Cycling of peripheral blood and marrow lymphocytes in cyclic neutropenia
(pre-B cells/cyclic hemopoiesis/regulatory factors)

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ABSTRACT A 16-month-old male patient with cyclic neutropenia was found to have cyclic fluctuations of monocytes, lymphocytes, platelets, and eosinophils in the peripheral blood. Changes in lymphocyte counts were not obviously related to B, T, or natural killer cells. All classes of immunoglobulins were elevated throughout the cycle. Studies of the marrow morphology revealed remarkable cyclic oscillations of lymphoid as well as myeloid lineage cells. Granulocyte-macrophage progenitors (CFU-c) cycled and were virtually absent 1 wk prior to the neutropenic nadir. The cyclic changes in marrow lymphoid cell numbers were primarily due to changes in numbers of surface immunoglobulin negative (sIg-) cytoplasmic Ig^- (cIg+) pre-B cells. Pre-B cell numbers cycled from normal to extraordinarily elevated values with the same periodicity but reciprocal to the neutrophil cycle. We propose that the primary defect in cyclic neutropenia may either be a periodic failure of an early myeloid differentiation factor or a blunted response of early myeloid precursors to a common hemopoietic growth factor. This may lead to periodic fluctuations in the production or delivery of growth factors (or factors) that influence early stages of differentiation of other hemopoietic cells, including pre-B cells. The essential periodic deficiency is consequently reflected in deficient production of CFU-c accompanied by excessive production or accumulation of pre-B cells (and probably other hemopoietic precursors) in the marrow.

Human cyclic neutropenia (HCN) is a rare blood dyscrasia characterized by periodic disappearance of neutrophils from the circulation with intervening periods of normal neutrophil counts (1-4). Symptoms of this disease during the neutropenic periods are usually mild with upper respiratory infections (URI) and aphthous stomatitis (4), but occasionally there are serious and even fatal infections (3). The neutropenic cycles vary in length from 19 to 23 days (4). Reciprocal oscillations of blood monocytes, cycling of the platelets and reticulocytes (2), and, occasionally, oscillations of eosinophils (1) have also been reported but are not consistent findings in all patients (4). In grey collie dogs, the animal model of this disease, the term canine cyclic hemopoiesis (CCH) is used because the neutropenia is always associated with cyclic monocytes, reticulocytosis, and thrombocytosis. The periodicity in CCH usually approximates 11-13 days (5).

Few studies have examined the relationship of lymphocytes to the neutrophil cycle in HCN or CCH. Whereas lymphocytes were reported to fluctuate periodically along with the neutrophils in some patients (2, 6), other investigators found no evidence of lymphocyte cycling (7). Two of three dogs studied with CCH had consistently reduced proliferative responses to phytohemagglutinin (PHA) when compared to normal dogs. In the remaining dog of this study, PHA responses were decreased when the peripheral neutrophil count was normal but well above normal levels during neutropenia (8). Reasons for these disparate and variable observations in both humans and dogs are not clear.

We report here the clinical and immunological findings on a patient with HCN, which includes a study of the lymphocyte subpopulations of the peripheral blood during the neutrophil cycle. Because cyclic neutropenia originates from a defect in the bone marrow (1-2, 4, 9-10), sequential bone marrow aspirations were studied. We were particularly interested in detecting changes in lymphoid cells, their phenotype, and their relationship to the cycles of myeloid cells and clonal myeloid precursors (granulocyte-macrophage colony-forming cells, CFU-c) in vitro. Work from this laboratory and others (11, 12) has suggested a close relationship of B lymphopoiesis to the marrow environment. For this reason, we paid attention to the early stages of development of B lymphocytes, which are normally largely confined to the marrow. Our observations on peripheral blood and marrow lymphocytes in this patient suggest an interpretation of the underlying defect in cyclic neutropenia.

MATERIAL AND METHODS

Case Report. A 16-month-old black male was referred to the Immunology Clinic of Oklahoma Children's Memorial Hospital with recurrent infections and possible immunodeficiency. He was born to a 26-year-old mother. The antenatal course was complicated by obesity, hypertension, gestational diabetes mellitus, and failure of normal induction of labor. The patient was delivered by cesarean section; birth weight: 10 pounds 2 ounces. The first infection reported was hordeolum at the age of 3 months. Since then, the patient has been sick every 2-3 wk, with fever, recurrent otitis media, URI, diarrhea, and, occasionally, aphthous stomatitis. The patient was hospitalized twice: with high fever and aphthous stomatitis at the age of 8 months and with high fever at the age of 14 months. No other family members had a history of recurrent infections.

