Neutrophilic Granulocytic Sarcoma and Acute Basophilic Leukemia in Cattle

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Abstract
A case of neutrophilic granulocytic sarcoma and a case of acute basophilic leukemia are described. The former was diagnosed in an 8-year-old Holstein cow with a large intrathoracic tumor mass (case 1), and the latter in a 14-month-old Holstein heifer with a rapid increase in white blood cell count (case 2). In case 1, the tumor mass was composed of agranular cells and more mature cells with eosinophilic granules staining positive for naphthol AS-D chloroacetate esterase (CAE) and macrophage, myeloid/histiocyte antigen (clone MAC387). In case 2, the tumor tissues consisted chiefly of myeloblastoid cells. However, differentiation to myelocytoid or metamyelocytoid cells was observed, and their intracytoplasmic eosinophilic granules were positive for tryptase (a marker for basophils and mast cells), but not for CAE (a marker for neutrophils and mast cells). Although reports of myeloid neoplasms are rare in cattle, as documented here, it is not difficult to diagnose them on the basis of histology, histochemistry and immunohistochemistry.

Discipline: Animal health
Additional key words: esterase staining, immunohistochemistry, myeloid leukemia, myeloid sarcoma

Introduction
In humans, granulocytic sarcoma (included in the classification of myeloid sarcoma) is a localized tumor of myeloblasts or immature myeloid cells, which can present before, simultaneously with or after a diagnosis of acute or chronic myeloid leukemia (Puranen et al. 2006, Chan 2007). Neutrophilic and eosinophilic granulocytic sarcomas have been documented in swine (Kadota et al. 1987, Brum et al. 2012). Due to the presence of distinct cytoplasmic granules easily detectable in sections stained with hematoxylin and eosin (HE), it is not difficult to make a diagnosis of eosinophilic leukemia, but to the best of our knowledge, no reports are available in cattle. Other myeloid leukemias may have been confused with lymphoid neoplasms in cattle and swine, because the reported cases are far fewer than expected from the abundance of normal hematopoietic cells (Kadota et al. 1987, Watanabe et al. 1998, Ikehata et al. 2011, Murayama et al. 2011, Ogihara et al. 2012).

In cattle, there are published cases of hematopoietic malignant neoplasia such as acute neutrophilic leukemia (Takayama et al. 1996), acute mastocytic-megakaryocytic leukemia (Ikehata et al. 2011), acute erythremic myelosis (pure erythroid leukemia) (Watanabe et al. 1998), mast cell sarcoma (Fujimoto et al. 2012), and histiocytic sarcoma (Anjiki et al. 2000). The reported cases of monocytic leukemia (Mackey et al. 1972), myelomonocytic leukemia (Woods et al. 1993) and mast cell leukemia (Trautwein & Stöber 1965) are devoid of convincing histological

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Received 9 November 2017; accepted 14 May 2018.
evidence. Though not examined in sufficient detail, the histology of two acute basophilic leukemias has been described previously (Takahashi et al. 2006, Murayama et al. 2011). An additional case has recently been reported to be composed of cells of the basophil series, based solely on the observation of purple intracytoplasmic granules in blood and bone marrow smears (Laabs et al. 2015). Unfortunately, in that paper, basophil tumor cells could not be distinguished from malignant mast cells, which also contain granules staining purple with Giemsa (Ikehata et al. 2011, Fujimoto et al. 2012). In the present study, we report a case of neutrophilic granulocytic sarcoma and another of acute basophilic leukemia. Giemsa and naphthol AS-D chloroacetate esterase (CAE) staining and immunohistochemistry were very helpful in their diagnosis.

Materials and methods

1. Animals

Case 1 was an 8-year-old Holstein cow that was slaughtered because of emaciation, anorexia, reduced milk production, brisket edema, and jugular vein distension. The white blood cell count (WBC) was 4,000/μl. An enzyme-linked immunosorbent assay for detecting bovine leukemia virus (BLV) antibodies was performed using a kit (JNC, Tokyo, Japan) with positive results. Case 2 was a 14-month-old Holstein heifer that was examined because of anorexia and edematous swelling of the udder. Enlarged lymph nodes were noted on rectal palpation. Blood examination revealed anemia (hematocrit, 15.7%) and thrombocytopenia (platelet count, 53,000/μl), but the WBC was 5,900/μl. Six days later, the animal had a temperature of 39.9°C, with a pulse rate of 120/minute. Multiple abdominal tumor masses were palpated on rectal examination. Nineteen days after the initial examination, the animal was euthanized because of severe emaciation, complete loss of appetite, and hematuria. Just before euthanasia, the hematocrit, platelet count, and WBC were 9.0%, 37,000/μl, and 12,700/μl, respectively. On blood smears stained with Diff-Quik (Silverman & Frable 1990), there were 87% atypical cells, 10% lymphocytes, and 3% neutrophils. The atypical cells were various in size, and all had purple granules in the cytoplasm. This stain was also performed on tissue smears. Real-time polymerase chain reaction for BLV provirus using a kit (TaKaRa, Shiga, Japan) revealed 4,160, 240, and 160 copies/100 ng DNA in samples from buffy coat, mesenteric, and lateral iliac lymph nodes, respectively.

