Cyclic Neutropenia: The Relationship Between Urine Granulocyte Colony Stimulating Activity and Neutrophil Count

By Aroop Mangalik and W. A. Robinson

Urinary colony stimulating activity (CSA) has been studied in a 70-yr-old female with chronic cyclic neutropenia of unknown cause. Changes in urine CSA were correlated with absolute granulocyte counts and were found to be highest when the granulocyte count was high or falling. It is suggested, on the basis of this and other data, that granulopoiesis, at least in part, may be regulated by a positive feedback control mechanism.

IN THE PAST FEW YEARS factors have been described from serum, urine, and tissues which will stimulate granulocyte colony formation in vitro. On the basis of a wide variety of evidence it has been suggested that these factors may represent the true granulopoietic substances capable of stimulating new maturation and production of this cell line in vivo as well as in vitro. Biochemical studies on the nature of the colony stimulating factor (CSF) from human urine, which has been the best characterized of all those studied, has shown it to be a glycoprotein with a molecular weight of about 40,000 which migrates electrophoretically as an alpha globulin. The major source of this factor remains unknown. It has been suggested that one source may be mature neutrophils themselves. While a wide variety of materials will stimulate colony formation from mouse bone marrow in the tissue culture systems used, the only effective source of stimulation of granulocyte colony formation by human bone marrow has been feeder layers of mature white blood cells (WBC) or products thereof. It has also been shown that the production of CSF by WBC feeder layers is the result of an active secretory process. These findings have led us to speculate about the possibility of “positive feedback” in the regulation of granulocyte production in which mature granulocytes secrete factors which stimulate new maturation and production of this cell line in vivo similar to that seen in vitro.

Confirmation of this suggestion has not been complete. One of the major areas of study and interest has been correlation between urinary CSF and peripheral neutrophil counts. It has been shown that in humans undergoing elective operative procedures, there is a close relationship between the rises

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and falls in peripheral neutrophil counts and urinary CSF, with the peak of the latter occurring 10–12 hr after the peak of granulocytes in the peripheral blood.10,11 It has also been shown that there is a sharp rise in urinary levels of CSA after WBC transfusion or administration of antineutrophilic serum.12,13 In gray collie dogs with spontaneous cyclic neutropenia, peaks of urinary CSA occurring between the peaks of the neutrophil count have been noted.14

No reports of CSA levels in humans with cyclic neutropenia have appeared, although one patient with chronic granulocytic leukemia in whom spontaneous cycling occurred has been reported.15 We have recently had the opportunity to study one adult patient over a 13-wk period with spontaneous cycling of the total granulocyte count from 0 to 1600 without treatment. In an attempt to determine the relationship between urinary CSA levels and peripheral neutrophil counts, with an eye toward determining the source of urinary CSA, correlative studies between these two parameters have been done. It has been shown that peaks of urinary CSA in this patient occurred at the time of the neutrophil peak in the blood or falling on the downslope of the neutrophil count. These studies, when taken with other data, suggest that neutrophils themselves may be one of the major sources of urinary CSA and have added further strength to the possibility that granulocyte production is in part regulated through a positive feedback system.

MATERIALS AND METHODS

The patient studied was a 70-yr-old white female referred to the University of Colorado Medical Center in November, 1971 with a 1-yr history of recurrent infections and a low WBC. Initial physical examination was normal except for mild splenomegaly. The initial WBC was normal and a bone marrow examination showed only mild granulocytic hyperplasia. It was elected to follow the patient at weekly intervals with total WBC and neutrophil counts. After 2 mo, a cyclic pattern in neutrophil counts was established. In February, 1972 a 13-wk investigative period was begun which is the basis for the present studies. During this time total WBC and neutrophil counts were done once per week. On the same day serum and a 24-hr urine were collected for determination of CSA. The patient's age and distance of her home from the hospital precluded more frequent study points.

Method of Assay of CSA

The method of urine collection and testing for urinary and serum CSA has been described in detail elsewhere.2 Twenty-four hour urine specimens were collected in sterile glass bottles. Fifty milliliter aliquots were removed and dialyzed for 72 hr against distilled water, sterilized by passing through Millipore filters, and stored at -20°C prior to assay. Serum was also dialyzed against distilled water for 72 hr. All assays were done on a single day. 0.15 ml of urine or 0.10 ml of serum was placed in the bottom of 35-mm plastic petri dishes to which was added 1 ml of modified McCoy's 5A medium (with 15% fetal calf serum) and 0.3% agar containing 75,000 nucleated mouse bone marrow cells. Each sample was run in triplicate plates. After mixing of the agar bone marrow cell medium and the test material, plates were incubated at 37°C with 100% humidity in an atmosphere of 7.5% CO2 in air. Colony counts were done on day 7 of incubation. Control urines of known CSA from three normal subjects were run simultaneously.

RESULTS

Figure 1 shows the relationship between the absolute neutrophil counts in this patient and urinary CSA over the 13-wk study period. It can be seen
that the neutrophil count varied between 0 and 1600 with approximately 3-wk periods between peaks.

Urinary CSA levels ranged from 5 to 60 with peaks occurring at approximately 3-wk intervals. Levels for normal human urine under the same conditions in our laboratory range from 0 to 20 with the majority being below 10. The differences noted in this study are significant using our present CSA testing methods. The first peak of urinary CSA in this study was correlated with the peripheral blood neutrophil peak. The subsequent peaks occurred at a time when the neutrophil count was low or falling. In both instances the CSA peak appears to be occurring on the downslope of the neutrophil curve rather than the nadir. More frequent points are, however, needed to establish this firmly.

Studies of serum CSA, both dialyzed and nondialyzed, were done at the same points. CSA was low in all samples and no cycling could be appreciated.