On the first visit, the physical examination was unremarkable except for evidence of URI and otitis media. Four weeks later, he had right otitis media and his leukocyte (WBC) count was 6,000 per mm^3 with no detectable neutrophils. Repeated WBC counts demonstrated that the patient had recurrent ep...
isodes of absolute neutropenia, with intervening periods of normal neutrophil counts. During our study, the patient had peripheral blood counts and differential counts every other day (at the same time) for 50 days and was examined every week. Detailed studies of the peripheral blood and marrow were done on days 13, 20, 27, 35, 42, and 49.

**Cell Suspensions.** Peripheral blood smears were stained with Wright/Giemsa. Peripheral blood lymphocytes (PBL) were separated on Ficoll-Hypaque (Histopaque 1077, Sigma) and washed twice in RPMI medium containing 5% heat-inactivated fetal calf serum. Cyto centrifuge preparations of PBL were stained with Jenner/Giemsa to count contaminating monocytes. Peripheral blood samples for flow cytometry analysis were exposed to the Ortho lysing reagent (0.15 M NH4Cl/0.01 M KHCO3/0.1 mM EDTA, pH 7.3, Ortho Diagnostic) at 24°C to remove erythrocytes. Bone marrow samples were aspirated from the posterior superior iliac crest into a heparinized syringe. Bone marrow cell suspensions were freed of erythrocytes by resuspending pelleted cells in 10 ml of Tris-HCl-buffered 0.15 M NH4Cl (pH 7.4) at 4°C until satisfactory erythrocyte lysis was achieved as described (13). Loss of nucleated cells with this procedure was always insignificant. Cells were washed twice with RPMI medium containing 5% fetal calf serum as above, and cyto centrifuge preparations were stained for morphological examination.

**Antibodies.** Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies OKT-3, OKT-4, OKT-8, and OKT-11 were obtained from Ortho Diagnostic. Monoclonal antibodies to human k and l light chains of Ig and antibodies from clone Leu-7 were generously donated by Becton-Dickenson. Monoclonal antibody Leu-7 has been shown to bind to cells with natural killer activity (NK cells) (14-15). Monoclonal anti-human k (clone 145-8) was donated by John Kearney. FITC-conjugated goat anti-mouse Fab and tetramethylrhodamine isothiocyanate-conjugated affinity-purified goat anti-human k for cytoplasmic staining were obtained from Southern Biotechnology Associates (Birmingham, AL).

**Immunofluorescence.** Cells for visual cell surface immunofluorescence were incubated with appropriate monoclonal antibodies as described in detail (13). Binding of the mouse monoclonal antibodies was detected with a FITC-conjugated goat anti-mouse Fab preparation that gave no detectable background staining on any of the samples studied. Cytoplasmic k chains of IgM (κμ) were detected in fixed cyto centrifuge preparations of FITC anti-cell-surface immunoglobulin (sIg)-stained cells by counterstaining with tetramethylrhodamine isothiocyanate-conjugated goat anti-human k as described (16). Visual immunofluorescence was scored on a Zeiss fluorescent Photomicroscope III under epi-illumination.

**Flow Cytometry.** Peripheral blood cells were stained with FITC-conjugated OKT-3, OKT-4, OKT-8, or OKT-11 monoclonal antibody (17) and were counted on an Ortho Cytofluorograph or Ortho Spectrum III (Raritan, NJ) gated on the lymphocyte window, which was determined by a combination of right-angle and forward-angle light scattering as described (18, 19).

**Serum Immunoglobulin Levels.** Serum immunoglobulins were determined on a rate nephelometer according to manufacturer's procedures (Beckman Immunochemistry Analyzer, Beckman Instruments).

**Serum Complement Levels.** The total hemolytic complement assay was carried out as described (20).

**Bone Marrow Cell Morphology.** Bone marrow differentials were the result of 200 cells scored in Wright/Giemsa-stained bone marrow smears. Independent differential counts were made by three investigators and were always in close agreement.

**CFU-c.** Bone marrow CFU-c were assayed as described (13) by using 10% CCT conditioned medium (GIBCO) as a source of colony-stimulating factors. Marrow cultures were established in soft agar at a concentration 2 × 10^5 cells per ml, incubated for 7 days at 37°C in 7% CO2 in humidified air, and scored for the presence of colonies consisting of >40 cells and clusters of 3–40 cells under a phase-inverted tissue culture microscope.

**RESULTS**

**Clinical Observations During the Study.** The patient had a cough during the first 2 wk of observation, with fever during the second week. On examination, there was throat redness and the submandibular lymph nodes were slightly enlarged. Respiratory syncytial virus was cultured from the nasopharynx on day 13. On day 27, the patient had diarrhea, runny nose, and red throat. A white exudate over the right tonsil grew Candida albicans. On day 35, he had a mild cough but was otherwise healthy. On days 20, 42, and 49 the patient was well with no remarkable findings. On day 49, he was on prophylactic treatment with Amoxicillin.