2. Histological, histochemical, and immunohistochemical examination

Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with HE, Giemsa, and CAE using paraarosaniline (Nakarai Chemicals, Kyoto, Japan) (Lennert & Feller 1992). Immunohistochemistry was carried out by the streptavidin-biotin complex/horseradish peroxidase (SAB) method on paraffin sections using a commercially available Histofine kit (Nichirei, Tokyo, Japan) and mouse monoclonal antibodies to macrophage, myeloid/histiocyte antigen (MMA) (clone MAC387; Dako A/S, Glostrup, Denmark), CD68 (clone EBM11; Dako A/S), macrophage (clone HAM56; Dako Corporation, Carpinteria, CA, USA), and tryptase (clone AA-1; Lab Vision, Fremont, CA, USA). Additionally, rabbit polyclonal antibodies to factor VIII-related antigen (Nichirei), hemoglobin (Lipshaw Immunon, Pittsburgh, PA, USA), CD3 (Dako A/S), CD20 (Spring Bioscience, Pleasanton, CA, USA), and CD31 (Spring Bioscience) were utilized. Antigen retrieval was performed by microwave heating in 10 mM citrate buffer, pH 6.0 (macrophage, tryptase) or 10 mM Tris buffer, pH 9.0 (CD31), at 90°C for 9 minutes or enzymatic digestion with 0.05% pepsin (Merck, Darmstadt, Germany) at 37°C for 25 minutes (MMA, CD68, factor VIII-related antigen, CD3). In case 2, small pieces of formalin-fixed tissues were post-fixed in 1% osmium tetroxide and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by electron microscopy (EM).

Results

1. Gross pathology

In case 1, a large flattened tumor mass (38 × 30 cm) was found adherent to the heart, which was displaced caudally, with no pericardium between them. There were several tumor nodules up to 2 cm in diameter on the auricles and in the kidneys. Several white areas, 3-5 mm in diameter, were present on the hepatic capsule. The bronchial lymph nodes were moderately enlarged, and on cut sections, the tissues were homogeneous and edematous, occasionally with necrotic foci or hemorrhagic dots. The medial iliac and hepatic lymph nodes were also enlarged.

In case 2, the medial retropharyngeal, deep cervical, tracheobronchial, mammary, medial and lateral iliac, and iliofemoral lymph nodes were markedly or moderately enlarged. The spleen was also enlarged to 60 × 16 cm, with bulging cut surfaces. The sternal and femoral bone marrow had a grayish-white color. There were multiple tumor nodules with hemorrhage on the urinary bladder...
mucosa, as well as three white areas up to 7 × 3 cm in size on the ventral border of the lungs. Arcuate vessels were clearly visible in the kidneys, with ureter wall thickening. The cerebral and spinal dura mater was partially thickened. On smears obtained from the deep cervical and lateral iliac nodes and stained with Diff-Quik, atypical cells were large in size, and a few of them contained basophilic granules with a tendency to accumulate.

2. Histological, histochemical, and immunohistochemical findings

On histological examination, in case 1, the thoracic flattened tumor mass was composed of diffusely proliferating tumor cells, which were also noted in nodules in the auricles and kidneys. The visceral pleura was extensively invaded by tumor cells. The bronchial lymph nodes were replaced by tumor tissues, but some normal lymphatic tissues were still present. The medial iliac and hepatic lymph nodes were slightly to moderately affected. There were small numbers of tumor cells in the medullary cord of the superficial cervical and mesenteric lymph nodes. Tumor cell accumulations were seen in places in the splenic red pulp but were rare in the liver. Cytologically, two types of cells, blast cells and more mature granular cells, were seen in tumor tissues (Fig. 1A). The blast cells predominated in number and were variable in size, with round, oval, or elongated nuclei with inconspicuous nucleoli and finely dispersed or stippled chromatin. The cytoplasm was moderate in amount in some larger cells, but scant in others. Many granular cells resembled the normal counterpart (neutrophil series), but atypical cells were noted as well (Fig. 1B). The cells

