Human Cyclic Neutropenia: Urinary Colony-stimulating Factor and Erythropoietin Levels

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Daily levels of urinary colony-stimulating factor (CSF) were measured in two patients with cyclic neutropenia. The CSF levels increased during the neutropenic period concomitant with the maximum peripheral monocytosis. Simultaneous erythropoietin (ESF) levels were measured in one of these patients. ESF also increased in the neutropenic period antecedent to the periodic reticulocytosis. These studies are compatible with a negative feedback system regulating both granulocytopoiesis and erythropoiesis. Whether the cyclic fluctuations of these substances are causally related to the cyclic marrow proliferation is uncertain.

Human cyclic neutropenia is characterized by the periodic disappearance of blood neutrophils at approximately 21-day intervals. There are also cyclic changes in the levels of the other formed elements of the blood: monocytes, lymphocytes, eosinophils, reticulocytes, and platelets. These changes are attributable to regular interruption and activation of marrow proliferation, that is, to cyclic hematopoiesis. Possible mechanisms to explain the basic hematopoietic derangement include a reduced size of the stem cell pool and consequent undampening of normal oscillations of blood cell counts, abnormalities of stem cell function, or cycling of short- or long-range humoral stimulators or inhibitors.

Grey collie dogs also have cyclic neutropenia due to cyclic hematopoiesis. In these animals there are cyclic fluctuations of colony-stimulating factor (CSF), a possible long-range humoral regulator of granulopoiesis. There are also cyclic changes in the levels of serum erythropoietin (ESF), a well-characterized long-range regulator of erythropoiesis. The CSF levels are high when blood granulocyte counts are low and fall during the periods of normal granulocyte levels. The ESF values are increased in the reticulocytopenic periods and lower when blood reticulocytes are elevated. The patterns of these fluctuations suggest that oscillations of long-range regulators may be part of the pathophysiologic mechanism of cyclic hematopoiesis.

To examine the role of CSF in human cyclic neutropenia, we have serially measured urinary CSF activity in two patients with cyclic neutropenia. Simultaneous serial ESF levels were determined in one of the patients. Both sub-
stances showed cyclic fluctuations which closely paralleled the pattern of changes found in the collie dogs.

MATERIALS AND METHODS

Patient 1 was a 67-yr-old white woman with symptoms suggestive of cyclic neutropenia dating to childhood. Daily blood counts over extended periods of time had established the diagnosis of cyclic neutropenia with a period of 21 days. A normal spleen had been removed prior to admission to the National Institutes of Health without altering the course of her disease. Patient 2 was an 8-yr-old boy with cyclic neutropenia well documented from early childhood. Both patients were well except for periodic aphthous stomatitis, occasional fever, and soft tissue infections during neutropenia. During the study periods no febrile episodes occurred, and neither patient had evidence of infection.

Daily white blood cell (WBC), red blood cell (RBC), and 100-400-cell differential counts were done with standard techniques. For reticulocyte counts, three drops of EDTA-anticoagulated blood and three drops of new methylene blue were mixed for 10 min and air-dried smears made. The percentage of reticulocytes was determined by examining 1000 RBCs in adjacent microscopic fields using a Miller disk (Bausch & Lomb, Inc., Rochester, N.Y.). Absolute reticulocyte counts were determined by multiplying the per cent reticulocytes by the RBC count. Total neutrophil and monocyte counts were plotted on a semi-log scale with all counts less than 100 graphed as 100. Reticulocyte counts were plotted arithmetically.

Daily 24-hr urines were collected without preservatives in 4-liter plastic bottles encased in dry ice. After storage at \(-10^\circ\text{C}\), an entire series of urines was thawed at room temperature, thoroughly mixed, and aliquoted for ESF or CSF assay.

CSF Assay

Twenty-four milliliters of urine were centrifuged for 20 min at 2000 g and the supernatant dialyzed in Visking tape (Union Carbide, Chicago, Ill.) at 4°C against 1 liter of distilled water (three changes) for 72 hr. Specimens were centrifuged again at 2000 g and the sediment discarded. The supernatant was filtered through Millipore filters (Millipore Corp., Bedford, Mass.; pore size, 0.45 mm) and stored at \(-10^\circ\text{C}\) until CSF was measured. Quantitative determination of protein in the dialyzed urine samples was done by the Lowry method.

The methods for urinary CSF assay have been described in detail elsewhere. The series from each patient was assayed on a single day. Each dialyzed urine sample (0.75 ml) was added to a plastic test tube containing 0.5 ml of mouse marrow (750,000 nucleated cells per ml), 2.5 ml of 1.6% methylcellulose, 0.5 ml of fetal calf serum, and 1.25 ml of modified McCoy's 5A medium. From each test tube, four 1.1-ml samples (75,000 nucleated cells and 0.15 ml urine) were pipetted into separate plastic petri dishes. Dishes were incubated at 37°C in 10% CO₂ in air and read at 7 days. All colonies with 20 or more cells were scored. A blank (McCoy's 5A) and two positive controls (supernatant from an L cell culture and pooled normal human urine) of known activity served as controls. CSF was expressed as the mean number of colonies per plate (mean of four plates) minus the mean of the blank.

Erythropoietin Assay

Details of urine processing and the assay procedure are described elsewhere. One-fourth of each daily urine pool was concentrated to 20 ml for assay, and each assay animal received two 1.0-ml injections of test material on consecutive days. ESF excretion was quantitated by comparing the response to a simultaneously determined ESF Standard B dose-response curve.