**Peripheral Blood Cell Counts.** The neutrophils cycled from zero to normal numbers in a cycle of 23 days (Fig. 1). The nadir lasted for approximately 5 days. During each cycle, there were oscillations in the other blood cells as well. The monocytes cycled reciprocally to the neutrophil numbers and reached a value of 5,000 per mm^3 during neutropenia and were <1,000 per mm^3 during the recovery phase. Eosinophils cycled from 52 per mm^3 to 1,260 per mm^3 but appeared to be slightly out of phase with the neutrophils. Platelet counts were found to be normal during neutropenia but thrombocytosis up to 805,000 per mm^3 was observed during the remainder of the cycle. Regular oscillations of the total WBC and the lymphocytes were observed and both approximated the neutrophil cycle. Lowest lymphocyte counts were found at the beginning of neutropenia but recovered more quickly than the neutrophil counts. Reticulocyte counts varied between 3.1 × 10^9 and 8.4 × 10^9 per mm^3 over the neutrophil cycle.

![Fig. 1.](image-url) Peripheral blood cell counts from a patient with cyclic neutropenia over the course of two complete cycles. Blood smears and cell counts were done at 2-day intervals. o, Neutrophils; Ç, lymphocytes.
Table 1. Peripheral blood lymphocyte subpopulations during neutropenic cycle

<table>
<thead>
<tr>
<th>Lymphocyte population</th>
<th>% of lymphocytes at day</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>13</td>
</tr>
<tr>
<td>slg'</td>
<td>22.7</td>
</tr>
<tr>
<td>OKT-3'</td>
<td>53.4</td>
</tr>
<tr>
<td>OKT-4'</td>
<td>30.3</td>
</tr>
<tr>
<td>OKT-8'</td>
<td>20.6</td>
</tr>
<tr>
<td>OKT-11'</td>
<td>58.2</td>
</tr>
<tr>
<td>Leu-7'</td>
<td>0.7</td>
</tr>
<tr>
<td>Non-B, non-T'</td>
<td>19.1</td>
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</tbody>
</table>

* Days of peripheral neutrophil nadir.

† Non-B, non-T cell percentages were calculated by subtracting slg' and OKT-11' cells from 100%.

Lymphocyte Subpopulations. As shown in Table 1, there were fluctuations in circulating slg' cells (B cells), OKT-3', OKT-4', OKT-8', and OKT-11' cells (T cells), and Leu-7' cells (NK cells) but the changes were not obviously related to neutropenia. The OKT-4/OKT-8 ratio did not vary significantly over the course of the cycles. However, the percentage and absolute number of non-B, non-T lymphocytes correlated directly with fluctuations in total lymphocyte count and closely with the onset of neutropenia (Fig. 1).

Serum Immunoglobulin Levels. Serum immunoglobulin levels of IgM, IgG, and IgA were slightly elevated throughout the cycle when compared to normal values for the age group. IgM levels were 193, 218, and 189 mg/dl (normal range [NR] = 43–173 mg/dl); IgG levels were 1,420, 1,240, and 1,080 mg/dl (NR = 345–1,213 mg/dl); IgA levels were 195, 183, and 142 mg/dl (NR = 14–106 mg/dl) on days 35, 42, and 49, respectively. They showed no evidence of fluctuation with the neutrophil cycles.

Complement. Total hemolytic complement values were found not to vary significantly from normal during the cycle. The values were 132, 70, and 77 CH50 units/ml on days 13, 20, and 27, respectively (NR = 80 ± 15 CH50 units/ml).

Bone Marrow Morphology. During the recovery phase of the neutropenic cycle, normal numbers of cells of the myeloid series were seen in the bone marrow and all stages of neutrophil development were present. Lymphocytes made up 20.5% and 7.0% of the marrow cells at this time (days 13 and 35, respectively) and were primarily small lymphocytes. As the marrow became increasingly depleted of immature and subsequently mature myeloid elements, there was a coincident increase in lymphoid cells (Fig. 2). At the neutrophenic nadir, the marrow was 60% and 64% lymphoid (on days 27 and 49, respectively). These lymphoid cells were distinct from those seen 2 wk earlier in having a larger size, increased nucleus-to-cytoplasmic ratio, and prominent nucleoli.