![Image](A) Bronchial lymph node. Well-differentiated tumor cells with lobulated nuclei and eosinophilic cytoplasmic granules are visible. Agranular tumor cells are frequently similar in size to the differentiated cells. HE. Bar = 5 μm. (B) Tumor adjacent to the heart. As in HE-stained sections, intracytoplasmic granules stain red. The arrow indicates an atypical granular tumor cell. Giemsa. Bar = 5 μm. (C) Bronchial lymph node. Differentiated tumor cells corresponding to promyelocytes have red granules and tend to accumulate. CAE. Bar = 50 μm. (D) Bronchial lymph node. In this field, there are MMA-positive tumor cells frequently with lobulated nuclei, admixing with MMA-negative immature cells. SAB. Bar = 5 μm.
were in various differentiation states, mainly at stages of myelocytes or metamyelocytes. These granular cells were positive for CAE (Fig. 1C) and were immunostained for MMA (Fig. 1D), but not for the other markers tested. Mitotic figures were plentiful in agranular blast cells, but few in granular cells.

In case 2, the bone marrow was nearly completely replaced by leukemia cells, which tended to invade the sternal periosteum. Residual normal hematopoietic cells were few in number and intermingled with tumor cells. Splenic sinuses were crowded with many neoplastic cells, with considerable extramedullary erythropoiesis. In the highly enlarged lymph nodes examined, neoplastic tissues predominated, with some remnants of surviving lymphatic tissues. Neoplastic involvement was moderate in less enlarged nodes. Several neoplastic cells were found within hepatic sinusoids. In addition to massive neoplastic growths, intravascular leukemia cells were conspicuous in the lungs. Neoplastic infiltration or accumulation was observed within cerebral and spinal epidural spaces, and in various organs such as salivary glands, mammary glands, heart, adrenal glands, kidneys, ureters, urinary bladder, uterus, and oviducts. Cytologically, the majority of leukemia cells were large blastoid cells with round to oval nuclei, small- to medium-sized nucleoli, slightly to moderately condensed chromatin, and moderate amounts of weakly basophilic cytoplasm. Intracytoplasmic granules were sometimes seen in these cells (Fig. 2A). More mature forms resembling myelocytes or metamyelocytes contained granules stained red with

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**Fig. 2.** Histology and immunohistochemistry in case 2

(A) Deep inguinal lymph node. Myeloblastoid cells and intervening reactive stroma are observed, and one of them contains granules in the cytoplasm (arrow). Giemsa. Bar = 5 μm. (B) Deep inguinal lymph node. Leukemia cells, with cytoplasm filled with eosinophilic granules, correspond to metamyelocytes or myelocytes, and are not easy to distinguish from relatively mature tumor cells of the neutrophil series (see Fig. 1A). HE. Bar = 5 μm. (C) Deep inguinal lymph node. Relatively mature leukemia cells have purple cytoplasmic granules (left) and are smaller in size than more immature cells (lower right). Giemsa. Bar = 10 μm. (D) Deep inguinal lymph node. An accumulation of relatively mature leukemia cells with tryptase-positive granules is seen in this field. SAB. Bar = 50 μm.
HE (Fig. 2B) and purple with Giemsa (Figs. 2A and 2C) and tended to accumulate (Figs. 2C and 2D). The granules were negative for tryptase (Fig. 2D), but not for CAE and other immunohistochemical markers for lymphohematopoietic cells. Most tumor tissues were characterized by stromal fibrosis, blood vessel hyperplasia detected by CD31 staining for vascular endothelial cells, prominent lymphocyte and macrophage infiltration, and slight eosinophil infiltration. Among these infiltrating cells, CD3 positive lymphocytes were the largest in number. Mitoses were seen mainly in large blastoid cells.

3. Ultrastructural findings

In case 2, blast cells with no or few intracytoplasmic granules were characterized by slightly condensed chromatin and poorly developed organelles. More mature cells with many granules had more condensed chromatin and slightly developed organelles (rough endoplasmic reticulum and mitochondria). The granules varied in electron density (Fig. 3A) and were particulate or finely granular (Fig. 3B). Golgi apparatus, glycogen granules, cytoplasmic projections, or abundant cytoplasmic filaments were observed in occasional cells with many granules.

