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Elevated autorosette formation by lymphocytes of dogs affected with cyclic neutropenia (CN)

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Summary. Cyclic neutropenia (CN) is an inherited disorder characterized by regularly recurring episodes of neutropenia in humans and Gray Collie dogs. Early thymic hypotrophy and lymphoid exhaustion in the CN dogs suggests there may be a lymphoid cell differentiation defect. Later manifestations of CN in dogs include arthritis, anaemia, glomerulonephritis, and amyloidosis which are often associated with autoimmune diseases. Several Coombs' antiglobulin tests were performed and did not detect autoantibodies, however, peripheral blood lymphocytes from CN dogs formed rosettes with their own erythrocytes while in normal dogs such rosettes were extremely rare. Furthermore, when lymphocytes from CN dogs were rosetted with erythrocytes from normal dogs, the numbers of allogeneic rosettes were comparable to those formed with autologous erythrocytes. These results suggest strongly that the rosetting lymphocytes are specific for common erythrocyte surface components. Although the physiological importance of the autorosetting phenomenon is not known, the frequency of autorosette formation in CN dogs, as reported here, suggests that it may be an early indication of developing autoimmune activity.

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INTRODUCTION

Cyclic neutropenia (CN) is an inherited disorder characterized by regularly recurring episodes of neutropenia in both humans and Gray Collie dogs (Rutledge, Hansen-Prüss & Thayer, 1930; Lund, Padgett & Ott, 1967). Dogs affected with CN experience a precipitous drop in the number of circulating neutrophils at approximately 12 day intervals. For about 3 days during each neutropenic episode, the dogs are highly susceptible to microbial infections. They commonly experience fever, anorexia, conjunctivitis, rhinitis, and other signs characteristic of intestinal or respiratory infections (Lund *et al.*, 1967; Dale, Alling & Wolff, 1972). Most CN dogs die by 1 year of age. However, with antibiotics and supplementary fluid treatment, they may survive longer, but recurrent infections continue. Among surviving dogs, arthritis, especially of the carpal joints, becomes apparent during the neutropenic episodes. Additionally, most of the dogs have moderate anaemia (Lund, 1969; Lange *et al.*, 1975), and postmortem examination commonly reveals amyloidosis and glomerulonephritis (Cheville, Cutlip & Moon, 1970). In both humans (Miyazaki,

1974) and dogs (Reynolds, Dale, Wolff & Johnson, 1971; T. J. Yang, unpublished observation) with CN, serum immunoglobulin levels are about twice those of normal individuals.

Since some of these later manifestations are also characteristic of many autoimmune states, several Coombs' antiglobulin tests were performed to determine whether autoimmune antibodies were present. Although no autoantibodies were detected, microscopic examination of both the antiglobulin-containing and the saline control pellets revealed a surprisingly high number of autorosettes formed by the lymphocytes of CN dogs. In contrast, identically treated lymphocytes of normal dogs formed few or none. Autorosetting techniques were then used to determine the incidence of autorosette formation and to quantify any differences between normal and CN dogs.

MATERIALS AND METHODS

Media

Serum-free RPMI1640 (Grand Island Biological Co., Grand Island, New York), supplemented with glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml) was used for all dilutions and washes.

Blood collection

Venous blood (3 ml) from two CN and two normal dogs and a CN-normal bone marrow transplant chimera (Jones, Yang, Dale & Lange, 1975; Angus, Wyand & Yang, 1978; Yang, 1978) was collected into heparinized tubes (preservative-free sodium heparin; Fellows Medical Manufacturing Co., Inc., Oak Park, Michigan), centrifuged for 15 min at 400 *g* at 4°, and the buffy coat and red blood cells removed for rosette formation.

Rosette preparation

Autorosettes were prepared according to the method of Sandilands, Gray, Cooney & Browning (1975). The erythrocytes were washed twice in medium and diluted to 0·5% of packed cells. The buffy coat was diluted to 3 ml in medium, layered over 3 ml cold Ficoll-Paque (Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey), and centrifuged for 30 min at 400 *g* at 4°. The interface was washed three times and diluted to 4×10^6 cells/ml in medium. Equal volumes (0·25 ml) of lymphocyte and erythrocyte suspensions were mixed and centrifuged at 200 *g* for 5 min at room temperature. The tubes were incubated for 2 h in an ice bath, gently resuspended, and the contents fixed by the addition of 0·5 ml of fresh 3% glutaraldehyde (Ladd Industries, Burlington, Vermont) for 20 min at room temperature. After addition of 5 ml of distilled water to each tube, they were inverted and centrifuged at 200 *g* for 5 min at room temperature. The pellet was gently resuspended in 0·25% trypan blue saline solution. A drop was placed on a slide and the coverslip ringed with melted vaseline. Over 500 lymphocytes were counted and rosettes reported as a percentage of total cells counted. This method results in preparations found to be stable for at least 24 h with no apparent disruption of the rosettes. Smears were also made and stained with Giemsa for identifying cell types (Angus, 1977).

RESULTS

A high percentage of lymphocytes from CN dogs 12 and 14 formed rosettes with their own erythrocytes, while few lymphocytes from normal dogs 06 and 23 and a CN dog with a normal bone marrow transplant 310 formed such rosettes (Table 1). During the preliminary microscopic examination of Coombs' test cell pellets, occasionally autorosettes were seen in preparations from normal dogs, but the CN dogs had three to ten times as many. The CN

Table 1. Percentage of autorosette forming lymphocytes in peripheral blood of normal and cyclic neutropenia (CN) dogs

Animal status and number	Autorosettes* (%)
Cyclic neutropenia, No. 14	8·5†
Cyclic neutropenia, No. 14	2·2‡
Cyclic neutropenia, No. 12	2·5†
Cyclic neutropenia, No. 12	1·0‡
Cyclic neutropenia-normal bone marrow transplant chimera, No. 310	0·0§
Normal Collie, No. 23	0·0
Normal mixed breed, No. 06	0·0

*Autorosettes were prepared according to the method of Sandilands *et al.* (1975)

†This result occurred during a neutropenic episode.