CFU-e. As shown in Fig. 3, during the week preceding neutropenia, (days 20 and 42), CFU-e were undetectable (<1 per 2 x 10⁶ cells plated) and clusters were infrequent (1 per 2 x 10⁶ cells plated on days 20 and 42, respectively). CFU-e were in the normal range during the neutropenic phase of the cycle (days 27 and 49). On days 13 and 35, in the recovery phase of peripheral neutrophils, intermediate CFU-e numbers were seen.

Bone Marrow Lymphoid Cell Subpopulations. Fluctuations in the proportion of slg' cells, OKT-3' cells, and Leu-7' cells in the marrow were not remarkable over the course of two cycles (Table 2). However, the frequency of cells with detectable cytoplasmic IgM+ chains (but not detectable cell surface IgM) increased by 100% during each cycle from normal values (1.6% and 0.7%) during the first week (days 13 and 35) to elevated levels during the second week (days 20 and 42) and further elevation during neutropenia (35% on day 27 and 46.8% on day 49, Fig. 2). The absolute numbers of pre-B cells per ml of marrow volume on days 35, 42, and 49 were 0.76 x 10⁶ per ml, 15.00 x 10⁶ per ml, and 25.00 x 10⁶ per ml, respectively (Fig. 4).
Cyclic neutropenia in this patient was accompanied by cyclic fluctuations of monocytes, eosinophils, platelets, and lymphocytes in the circulation. The marked difference between the oscillations of the neutrophils and other circulating cells was that the neutrophils cycled from normal levels to zero, whereas other blood cells oscillated from normal to elevated levels.

Of particular interest to us was the cyclic pattern of peripheral lymphocyte counts that approximated that of the neutrophils. The lowest lymphocyte numbers were consistently found during the neutrophil nadirs. Guerry et al. (2) described a patient with periodic lymphocyte fluctuations. Andrews et al. (7) demonstrated in one patient with HCN that there was no evidence of periodicity of the lymphocyte counts and that the T-lymphocyte subpopulation appeared functionally normal as measured by PHA responses. Borkowsky et al. (6) demonstrated T-cell lymphopenia and reduced PHA (but not pokeweed mitogen) responses during the neutrophil nadirs in two patients. Total lymphocyte counts were not reported.

In our patient, there was no T-cell lymphopenia and the response to PHA, concanavalin A, or pokeweed mitogen was not reduced during the neutrophil nadir (unpublished data). No cyclic oscillations in the phenotypically identified helper T-cell (OKT-4+) or suppressor cell T-cell (OKT-8+) populations were seen, although fluctuations in the T-cell subsets were found (Table 1). Changes in B cell and Leu-7+ cell numbers were seen but were also not correlated with cyclic oscillations of the lymphocytes or neutrophils. High immunoglobulin levels were observed in this patient, as has been previously reported in both HCN and CCH (6, 8). This may reflect hyperactivity of the humoral immune system due to recurrent infections or may be related to the primary defect in this disease. Only non-B, non-T lymphocytes were found to have fluctuations that correlated with the lymphocyte cycle.

Changes in neutrophil counts have been shown to reflect changes in myeloid precursors in the marrow. However, our understanding of temporal changes in circulating lymphocyte numbers is complicated because lymphocytes are known to proliferate in response to antigens and disappearance of the lymphocyte component of immunity may have secondary effects on both lymphocytes and monocytes in the periphery.

The frequency and number of morphologically identified myeloid cells in the marrow cycled in direct correlation with the number of circulating neutrophils (Fig. 2). Committed progenitors of the myeloid series (CFU-c) cycled such that their apparent nadir value preceded the nadir of mature neutrophils by 1 wk (Fig. 3). This CFU-c nadir was followed by elevation to normal numbers during the period of circulating neutropenia. Cycling of CFU-c has been previously reported in both patients with HCN (10, 21) and dogs with CCH (22, 23) and was anticipated by observations on the cycling of morphologically recognizable myeloid progenitors in HCN originally described by Page and Gage (1).

Changes in morphology of the bone marrow during the neutrophil cycles were impressive. The percentages of myeloid cells in the marrow were low during neutropenia (10.0% and 11.5%), whereas percentages of lymphoid cells at the same time were extraordinarily high (60% and 64%) and morphologically immature lymphoid cells predominated. This was so striking, that examination of marrow cell morphology only during the periods of neutrophil nadir could lead to the erroneous conclusion that leukemic changes had occurred in this patient. One week after the peripheral neutrophil nadirs (days 13 and 35), the marrow appeared completely normal.