RESULTS

The simultaneous reticulocyte, ESF, neutrophil, monocyte, and CSF levels for patient 1 are presented in Fig. 1. In each cycle, urinary CSF rose during the neutropenia period and peaked just prior to, or with, the return of periph-
Fig. 1. Simultaneous daily reticulocyte, erythropoietin, neutrophil, monocyte, and colony-stimulating factor levels for patient 1. For the first 10 days for which reticulocyte counts are shown (study days 10–19), no ESF was detectable. No ESF was detectable also on study days 21, 30–33, 36–40, and 49. CSF was not detectable on days 2, 6, 18, 23, 28, 30, 33, 36, 40, and 41. The open circles (○) represent days when no data were available. Neutrophil and monocyte counts of 100 or less are graphed as 100.

eral blood neutrophils and just after the rise in the blood monocyte count. CSF fell during the period of highest blood neutrophil numbers. Peak CSF activity ranged from 18 to 26 colonies per plate. Normal human urine stimulated a mean of $1.4 \pm 0.6$ colonies per plate in this assay. Average urine volumes during the periods of highest and lowest CSF activity were the same (1354 ± 78.6 ml and 1360 ± 70.7 ml, respectively). The protein content of the six urines with the highest CSF and those with the lowest also were not significantly different ($p > 0.05$, Student’s $t$ test). For these reasons CSF levels were not corrected for original urine volume or postdialysis protein content.

To investigate the possibility of an excess of inhibitor levels present in urines of low activity, mixing of the dialyzed urine samples with peak and trough activity was done. Only a dilutional effect was seen.

The pattern of ESF excretion was similar to that of CSF. ESF excretion was unmeasurable on 17 of 20 days during the periods of highest neutrophil counts. ESF appeared early in neutropenia and persisted until neutrophil
recovery was established. Increasing ESF excretion was associated with increasing reticulocyte levels, and its fall was followed in a variable period by reduced reticulocyte numbers. During the whole study period the patient was mildly anemic (mean hematocrit of 33.4\(^\circ\) ± 0.3\(^\circ\)).

Levels of urinary CSF for three cycles in the second patient with cyclic neutropenia are shown in Fig. 2. The pattern of CSF fluctuation vis-a-vis neutrophil and monocyte counts is essentially the same as in patient 1, although much higher CSF levels (65 colonies per plate) were obtained. ESF levels were not measured in patient 2.

**DISCUSSION**

The first studies of urinary CSF in a patient with cyclic neutropenia have recently been reported.\(^{15}\) While the data seemed to show a positive correlation between peripheral blood granulocyte count and CSF, it was pointed out that irregular and infrequent data collection did not allow for completely satisfactory analysis of the relationship between these two. In the studies reported here we have determined daily CSF levels in two patients with cyclic neutropenia. The pattern of fluctuation of human urinary CSF exactly parallels that in grey collie dogs with cyclic neutropenia.\(^{9}\) In both dog and man, levels of CSF
rise during neutropenia, peak with the return of blood neutrophils, and fall to low levels after the peripheral blood is repopulated with neutrophils.

The monocyte has been shown to be the important cell in the generation of CSF, and it is of interest that for our two patients the level of urinary CSF appears to correlate with the peripheral blood monocyte level. One might postulate that levels of CSA vary inversely in response to neutrophil levels in a negative feedback control system. In such a system, the monocyte would be involved in generation of the trophic substance. Thus, the periodic oscillations of urinary CSF in this disease may be regarded as an appropriate, physiologic, but secondary response to a primary alteration in the production of neutrophils. The temporal relationship of CSF and neutrophil levels suggests a biologic role for CSF, but our data do not prove a causal relationship.

The cycling of ESF excretion with approximately the same phase relationships as CSF also parallels the findings in the grey collie dog. Increases in ESF are followed by increasing reticulocyte counts and falling levels of ESF, with a decreasing number of reticulocytes. It is not apparent from our data why ESF excretion decreases during the peak of the blood neutrophil count. In the dogs it was found that ESF was decreased during the neutropenic periods and that ESF levels paralleled the blood neutrophil counts. This relationship raised the question of whether the ESF production was inhibited during neutropenia because of concomitant infection. In the patient, the increase in ESF occurred during neutropenia, indicating that subclinical infection or inflammation accompanying neutropenia is not a likely cause for the ESF fluctuations observed. Other possible causes of the ESF changes are a primary disturbance in ESF generation, exaggerated feedback control of ESF synthesis, and release or increased ESF utilization by an expanded erythroid marrow. Since a normal hematocrit was not achieved, physiologic damping of ESF production is not a likely explanation. Because ESF and CSF appear to have distinct biologic properties in the assay systems used, it is also unlikely that the simultaneous peaks of ESF and CSF activity reflected fluctuations of a single hematopoietic-stimulating substance.

We have not examined, except by the relatively crude methods of mixing, the possibility of cyclic inhibition of hematopoiesis as pathogenetically important. An excess of CSF seems unlikely from the mixing experiment performed with one patient's urine. An exhaustive search for ESF inhibition in the canine disease failed to demonstrate either inhibitors of ESF or hemoglobin synthesis.

Our data do not support the hypothesis of positive feedback as operative in the control of granulocytopenia in human cyclic neutropenia. Rather, it is consonant with a negative feedback control system in which levels of the CSF are inversely related to the blood granulocyte count. Periodic oscillation of reticulocyte levels may be determined by cyclic fluctuations of ESF, the cause of which is as yet obscure.

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