Perforin-Like Immunoreactivity in Feline Globule Leukocytes and Their Distribution

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(Received 23 February 1994/Accepted 18 August 1994)

ABSTRACT. Distributional and immunohistochemical characteristics of the feline globule leukocyte (GL) were investigated by light microscopy. The GL, which contained eosinophilic large granules in the cytoplasm, was frequently found in the epithelia of the intestine and gall bladder, and less frequently found in those of the gastric pit, intrahepatic bile duct, and interlobular secretory duct of the pancreas. No GL was seen in the respiratory and urogenital organs. The GLs composed a homogeneous cell population including no mast cells according to the following histochemical stainings; phosphotungstic acid hematoxylin, alcian blue and peroxidase. The feline GL showed perforin-like immunoreactivity to anti-human perforin monoclonal antibody, but did not show histamine-immunoreactivity to anti-histamine polyclonal antibody. The results suggest that the feline GL is a lineage of large granular lymphocytes. The epitheliotropism and the characteristics as granular lymphocytes of the feline GLs were similar to those of the intestinal $\gamma\delta$ T cells of the mouse.—**KEY WORDS:** feline, globule leukocyte, large granular lymphocyte, perforin, $\gamma\delta$ T cell.

J. Vet. Med. Sci. 56(6): 1101–1105, 1994

Globule leukocytes (GLs) are commonly found in the epithelia of the intestinal, respiratory, and urogenital tracts of many animal species as well as human and birds [1, 11, 18]. The GL is morphologically characterized by eosinophilic large cytoplasmic granules and a lymphocyte-like nucleus. Current theories propose that in some ruminants and laboratory rodents, particularly the mouse and rat, GLs originate from mucosal mast cells rather than lymphocytes [6, 14, 16]. However, some authors claim that large granular lymphocytes (LGL) are a possible source of GLs in mammals [2–4]. It was confirmed by an immunohistochemical study using anti-chicken thymocyte serum that chicken GLs are intraepithelial granulated T lymphocytes [10]. Thus, the origin of GLs in mammals remains to be determined. As the cat is an important animal species in comparative anatomy as well as veterinary clinic and has GL with particularly large cytoplasmic granules [17], we have chosen the cat to study GLs.

In this study, we have used a mouse anti-human perforin monoclonal antibody (mAb) to examine the cellular origin of the feline GL. Perforin, also termed as pore-forming protein, is one of the granule contents of the granulated lymphocytes including natural killer (NK) cells, cytotoxic T lymphocytes (CTLs) and $\gamma\delta$ T cells in human, rats and mice, which play an important role in killing target cells [19].

In the present paper, we report a perforin-like immunoreactivity in the granules of the feline GLs, suggest a LGL lineage of the feline GLs, and discuss similarities of the feline GLs to murine $\gamma\delta$ T cells.

MATERIALS AND METHODS

Animals and Tissue preparation: Seven adult cats consisting of 3 intact males, 2 intact females and 2 spayed females were obtained from the Animal and Epidemic Control Center of Sapporo City (Sapporo, Japan). The accurate ages of the cats were unknown. But as no tooth

loss was observed, all the cats were identified as young. They were healthy by physical examination. All the cats were euthanized with an overdose of sodium pentobarbital (Nembutal, Abbot Laboratories, North Chicago, U.S.A.). By necropsy, all the cats were confirmed to be free from pathological changes and internal parasites.

Tissue samples were collected from the following regions; caudal lobe of the lung, thoracic parts of the trachea and esophagus, body of the stomach, duodenum, jejunum, ileum, cecum, colon, gall bladder, left lateral lobe of the liver, right lobe of the pancreas, non-pregnant uterus, kidney, urinary bladder, thymus, and spleen. Pregnant-uteri were collected from another series of 3 cats of spayed at Hokkaido University Veterinary Medicine Teaching Hospital. These cats were at the stage of late pregnancy, and their fetuses were 9 to 11 cm in crown-rump length.

Segments of these tissues were fixed in Bouin's solution for 6 hr, Carnoy's solution for 3 hr or 4% paraformaldehyde (PFA) in 0.2 M phosphate-buffered saline (PBS) overnight, then dehydrated, cleared and embedded in paraffin. Two- μ m sections were cut with a conventional microtome, and mounted on glass slides. Dried sections were deparaffinized, rehydrated, and stained according to the following methods:

Histochemistry: Sections prepared from tissues fixed in Bouin's solution were stained with hematoxylin and eosin (H&E) and phosphotungstic acid hematoxylin (PTAH) to detect GLs [17]. Other sections prepared from tissues fixed in Carnoy's solution were stained with alcian blue (0.1% w/v in 0.7 N HCl) and safranin O (0.5% w/v in 0.125 N HCl) to detect mast cells [12]. In addition, peroxidase staining was performed by the following procedure: The dissected tissues were rapidly frozen in liquid nitrogen. Then cryostat sections of 6 μ m were cut and mounted onto gelatin-coated slides. The sections were fixed in 10% neutral buffered formalin for 30 min. After rinsing in PBS, the sections were developed with diami-

nobenzidine (DAB) and slightly counterstained with hematoxylin.