As shown in Table 2, marrow B, T, and Leu-7+ cells oscillated with no apparent periodicity or obvious correlation with the cycling of marrow lymphoid cells. However, phenotypic pre-B cells did cycle and the numbers correlated with the marrow lymphoid cell cycle. The cycling of lymphoid cells and pre-B cells in the marrow occurred in synchrony and both were reciprocal to the cycles of total marrow myeloid cells and circulating neutrophils (Fig. 2). This lymphoid cell cycle was not due to changes in the marrow cellularity nor to dilution by peripheral blood in the aspirate (Fig. 4). The frequencies of pre-B cells were within reported normal values (13, 16) 1 wk after the neutrophil nadirs and then increased over the following 2 wk to 35.0% and 46.8% on days 27 and 49 (Table 2). To the best of our knowledge, periodic cycles of marrow lymphoid cells and the finding that pre-B cells were the predominant component of this cycle have not been described previously.

Large pre-B cells are the final proliferating cell compartment in the hemopoietic production of B lymphocytes (24–26). They appear to leave this compartment after a single division and are normally unable to self-renew (26). This suggests that the cyclic wave of pre-B cells we observed in this patient reflected cyclic changes in early B lineage progenitors from which pre-B cells derive (26, 27). However, additional studies will be required to

**DISCUSSION**

**Table 2. Bone marrow lymphocyte subpopulations during neutropenic cycle**

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<tr>
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<th>% of nucleated cells at day</th>
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<tr>
<td></td>
<td>13</td>
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<tr>
<td>cu+ slg-</td>
<td>1.0</td>
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<tr>
<td>slg+</td>
<td>9.4</td>
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<tr>
<td>OKT-3+</td>
<td>5.9</td>
</tr>
<tr>
<td>Leu-7+</td>
<td>ND</td>
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</table>

ND, not determined. *Days of peripheral neutrophil nadir.

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rule out alternative hypotheses that pre-B cells in this patient are abnormally capable of self-renewal periodically or that they are failing to mature normally. Some changes in the kinetics of B-lymphocyte formation may have occurred in this individual because equivalent elevations of B cell numbers were not observed in the marrow (Fig. 4). However, this may reflect only an accelerated transport of differentiated progeny to the periphery.

CCH in dogs is associated with cycling of marrow precursors of myeloid and erythroid cells (22, 23) and HCN is associated with cycling of myeloid and megakaryocyte progenitors (21). The hemopoietic defect in this patient resulted in profound alterations of both myeloid lineage cells and B-lineage cells in the bone marrow. Although details of the lesion in this experiment of nature remain speculative, the essential defect certainly involves stages of hemopoiesis earlier in differentiation than CFU-c or pre-B cells. Most importantly, this defect leads to cyclic changes in the differentiation of both myeloid and B-lymphoid cells.

This cyclic alteration of early hemopoiesis must reside either in the pluripotential hemopoietic stem cells or in factors that relate to both myeloid and lymphoid cells. Lange and Jones (9) have suggested that the pattern of cells found to cycle with patients with HCN and dogs with CCH are related to the stem cells involved in the disease and that the expression at the level of differentiated progeny is likely modified by hemopoietic regulatory molecules. We remain intrigued by the striking difference between the bone marrow myeloid and lymphoid cells in this patient—that is, myeloid progenitors cycled from normal to undetectable levels, whereas B-lymphocyte precursors cycled from normal to greatly elevated numbers. These observations suggest as an alternative hypothesis that the primary defect in cyclic neutropenia may be in the production or reception of factors that govern early steps in differential hemopoiesis. This difference may relate to competition of lineage-specific early differentiation factors on an uncommitted stem cell pool such that the failure of one factor leads to excesses in response to other factors. The consequences would be defective response of the neutrophil lineage and cyclic overproduction of other lineages. Alternatively, it is possible that these observations could result from the action of a single early hemopoietic factor that regulates all of the committed lineages in the marrow. The cyclical defect in this latter case would be in the expression of appropriate receptors on myeloid progenitors. This may be accompanied by altered feedback regulation of the postulated single factor.

If this patient is not unique and lymphoid cell cycling is found in other patients with HCN and in dogs with CCH, this disease may be a model important for further study and understanding of control of B-cell lymphopoiesis in the marrow. One of the factors we were unable to control in this study was the effect of repeated infections on the marrow lymphocyte population. Although the clinical course of this patient did not suggest infection was of primary importance in the cyclic changes we observed, subtle differences in marrow B cells (28) and pre-B cells (29) have been associated with antigenic challenge. Resolution of the effect of infection on cyclic hemopoiesis will likely come from studies of dogs with CCH. Certainly the hypothesis that patients with HCN and dogs with CCH have an underlying abnormality in the regulation of hemopoiesis, including lymphopoiesis, as suggested here should be investigated further in the grey collie model.

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