The Inhibitory Effect of Circulating Lymphocytes on Granulopoiesis in Human Cyclic Neutropenia in Vitro

MASAMITSU INOUE, KATSUNORI YAMADA, YOJI ISHIDA,
KENJI SHINOHARA, TOSHIO KANEKO and NOBORU
MATSUMOTO*

*The Third Department of Internal Medicine, Yamaguchi
University School of Medicine and *School of Allied Health
Science, Yamaguchi University, Ube 755*

INOUE, M., YAMADA, K., ISHIDA, Y., SHINOHARA, K., KANEKO, T. and MATSUMOTO, N. *The Inhibitory Effect of Circulating Lymphocytes on Granulopoiesis in Human Cyclic Neutropenia in Vitro*. Tohoku J. exp. Med., 1984, **143** (2), 213-220 — The effect of peripheral mononuclear cells (MNCs), lymphocytes and sera of a patient with typical cyclic neutropenia (CN) upon the normal colony forming unit in culture (CFU-C) growth was examined. MNCs and lymphocytes obtained during the recovery phase of the cycles inhibited CFU-C growth. In contrast, these cells obtained during the neutropenic phase revealed no suppressive effect. Serum inhibitors of granulopoiesis were not detected at any time during the cycle. These findings suggest that MNCs, especially lymphocytes, may be one of the contributory factors for the development of CN in this case. ——— cyclic neutropenia ; CFU-C ; lymphocytes

Cyclic neutropenia (CN), observed in both humans and gray collie dogs, is characterized by the periodic oscillation of circulating neutrophils. The cause of cycling has been shown to be fluctuating production of cells by the bone marrow in both humans and dogs (Dale et al. 1972a, b ; Guerry et al. 1973 ; Patt et al. 1973 ; Meuret and Fliedner 1974). In gray collie dogs, a congenital disorder of the pluripotential stem cells has been confirmed by bone marrow transplantation (Dale and Graw 1974 ; Weiden et al. 1974 ; Jones et al. 1975), and cyclic production of all blood elements has been shown in the animal (Dale et al. 1972b ; Weiden et al. 1974). In human, however, the exact etiology of CN remained unclear and this condition, which appears to be acquired in some individuals and congenital in others, may occur by several pathogenic mechanisms (Wright et al. 1981).

The concept of negative feedback regulation in myelopoiesis with respect to the etiology of CN is appealing. There is experimental evidence that differentiated neutrophils and their products may suppress the proliferative capacity of

myeloid progenitor cells (Broxmeyer 1978 ; Herman et al. 1978). Recently, Barr and Stevens (1982) have reported a significant role of T lymphocytes subpopulations in the regulation of human granulopoiesis. In canine cyclic hematopoiesis, it is reported that bone marrow adherent cells from the dogs with this disease alternatively inhibit and stimulate in vitro granulopoiesis of normal dog marrow (Jones and Jolly 1982). Stimulated by these observations, we have studied the effect of circulating mononuclear cells, lymphocytes and serum from a patient with CN on the colony forming unit in culture (CFU-C) growth of the normal bone marrow. Our results suggest that the lymphocytes of this case is one of candidates for inhibitors on granulopoiesis.

MATERIALS AND METHODS

Patient

The patient studied was a 24-year-old female who has had episodes of fever, malaise and aphthous stomatitis every 21 days from the age of 10. When she was first admitted to the Yamaguchi University Hospital at the age of 19, a diagnosis of CN and cystinuria was established. Therapy with prednisolone in a dose of 20 mg every other day for three months and lithium carbonate 600 mg daily for three months was ineffective. She is currently seen at the out-patient clinic and a short course of antibiotics has been prescribed during neutrophilic nadirs. Her parents and an elder sister have neither hematological problems nor cystinuria.

Separation of circulating mononuclear cells (MNCs) and lymphocytes

Patient's blood was obtained from the antecubital vein into heparinized syringe. MNCs were collected by Ficoll-Metrizoate (Nyegaard & Co. Oslo) gradient. After washing two times MNCs were diluted to 1×10^6 /ml in alpha-medium. Lymphocytes were harvested by removal of adherent cells after incubation of MNCs in a glass petri dish at 37°C for 30 min.

Preparation of bone marrow cell suspension

Bone marrow cells were obtained from the patient and normal volunteers into heparinized syringe and diluted in alpha-medium. The heparinized samples were treated by Ficoll-Metrizoate centrifugation to obtain a mononuclear cell suspension, which was then diluted to the final concentration of 2×10^6 /ml. Patient's bone marrow was aspirated before and just after the peak of neutrophil count, and at the time of neutrophilic nadir. Bone marrow cells from normal volunteers were used for co-culture assay with patient's MNCs and lymphocytes.

CFU-C assay

Bone marrow culture was performed using semisolid agar culture methods of Robinson et al. (1967). One ml of solution containing 10% of human placental conditioned medium as the source of colony stimulating activity (Burgess et al. 1977), 20% fetal calf serum, 0.3% Bacto-agar (Difco) and 2×10^5 marrow mononuclear cells was placed in a 35 mm plastic culture dish (Lux), and incubated at 37°C in a humidified atmosphere of 5% CO₂. After 7 days incubation, colonies of more than 40 cells were enumerated with an inverted microscope.