Immunohistochemistry: For perforin-immunostaining, sections prepared from the tissues fixed in 4% PFA/PBS were rinsed in 50 mM Tris-HCl buffer (pH 7.6) and then treated with 0.5% periodic acid for 15 min. After rinsing in Tris buffer, the sections were blocked with 5% normal goat serum in PBS for 30 min at room temperature (RT) and incubated with a mouse anti-human perforin monoclonal antibody (mAb), 1B4 at a dilution of 1:400, at 4°C over night. This mAb strongly crossreacts with mouse and rat perforin. After rinsing, the sections were incubated with biotinylated goat anti-mouse IgG (Tago, Inc., U.S.A.) at a dilution of 1:500 for 1 hr at RT. Following further rinsing, the sections were reincubated with ABC for 30 min. After rinsing, the sections were developed with DAB and slightly counterstained with hematoxylin. As a negative control, non-immunized mouse ascites (Cederlane, Canada) was used replacing the primary antiserum at a dilution of 1:100.

For histamine-immunostaining, sections prepared from tissues fixed in Bouin's solution were subjected to the peroxidase-antiperoxidase (PAP) procedure. The sections

were treated with 0.3% H₂O₂ in methanol for 30 min. After rinsing in PBS, the sections were blocked with 5% normal goat serum for 30 min at RT and incubated with rabbit anti-histamine polyclonal antibody (Chemicon, California, U.S.A.) at dilution of 1:300 at 4°C over night. The sections were rinsed, then incubated with goat anti-rabbit IgG at dilution of 1:200 for 1 hr at RT. Following further rinsing, the sections were reincubated with PAP complex for 1 hr at RT. After rinsing, the sections were developed with DAB and slightly counterstained with hematoxylin. As a negative control, non-immunized rabbit serum was used replacing the primary antiserum at dilution of 1:100.

RESULTS

Distribution of feline GLs and histochemistry: The GL was characterized by eosinophilic large granules in the cytoplasm (Fig. 1a). In this study, distribution of GLs was restricted to the gastrointestinal tract and its associated ducts, such as the bile ducts and the pancreatic secretory duct, and to the spleen of all the cats. No GL was observed in the thymus, respiratory organs and urogenital

Fig. 1. a: Feline globule leukocytes (arrows) in the villus epithelium of the jejunum. The cells have prominent eosinophilic large granules in their cytoplasm. H & E staining. $\times 700$.
b: Perforin-like immunoreactive cells (arrowheads) in the villus and crypt epithelia of the jejunum. These cells were confirmed as globule leukocytes by examination of adjacent serial sections stained with H & E. Perforin-immunoperoxidase staining. $\times 700$.

organs including the pregnant and non-pregnant uteri.

In the gastrointestinal tract, GLs were frequently seen in the epithelia of the duodenum, jejunum, ileum, cecum and colon. Very few number of GLs were seen in the epithelium of the stomach with their distribution limited to the gastric pit. In the small intestine, GLs were concentrated at the base of the villus and at the opening portion of the crypt, whereas in the cecum and colon they were randomly distributed. However, GLs were hardly seen in the lamina propria of the small intestine. Their distribution and frequency both in the epithelium and in the lamina propria varied to some degree between the different cats used in this study. Histological examination revealed no parasitic infection in the gastrointestinal tract, gall bladder and liver, and no erosion, no ulcer and no hyperplasia of epithelial components. Thus, the cause of the distributinal and frequency variation remained unknown. The exact correlation of the frequency between epithelial GLs and lamina propria GLs is currently under a histometric study.

In the biliary system, GLs were frequently found in the epithelium of the gall bladder, and less frequently found in the interlobular bile duct of the liver. In the duct system of the pancreas, a few GLs were seen in the interlobular

secretory duct. There was no correlation of the frequency between biliary GLs and pancreatic GLs. No GL was detected in the subepithelium of the bile duct and the secretory duct of the pancreas.

GLs were not observed in the splenic white pulp, but were rarely seen in the red pulp.

The cytoplasmic granules of the GL in various tissues were exclusively stained brownish black with PTAH, so that the GLs in the spleen and the lamina propria of intestine were easily confirmed by their stainability. But they were not stained with alcian blue and peroxidase in contrast to mucosal mast cells.

