1) bone tissue from control dogs, 2) omission of the primary antibody, link antibody, or ABC, or 3) incubation of the sections with control rabbit serum instead of the primary antibody. Immunostaining was done in batches, and in each series, the same tissue block from a CDV-positive animal was included as internal control for staining specificity and intensity.

**Results**

**Distribution of viral antigen**

Viral antigen was visualized as a dark brown precipitation product within cytoplasm and nuclei of bone and bone marrow cells. In areas of marrow necrosis, positively stained canine distemper virus (CDV) antigen had a fine granular appearance.

Viral antigen distribution and cell tropism were similar in the primary and secondary spongiosa and varied with number of days postinfection; however, in the zone of secondary spongiosa immunostaining was more prominent in bone marrow than in bone cells. Findings in both compartments are condensed to one description in Fig. 1.

Both bone tissue and bone marrow were virus negative when examined on postinfection day (PID) 4. At PID 5–7, a few immunopositive bone marrow cells were detected in the marrow stroma associated with vascular channels (Fig. 2). Their number increased steadily, and between PID 8 and PID 14 numerous cells in the bone marrow were immunopositive (Fig. 3). Viral antigen was observed in many different cell types, including myeloid and erythroid cells, megakaryocytes, vascular endothelium, and mononuclear intravascular cells. Between PID 20 and PID 36, the number of immunopositive bone marrow cells declined slightly.

In osseous tissue, viral antigen was first detected at
This lesion was observed in four animals after PID 25. Marrow cells among the metaphyseal trabeculae was interpreted as depletion/atrophy of the bone marrow. Evidence of empty lacunae as indication for necrotic osteocytes, and there was no evidence of empty lacunae as indication for necrotic osteocytes. Between PID 26 and PID 32, was characterized by long straight cartilage cores with variable amounts of apposed bony matrix (Figs. 7, 8). There was no bridging between trabeculae with formation of thicker secondary spongiosa more distal than normal in the metaphysis. The metaphyseal bone lesion was not accompanied by inflammatory cells, and there was no evidence of empty lacunae as indication for necrotic osteocytes.

Discussion

The present study confirms and extends previous observations on CDV infection and associated lesions of bone tissue. Light microscopic findings varied at different days postinfection (Table 1, Fig. 6). The earliest change detected was necrosis of osteoclasts, which was characterized by pyknosis and karyorrhexis. This lesion, which was not detected until PID 8, was usually of minimal severity. The percentage of dogs affected was highest at PID 8–14 and subsequently declined until PID 41. Between PID 23 and PID 32, necrosis and atrophy of osteoblasts and bone marrow cells were present. The necrosis was characterized by pyknosis, karyorrhexis, and increased cytoplasmic eosinophilia. Osteoblast necrosis was an infrequent finding and usually of mild severity. Mild to moderate osteoblast atrophy, characterized by flattened lining cells on primary spongiosa trabeculae, was frequently observed. On PID 23 and PID 24, mild to moderate bone marrow necrosis was present in two dogs. The finding of fewer than normal marrow cells among the metaphyseal trabeculae was interpreted as depletion/atrophy of the bone marrow. This lesion was observed in four animals after PID 25. Persistence of primary trabeculae, most prominent between PID 26 and PID 32, was characterized by long straight cartilage cores with variable amounts of apposed bony matrix (Figs. 7, 8). There was no bridging between trabeculae with formation of thicker secondary spongiosa more distal than normal in the metaphysis. The metaphyseal bone lesion was not accompanied by inflammatory cells, and there was no evidence of empty lacunae as indication for necrotic osteocytes.

Histologic findings

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Discussion

The present study confirms and extends previous observations on CDV infection and associated lesions of bone tissue. Although the age of the animals varied moderately at different days postinfection, lesions and viral antigen distribution were similar. CDV antigen was demonstrated in marrow cells, osteoblasts, osteoclasts, and osteocytes. The close temporal relationship suggests that the proximate source of virus to osseous tissues is the virus-infected bone marrow. At the end of the observation period, CDV antigen appeared more prominent in metaphyseal osteoclasts. Associated metaphyseal bone lesions, most prominent between PID 8 and PID 32, were characterized by osteoclast necrosis and persistence of primary spongiosa. In addition, atrophy and necrosis of bone lining and bone marrow were noted. Following viral clearance at PID 41, most bone lesions had resolved.