‡This result occurred during a non-neutropenic stage.

§No autorosettes seen from among 500 mononuclear cells counted.

Table 2. Percentage of rosette formation between lymphocytes and autologous or allogeneic erythrocytes

Lymphocyte source	Erythrocyte source	Rosettes* (%)
CN, No. 14†	CN, No. 14	2·2‡
CN, No. 14	Normal, No. 23	2·2
CN, No. 12	CN, No. 12	1·0
CN, No. 12	Normal, No. 23	0·5
Normal, No. 23	Normal, No. 23	0·3
Normal, No. 23	CN, No. 14	0·2

*Rosettes were prepared according to the method of Sandilands *et al.* (1975).

†CN = dogs affected with cyclic neutropenia.

‡This result occurred during a non-neutropenic, non-fever stage.

dog, CN14, with the more severe clinical signs had 8·5% autorosette-forming cells during the nadir of neutrophil counts (Table 1). During a non-neutropenic period, the number of autorosettes in CN14 was decreased to 2·2%, the level found for the more healthy CN dog 12 (Table 2). Normal dogs always formed fewer than 0·3% autorosettes (Tables 1 and 2). In order to exclude the possibility that the elevated autorosette formation during neutropenia was due to immune specific lymphocytes attaching to erythrocytes having bound bacterial antigens (Duffus & Allan, 1969), or immunoglobulin-coated erythrocytes, as occur in malarial infection in mice (Lustig, Nussenzweig & Nussenzweig, 1977), lymphocytes from neutropenic dogs were rosetted with erythrocytes from normal dogs. The levels of allogeneic rosettes were comparable to those of the autologous ones in CN dogs (Table 2), indicating that the autorosettes were the result of binding of lymphocytes to common surface components present on both autologous and allogeneic erythrocytes.

DISCUSSION

Although the physiological importance of the autorosetting phenomenon is not known, it has been much discussed. Autorosettes have been frequently observed in thymocyte preparations from various species: man, mice, rats, rabbits, pigs, cattle, sheep, and guinea-pigs (Micklem, Asfi, Staines & Anderson, 1970; Baxley, Bishop, Cooper & Wortis, 1973; Sandilands, 1974; Kaplan, 1975). In peripheral blood however, autorosettes are less common but are formed by a small percentage of T lymphocytes (Sandilands, *et al.*, 1974; Kaplan, 1975; Charreire & Bach, 1975).

In the present study, lymphocytes from CN dogs formed autorosettes more frequently than lymphocytes from normal dogs (Table 1). Since CN dogs manifest some symptoms such as arthritis and glomerulonephritis (Cheville, 1968; Lund, 1969), which are often associated with autoimmune disease, it is tempting to speculate that the increased numbers of autorosetting cells may be related to autoimmunity.

Postmortem examination of CN dogs reveals early thymic hypotrophy (Angus *et al.*, 1978) and lymphoid exhaustion (Cheville, 1968; Gregory, Machado & Jones, 1977), suggesting that their increased autorosette formation might be similar to that of adult thymectomized and nude (athymic) mice, where the spleen cells are found to have higher than normal numbers of autorosetting cells (Charreire & Bach, 1975). Since thymic factor is believed to be required for the maturation of T lymphocytes to cells which can distinguish self from non-self (Goldstein, Guha, Howe & White, 1971) the elevated autorosette formation in CN dogs may be a consequence of their thymic hypotrophy.

Burnet (1969) suggested that the thymus was the major site of stem cell differentiation to immunocytes and also the primary site of censorship of self-reactive immunocytes. He stated that autoimmune disease has been associated with thymic atrophy in a patient with systemic lupus erythromatosus, and in F_1 NZB × NZW mice, where varying levels of thymic cortex atrophy are found. Since the thymus in CN dogs is hypotrophic, it may, therefore, be unable to act as a censor of immunocytes reactive against self-antigens. These cells could then proliferate, resulting in various autoimmune states.

There are several reasons to suspect a defect in lymphoid cell differentiation in CN dogs: (1) Their early thymic hypotrophy and lymphoid exhaustion; (2) The diminished blastogenic response of lymphocytes from some CN dogs to T cell mitogens (Angus *et al.*, 1978); and (3) The evidence provided by reciprocal bone marrow transplantation that the major cause of the CN syndrome is a defect in bone marrow stem cell differentiation (Dale & Graw,

1974; Weiden, Robinett, Graham, Adamson & Storb, 1974; Jones *et al.*, 1975). A defect of lymphoid cell differentiation could result in an increase in self-reacting lymphocytes. The finding by Sandilands *et al.* (1974) that there is an increase in autorosette formation in melanoma patients supports the hypothesis that suppressor cells are somehow defective.

The elevated autorosette formation in the CN dogs may be an early sign of a defect of differentiation or maturation of lymphocytes, or a defect of the censorship mechanism. The resultant proliferation of self-reactive clones or failure to suppress the proliferation of altered cells might result in active autoimmune disease or cancer with time. Indeed, the one CN dog known to survive 2–1/2 years died from hepatic carcinoma with multiple metastases (Lund, 1969).

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