Co-culture study

Bone marrow mononuclear cells (2×10^5 /ml) from normal donors were mixed with

patient's MNCs, lymphocytes ($1 \times 10^5/\text{ml}$), or serum at the final concentration of 10% in a semisolid agar solution and incubated for 7 days as described above. CFU-C assay was performed in quadruplicate, and the results were expressed as percent of control colony growth. As control CFU-C assay, marrow cells from the same normal subjects were cultured without adding MNCs, lymphocytes or serum. Statistical evaluation of results was performed with Student's *t* test.

RESULTS

The oscillation of circulating neutrophils, lymphocytes and monocytes, and the number of CFU-C colonies during the cycle are shown in Fig. 1. Neutrophil counts regularly fluctuated with approximately 21-day periodicity. During the neutropenic periods, neutrophils completely disappeared from the peripheral blood for 3–5 days. The cyclic oscillation of monocyte counts was also observed, and the duration of monocytes cycle was the same as that of neutrophils, peak monocyte counts occurring near the end of neutropenic periods. Cyclic fluctuations of lymphocyte, reticulocyte and platelet counts were not observed in this case.

Bone marrow CFU-C growth of this patient showed the tendency to fluctuate (Fig. 1). The numbers of CFU-C were 88.0 ± 12.7 , 71.3 ± 11.5 and 39.8 ± 6.7 just before and after the peak of neutrophil counts, and at the neutrophilic nadir respectively. Thus, the number of CFU-C was significantly reduced at the neutropenic period compared to that of the recovery phase from neutropenia. However, even at the time of neutrophilic nadir, the number of CFU-C was not decreased, compared with those of hematologically normal subjects ($120\text{--}40/2 \times$

Fig. 1. Fluctuation in the number of CFU-C colonies of the patient's bone marrow cells (upper panel) and serial neutrophil, monocyte and lymphocyte counts (lower panel) during the cycle. Bars represent standard deviation of means. ●—●, neutrophils; ○—○, monocytes; △—△, lymphocytes.

10^5 marrow cells).

As shown in Fig. 2, co-culture studies revealed the inhibitory effect of patient's MNCs and lymphocytes on the normal CFU-C growth. When the peripheral neutrophil count was more than 0.3×10^9 /liter, addition of MNCs and lymphocytes reduced the normal CFU-C growth to $53.0 \pm 4.8\%$ and $54.9 \pm 11.8\%$ respectively. However, MNCs and lymphocytes obtained at the neutrophilic nadir showed no significant suppression of CFU-C growth ($84.0 \pm 3.3\%$ and $108.7 \pm 7.7\%$ respectively). As these results suggested that the peripheral lymphocytes of this case might be a possible regulator of granulopoiesis, the T-cell subset was determined with monoclonal antibodies. The number of peripheral T cells (OKT3), inducer-helper T cells (OKT4), cytotoxic-suppressor T cells (OKT8) and B lymphocytes (OKIa) showed no significant fluctuation during the cycle. However, there was a tendency that cytotoxic-suppressor T cells were slightly increased between the neutrophilic nadirs ($33.6 \pm 2.3\%$), compared with those at the time of neutropenia ($28.3 \pm 2.1\%$). To examine the effect of soluble factors derived from T lymphocytes, peripheral T cells obtained at the neutrophilic nadir and between nadirs were incubated for 48 hr at 37°C in the presence or absence of Con A (Wako Chemical). These supernatants, however, revealed neither inhibitory nor stimulative effect on the normal CFU-C growth (data are not shown). Of interest was the absence of response of T lymphocytes to mitogens. The response of peripheral T lymphocytes of this patient to Con A and PHA-P was both one third of the normal control at any time during the cycle.

As shown in Fig. 2, addition of the patient's serum obtained at the neutropenic period and recovery phase had no influence on the growth of CFU-C from the normal bone marrows ($108.4 \pm 22.0\%$ at neutropenic period, and $100.3 \pm 25.9\%$

Fig. 2. Effects of patient's peripheral mononuclear cells (MNC), lymphocytes (Lym) and sera on the CFU-C growth from normal bone marrow (NBM). Mononuclear cells and lymphocytes obtained between neutrophilic nadirs significantly inhibit normal CFU-C growth (* $p < 0.01$).

at the recovery phase).