Many CDV antigen-positive bone marrow cells have been observed following aerosol infection of pathogen-free Beagles in the early phases of infection. In addition, CDV nucleoprotein and phosphoprotein genes were demonstrated in marrow cells, osteoclasts, osteocytes, and osteoclasts of distemper-infected dogs. In areas with reduced active bone formation and remodeling, such as the secondary spongiosa, viral antigen was less prominent in bone cells. These findings may indicate that metabolically active bone cells are more susceptible to CDV infection than are resting bone cells. The chondrocytes of the growth plate were negative for CDV antigen, suggesting that these cells are refractory to infection or do not allow productive infection with virus protein synthesis. Although, osteoclast necrosis was still demonstrated at PID 41, immunocytochemically CDV antigen was no longer present in these cells. Similarly, there was no immunocytochemical indication of viral persistence in bone cells; however, this observation needs to be pursued by more sensitive methods such as in situ hybridization and polymerase chain reaction and by inclusion in the study of more dogs in late convalescence. Although some bones were stored for up to 4 years in aldehyde solutions prior to decalcification and tissue blocks were used several years after embedding in par-
There was a strong correlation between occurrence of CDV antigen and osseous lesions. The first and most frequently observed change, necrosis of osteoclasts, was associated with strong virus-specific immunostaining of these cells, indicating a direct virus-induced cytolytic effect. In this context, persistence of the primary trabeculae is not an unexpected complication of failure to model and is called a growth retardation lattice. The retention of primary trabeculae secondary to inhibition of osteoclasis is likely the pathogenesis of the observed lesions. Surprisingly, although osteoblast necrosis and atrophy were observed, no morphological consequences of these lesions, i.e., osteopenia, were found. The osteosclerosis might be a reflection of the imbalance between formation and resorption at earlier stages when osteoclast necrosis was already present and osteoblasts were still intact.

Whether distemper-associated bone lesions occur as a separate entity in dogs without signs of systemic disease remains to be determined. A possible role of CDV has been suggested in inflammatory joint diseases of the dog. In several reports, an infectious cause has been suspected in hypertrophic osteodystrophy (HOD), a metaphyseal bone disease in young dogs. However, changes characterized by subepiphyseal hemorrhages, infiltration of neutrophilic inflammatory cells, and trabecular microfractures are different from CDV-induced metaphyseal bone lesions as observed in the present study. However, distemper virus transcripts have been demonstrated in bone cells of dogs with HOD, suggesting that CDV-associated bone lesions may appear differently in naturally occurring disease. In distemper-infected dogs, both sense and antisense probes bound to bone cells using in situ hybridization, whereas in HOD, as in human Pagetic bone, only CDV-N gene-specific antisense probes hybridized with CDV RNA. These findings led to the suggestion that viral genome is not present in large amounts, maybe due to genomic mutations that still allow transcription but preclude active replication.
The metaphysis of young dogs represents an area of greatly enhanced osteoblastic and osteoclastic activity and resembles, metabolically, Paget's disease bone tissue. In the latter, disorganized woven bone, reduced medullary spaces full of vascularized fibrous tissue, lymphomonocytic infiltration, and increased osteoclastic activity have been observed. In the dog, however, CDV-associated bone lesions were transient and were characterized by osteoclast necrosis, persistence of primary spongiosa, and immunocytochemically there was no evidence of viral persistence in bone cells. As described for Paget's disease of bone, CDV was found in osteoblasts, osteoclasts, and osteocytes of dogs.

This study shows a strong correlation between CDV infection and osseous lesions in young dogs. Bone infection appears to be part of a generalized viremia; therefore, the biological significance of the observed bone lesion remains to be determined. However, the findings indicate that CDV infection of young dogs represents a useful model to study the pathogenesis of paramyxovirus-induced bone lesions.

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References