**DISCUSSION**

The present studies have indicated that cycling of urinary CSA may occur in some patients with cyclic neutropenia. To our knowledge this is the first report of this in humans. Colony growth of bone marrow cells from patients with neutropenia has been studied by several authors, but changes in this parameter during neutrophil cycles have not been reported. The meaning of the present findings must await final characterization of the urinary CSF but suggest that it may be related to neutrophil production and may represent or reflect the presence of a granulopoietin. The lack of frequent time points in the present study make interpretation of the relationship between these two

**Fig. 1.** Relationship between the absolute total peripheral neutrophil count and urinary CSA in a 70-yr-old female. Neutrophil counts done at weekly intervals are shown by the dotted line. Urinary CSA levels are shown on the solid line. Each point on the CSA curve is the mean colony count of three plates with standard error of mean. Urine collections for the points obtained were during the 24 hr preceding the neutrophil count.
factors (urinary CSA and neutrophil counts) difficult. In the present study this was the result of personal practical factors which could not be overcome. While it is possible that peaks of either neutrophils or CSA occurred between the weekly time points studied this does not detract from the major finding that the CSA peaks were found in all three cycles studied when the neutrophil count was high or falling. This finding along with other data to be discussed has suggested to us that neutrophils themselves may be one of the major sources of urinary CSA.

Most workers in the past have attempted to correlate peripheral neutrophil counts with proposed granulopoietic factors in a schema of "negative feedback" control. In such a system it would be expected that granulopoietic factors would be high when the granulocyte count is low, and low when the granulocyte count is high. On a theoretical basis it seems unlikely that such a system is operative in controlling granulocyte production. In hematologically normal humans, a rise in peripheral granulocyte count, as for example in bacterial infection, is the time at which granulocyte utilization is increased and production needs to be stimulated rather than dampened. The rate of granulocyte utilization and breakdown would seem to be the best possible determinant for new production to supply needs and replenish stores. While it is conceivable that sensory mechanisms outside the hematopoietic system itself could recognize this and signal the need for increased production, this seems to us uneconomical. We would suggest that the major source of factors stimulating new maturation and production of this cell line during times of increased stress and utilization is from mature neutrophils themselves as they senesce or are broken down in vivo.

The evidence that neutrophils serve as a source of CSA (or granulopoietin) comes from a number of areas. The only effective source of stimulation of granulocyte colony formation from human bone marrow is WBC feeder layers or conditioned medium from these or human spleen.8-19 The exact cell responsible for the production of CSA in these systems has not been determined. There is evidence on the basis of cell separation studies that the monocyte may be a major source20 of CSA. The small number of monocytes present in most normal human WBC feeder layers and the prolonged handling of neutrophils during cell separation studies with possible resultant loss of CSA necessitate further studies. Studies of urinary CSA in hematologically normal patients undergoing elective operative procedures have shown that the levels of CSA in these patients are highest 10-12 hr after the peak of neutrophils in the peripheral blood and not at low points of neutrophil count.10,11 In patients with aplastic anemia and acute granulocytic leukemia in whom the level of mature granulocytes is very low, only small amounts of CSA are found in the urine.2,18 CSA levels in these patients rise as the neutrophil count rises in the peripheral blood. It has also been demonstrated that sharp peaks of urinary CSA occur in these patients following WBC infusions.18 Finally, the evidence presented here in a patient with idiopathic cyclic neutropenia suggests that the neutrophils themselves are a major source of urinary CSA.

There are many objections to the concept of positive feedback regulation.
In such a system, if the mature cell line producing granulopoietic substances is ever entirely destroyed the system cannot reinstate itself. This has been an objection raised by many. We would suggest that such a mechanism may, in fact, be operative and may be the major problem in certain granulocytopenic and aplastic states. We have recently had the opportunity to study one patient with prolonged aplastic anemia in whom urinary CSA levels were consistently low. In this patient the number of colony-forming cells in the bone marrow was only slightly reduced suggesting that the major defect resided in the inability to produce granulopoietic substances.\textsuperscript{18} The patient was treated with infusion of large numbers of buffy coat WBC, with subsequent recovery of granulocyte production. The urinary CSA showed an increase corresponding to granulocyte recovery.\textsuperscript{21} This patient will be reported in detail elsewhere.

Inhibitory factors or arms must also exist in any such system to prevent uncontrolled production. Evidence for inhibitory factors affecting granulocyte production exists but these have not been well worked out. Haskill et al., using cell separation techniques, indicate that mature granulocyte forms in the bone marrow have an inhibitory effect on colony formation in vitro.\textsuperscript{22} Shad-duck et al., using a white cell preparation from rat peritoneal exudate in a liquid culture, have also found evidence for inhibitory activity.\textsuperscript{23} Mixing of the supernatant with preparations of known CSA potency revealed a marked inhibition in the 24-hr culture which decreased considerably on the seventh day of culture. The earlier culture system was dominated by the earlier granulocytic cells, while on day 7 most of the cells were mature neutrophils. Both of these studies confirm the presence of CSA in the mature granulocytes and suggest an inhibitory activity of the earlier forms.

It is likely that more than one mechanism is involved in determining the rate of production and release of granulocytes from the bone marrow, as suggested by the studies of Morley.\textsuperscript{24} It is also likely that the major arm of regulation lies in factors stimulating maturation and production of this cell line. Whether the urinary CSF represents such a factor remains to be determined but it is the best candidate thus far for such a role. Further studies in humans are needed to determine the relationship between urinary CSA and granulocyte production and breakdown, but our data suggest a neutrophil source. The concept presented of a positive feedback system of cellular regulation, if proven, may be unique in our present understanding of biologic cellular regulation.

REFERENCES


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