DISCUSSION

The mechanism of cyclic hematopoiesis in gray collie dogs has been studied extensively, and it is generally accepted that the disorder is the result of periodic pluripotential stem cell failure (Patt et al. 1973 ; Weiden et al. 1974). Recently Krance and coworkers (1982) reported a patient who acquired CN from her histocompatible sibling donor following bone marrow transplantation as treatment for acute lymphoblastic leukemia. Their clinical observations provided strong evidence to support the concept that human CN is also caused by a regulatory defect in hematopoietic stem cell. However, there still remains a possibility that other components of the marrow are also responsible for the development of CN, because not only the pluripotential stem cells but also other blood elements including lymphocytes and fibroblasts are also transferred to the recipient by bone marrow transplantation. As pointed out by Wright et al. (1981), inferences from canine cyclic hematopoiesis must be applied to human CN with some caution. Indeed, there are several differences between these two diseases. The period length is 21 days in man and 12 days in dogs. The human disease appears to be acquired in at least some cases, and cyclic hematopoiesis in dogs is an autosomal recessive disorder. In gray collie dogs, the cyclic fluctuation of the neutrophil is usually associated with fluctuations in lymphocytes, platelets and reticulocytes (Lund et al. 1967 ; Dale et al. 1972a, b ; Patt et al. 1973). On the contrary, cycling in the counts of blood elements other than neutrophils is observed in the limited patients (Wright et al. 1981). Finally, it is shown that lithium carbonate therapy abrogates the recurrent neutropenia in canine cyclic hematopoiesis (Hammond and Dale 1982), but this therapy is ineffective for human CN (Hammond et al. 1983). From these observations, it is suggested that the biochemical basis of human CN differs from that of canine cyclic hematopoiesis (Hammond et al. 1983), and that more than one mechanism is involved in determining the rate of production and release of granulocytes from the bone marrow (Morley and Stohlman 1970).

Studies of patients with CN showed that neutrophils have a normal survival in circulation and that periodic interruption of marrow cell production causes cycling of neutrophil counts (Guerry et al. 1973 ; Meuret and Flidner 1974). As one of important humoral regulators of granulopoiesis, the production of colony-stimulating factor (CSF) has been studied in human CN (Greenberg et al. 1976 ; Mangalik and Robinson 1973 ; Mizoguchi et al. 1976 ; Moore et al. 1974). These observations have shown that cycling in the peak levels of CSF in the plasma and urine coincides with the peak blood monocytes numbers. Moore et al. (1974) have proposed a "monocyte feedback model" to explain the alternate cycling of monocytes and neutrophils in CN. In their model, levels of CSF favor monocytopenia. As monocytes increase, they produce more CSF, resulting in increased

differentiation of neutrophils and a decrease in monocytes. The latter results in a fall in CSF and thus limits granulopoiesis. This mechanism, however, would not explain the simultaneous cycling of monocytes and neutrophils seen in the patient reported by Adams and Liu (1982). Furthermore, CSF has been found to cycle out of phase with blood neutrophils even when the monocyte count has remained relatively constant (Chikkappa et al. 1980). Thus, the significance of CSF with respect to the etiology of CN remains undetermined.

Recently, cellular regulation of erythropoiesis (Haq et al. 1983) and granulopoiesis (Barr and Stevens 1982) by lymphocytes has been reported. Barr and Stevens (1982) examined the influence of helper and suppressor T cells in co-culture with autologous hematopoietic progenitor cells. They found that helper T cells produce an increase and suppressor cells a decrease in the number of granulocyte-macrophage colonies. Their results suggest that cellular regulation of human granulopoiesis is effected, at least in part, by lymphocyte subpopulations. To the best of our knowledge, the pathogenetic role of lymphocytes in human CN has not been described. In the present study, the peripheral lymphocytes obtained during the recovery phase of the cycles inhibited the normal CFU-C growth, while those obtained at the neutropenic period revealed no inhibitory effect. These findings suggest that the peripheral lymphocytes is one of probable regulators of granulopoiesis, at least in this case. Considering the stimulative and inhibitory effects on the granulopoiesis by T lymphocyte subpopulations reported by Barr and Stevens (1982), we prospected that lymphocyte subsets might fluctuate during the cycles, and that the lymphocytes obtained at the recovery phase might produce an inhibitory substance *in vitro* after Con A activation. Contrary to our expectation, lymphocyte subpopulations identified by monoclonal antibodies showed no significant fluctuation. In addition, soluble factors which are produced by activated lymphocytes and inhibit CFU-C growth were not demonstrated. Therefore, the mechanism by which the peripheral lymphocytes inhibit the normal CFU-C growth remains unknown. In this case, the numbers of bone marrow CFU-C fluctuated during the cycles, but comparing to the numbers of CFU-C from normal marrows, those of CFU-C of this patient were not decreased even at the time of neutrophilic nadirs. This finding suggests that the result of *in vitro* granulopoiesis does not necessarily reflect the *in vivo* granulopoiesis in human CN. Although these studies showed the periodic inhibition of *in vitro* granulopoiesis by the lymphocytes, it is not possible to decide at the present time whether or not the circulating lymphocytes play a crucial role in the development of CN. During the preparation of this manuscript, Osborne et al. (1983) reported abnormal purine and pyrimidine metabolism in canine cyclic hematopoiesis, suggesting a possibility that this metabolic derangement is primarily responsible for the defective neutrophil proliferation. As one of future problems, biochemical approaches may be required to clarify a pathogenetic mechanism of this unusual disease.

Acknowledgment

This study was supported in part by the Research Grant for the Intractable Diseases from the Ministry of Health and Welfare of Japan.

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