Immunohistochemistry: Perforin-like immunoreactivity was observed in intraepithelial mononuclear cells of various tissues where the GLs were distributed, and the reactivity was obviously intense in cytoplasmic granules (Fig. 1b). These intraepithelial perforin-like immunoreactive cells were confirmed as GLs by observation of adjacent serial sections stained with H&E. Other immunoreactive cells to anti-perforin mAb were rarely seen in the lamina propria of the intestinal mucosa and in the splenic red pulp. But we failed to confirm these cells as GLs by examination of adjacent serial sections stained with H&E. These cells had clear or very faint pinkish

Fig. 2. a: A histamin-immunoreactive cell (arrowhead) in the lamina propria of the jejunum. Histamin-immunoperoxidase staining. $\times 700$.
b: A globule leukocyte showing no histamin-immunoreactivity (arrowhead). Jejunum. Histamin-immunoperoxidase staining. $\times 700$.

cytoplasm and lymphocyte-like nuclei, and also, the cells showed a diffuse pattern of immunoreactivity. In the parenchyma of the liver and in the splenic white pulp, perforin-like immunoreactive cells were not seen. No reaction was detected in discrete mononuclear cells including GLs in sections of the intestines and spleen by non-immunized mouse ascites used as negative control.

Histamine-immunoreactive cells were located in the lamina propria and submucosa of the intestine, but intraepithelial mononuclear cells including GLs showed no reactivity (Figs. 2a and 2b).

DISCUSSION

The distribution, histochemical characteristics and ultrastructure of the GL in healthy cats have been reported by Takeuchi *et al.* [17].

In this study, the GL was seen in the gastrointestinal tract, gall bladder, and spleen. These results were in agreement with those of Takeuchi. However, we noticed, for the first time, GL in the intrahepatic bile duct and the secretory duct of the pancreas. Although rare GLs have been reported in the tracheal and bronchial epithelia of the cat by Takeuchi, we failed to detect GLs in these organs.

Previous studies on human and mouse perforin have revealed that LGLs including NK cells, $\alpha\beta$ T cell receptor (TcR)-positive cytotoxic T lymphocytes (CTLs) and $\gamma\delta$ TcR-positive T lymphocytes ($\gamma\delta$ T cells) express this lytic factor in their granules [9, 15]. Although cat perforin has not been isolated, the intense immunoreactivity of feline GLs to the mouse anti-human perforin mAb indicates the existence of perforin or perforin-like protein in the cat. Expression of perforin is limited to the LGLs in some animal species. Therefore, perforin-like immunoreactivity in the feline GL suggests a LGL lineage of the feline GL. As mentioned before, LGLs are classified into 3 cell types, NK cells ($CD3^-$), CTLs ($CD3^+$) and $\gamma\delta$ T cells ($CD3^+$). It is necessary to determine which cell type is identical with the feline GL. In the 3 cell types, $\gamma\delta$ T cells have been found to differ dramatically from CTLs and NK cells in property of specific homing to epithelial surfaces [7, 8]. The epitheliotropism and the characteristics as granular lymphocytes of the feline GL are similar to those of $\gamma\delta$ T cells of other animals, particularly mice and sheep [5, 13].

The present study revealed another perforin-immunoreactive cells in the lamina propria of the intestine and in the splenic red pulp, which were not identical with GLs by observation of adjacent serial sections stained with H&E. They also showed an immunoreactive pattern different from that of GLs. The GL has been thought to migrate from the lamina propria to the epithelium [2, 11]. It is necessary to investigate whether the perforin-immunoreactive cells are precursors of GLs or other cell type of LGL lineage.

In the mouse and rat, since it is known that GLs as well as lamina propria mast cells are stainable with alcian blue following fixation with Carnoy's solution [12] and have

serotonin [6], many investigators suggest that GLs are derived from mast cells. In this study, we performed alcian blue staining, peroxidase staining and histamine-immunostaining, but GL showed results different from those of mast cells. We have confirmed that both granulated lymphocytes and mast cells within the gastrointestinal epithelium of the mouse possess large eosinophilic granules in their cytoplasm, identifying both cells as murine GLs (Konno A, unpublished data). This heterogeneity of murine GLs, probably like that of rat GLs, may be the cause of the controversy on the derivation of the GL.

Further investigations are certainly required to define the immunological surface phenotype, function and fate of the feline GL.

ACKNOWLEDGEMENT. Dr. M. Nakata (Sumitomo Electric Industries Corporation, Japan) is gratefully acknowledged for the gift of monoclonal antibody directed to human perforin.

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