Discussion

In general, acute leukemia is histologically characterized by the presence of many leukemia cells within splenic sinuses, hepatic sinusoids and pulmonary capillaries at relatively early stages of tumor development (Takayama et al. 1996, Watanabe et al. 1998, Kagawa et al. 2009, Ikehata et al. 2011, Yokota et al. 2015). Although agranular tumor cells predominated in case 1, differentiation to more mature forms was evident. The mature cells had granules stained red with Giemsa and CAE (a marker for neutrophils and mast cells) and positive for MMA (clone MAC387), but not for macrophage markers, CD68 and macrophage (clone HAM56). These features correspond to those of neutrophilic leukemia (Takayama et al. 1996, Anjiki et al. 2000). Although this case was judged to be at an advanced stage of disease from clinical and macroscopic findings, the WBC was relatively low. In addition, localized neoplastic lesions were observed in the spleen, liver, and lungs, and a large tumor mass was seen adjacent to the heart. Based on these findings, a diagnosis of neutrophilic granulocytic sarcoma of thoracic cavity origin was made (Puranen et al. 2006, Chan 2007). In de novo myeloid sarcomas in humans, which occur without evidence of pathological involvement of the bone marrow and peripheral blood (Campidelli et al. 2009), patients may never develop acute myeloid leukemia (Riddle & Olsen 2012). In case 1, it is highly probable that a large tumor mass adherent to the heart caused circulatory disturbances leading to brisket edema, jugular vein distension, and emaciation. The animal’s very bad condition indicates that this type of sarcoma does not terminate as an acute leukemia.

In contrast to case 1, the bone marrow, spleen, and lungs were heavily infiltrated with leukemia cells in case 2. Due to the fact that intracytoplasmic granules stained purple with Giemsa and positive for tryptase (a marker for basophils and mast cells) but negative for CAE, a diagnosis of acute basophilic leukemia was established (Takahashi et al. 2006, Murayama et al. 2011). This diagnosis is supported by ultrastructural observations.
of intracytoplasmic granules with a particulate content. Such granules are characteristic of basophilic leukemia in cattle (Takahashi et al. 2006), whereas granules with a homogeneous content are observed in mast cell tumors (Madewell et al. 1984, Iwabuchi et al. 1987, Ikehata et al. 2011).

In routine histological sections, it is not easy to identify eosinophilic granules in acute granulocytic leukemias or to distinguish between the current two cases. Additionally, normal basophils can be readily confused with neutrophils or eosinophils in cattle (Kawashima et al. 2016) and cats (Fairley & Shackleton 2013). In contrast, Giemsa staining for histology is helpful in distinguishing between granules of basophil or mast cell neoplasms and those of neutrophilic or eosinophilic granulocytic leukemia, or granular T cell lymphomas (Takayama et al. 1996, Nozaki et al. 2006, Naitou et al. 2007, Ikehata et al. 2011, Fujimoto et al. 2012).

In case 1, some tumor cells were capable of differentiating into fully mature cells, and tumor cells corresponding to metamyelocytes, myelocytes, or promyelocytes were also observed. However, agranular tumor cells, which were frequently of a similar size to granular cells, were morphologically dissimilar to myeloblasts. Such cytological deviation from the normal may be linked to a loss of the ability to enter blood vessels. In contrast, in case 2, nearly all leukemia cells resembled their normal counterparts, and discrete differentiation up to the stage of metamyelocytes or myelocytes was detected. The disparity between the numbers of granular cells in blood smears and in tissue sections implies that more mature cells are liable to invade the bloodstream (Laabs et al. 2015), although these cells were mitotically less active.

Unlike in the previously reported cases (Takahashi et al. 2006, Murayama et al. 2011), reactive infiltration of many lymphocytes and macrophages was seen in case 2, and fibrosis and mild eosinophil infiltration were also noted. In addition, the number of leukemia cells with granules was much larger than in the previous cases. Marked eosinophil infiltration is observed in bovine well-differentiated (Fujimoto et al. 2012) but not in less-differentiated cutaneous mastocytoma (unpublished data). Mast cell degranulation causes the development of pulmonary fibrosis in humans (Veerappan et al. 2013), and intestinal sclerosing mast cell tumors have been reported in cats (Halsey et al. 2010). The stromal reactions accompanying leukemia in case 2, which are probably related to cytoplasmic granules or cytokines, suggest the presence of similar functions in normal bovine basophils.

The current cases were neoplasms of the myeloid series and were considered to have no relation to BLV. The relatively low numbers of viral copies in the lymph nodes of case 2 support this view (Somura et al. 2014). However, large numbers of copies cannot always be linked to a diagnosis of BLV-associated lymphoma, since they may be observed also in healthy cattle infected with BLV (Chiba et al. 2014). Likewise, a diagnosis of basophilic leukemia could not be established by demonstrating the presence of purple cytoplasmic granules in blood smears (Laabs et al. 2015), because similar granules are also seen in malignant mast cell tumors (Ikehata et al. 2011, Fujimoto et al. 2012). Bilineal leukemia or sarcoma is not rare in cattle and swine (Kadota et al. 1987, Ikehata et al. 2011, Fujimoto et al. 2012), and it is not easy to detect the secondary predominating lineage on blood smears. Virological or blood examination is only supplementary for the diagnosis of lymphohematopoietic neoplasms in cattle. A definitive diagnosis of a neoplasm should be made on the basis of histology, histochemistry, and immunohistochemistry, especially before establishing it as a distinct disease entity.

References


