Proliferation Kinetics of Sézary Cells

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The proliferation kinetics of neoplastic T cells arising in Sézary syndrome (Sézary cells) are still poorly understood. Kinetic studies with $^3$H-thymidine as a DNA precursor revealed a low incorporation rate of the nucleotide into Sézary cells obtained from the peripheral blood versus Sézary cells from the skin. Using the double-label autoradiography we determined the duration of single cell cycle phases of blood and cutaneous Sézary cells. The results indicate the almost complete lack of proliferative activity in the blood but a considerable portion of proliferatively active Sézary cells in skin infiltrates. The removal of a large mass of quiescent blood cells by leukapheresis did not affect the proliferative state of the residual peripheral cell population implying that the procedure did not induce the migration of proliferating skin cells towards the blood. Terminally, the disease underwent transition into immunoblastic lymphoma. At this time, the kinetic behavior of the peripheral immunoblasts showed great similarity to that of cutaneous Sézary cells. The findings point towards a common extravascular production site of Sézary cells and immunoblasts probably located in the lymphatic tissue.

In recent years, the Sézary syndrome (SS) has attracted considerable interest from the hematologic, immunologic, and dermatologic points of view because the cells involved in this disease offer the unique opportunity of providing important insights into lymphocyte physiology. It is now generally accepted that the Sézary cells (Sc) found in peripheral blood, bone marrow, skin infiltrates, and throughout the lymphoreticular system represent a clonal population of neoplastic T lymphocytes. Their morphologic appearance is characterized by a convoluted or cerebriform nucleus. The histologic manifestation in the skin is identical to that in mycosis fungoides, and many investigators support the concept that SS is a leukemic variant of the latter.

The original site of Sc production is still unknown. Kinetic studies in vivo or in vitro, which would help to identify the site of proliferation of the neoplastic T cells, have been scant. Chandra et al. found a relatively high $^3$H-thymidine labeling index of cutaneous Sc after intradermal $^3$H-thymidine injections, but a lack of $^3$H-thymidine uptake during a 1-hr incubation of isolated circulating Sc. Similarly, Bosman and Van Vloten observed a more rapid proliferative activity of Sc obtained from skin infiltrates as compared to peripheral Sc. Shackney et al. suggested on the basis of in vivo investigations with pulse doses of $^3$H-thymidine that the skin was not the primary site of Sc production. In a recent study, Miller et al. scanned Sc, labeled with $^{11}$In oxine, after intravenous injection in the body of their patient and observed that the cells accumulated in the skin and did not reenter the circulation. Bunn et al. concluded from their analyses that the neoplastic cells in mycosis fungoides and SS proliferate preferentially in lymph nodes and from there migrate into the blood stream.

The present study was mainly designed to further evaluate cell-cycle parameters of Sc in a case of SS developed from mycosis fungoides, which terminally underwent transition into lymphoma of high-grade malignancy. Using a double-label autoradiography technique, the proliferative capacity of Sc isolated from peripheral blood as well as skin infiltrates was studied in the different stages of the disease. In addition, the effect of extensive leukapheresis on the proliferative behavior of the residual peripheral Sc population was determined.

MATERIALS AND METHODS

Isolation of Peripheral Blood Lymphocytes

Peripheral blood lymphocytes were isolated from heparinized blood after 1:1 dilution in normal saline, layered onto Ficoll-Hypaque gradients, and centrifuged at 400g for 20 min.

Preparation of Skin Cells

Specimens of skin biopsies were carefully minced and kept in 0.2% trypsin solution for 1.5 hr at 4°C. Subsequently, connective epidermal tissue and adhesive collagen fibers were detached with forceps and removed by passage through a close-meshed gauze. From the resulting suspension, viable cells were separated by Ficoll-Hypaque density centrifugation, washed, and dispersed in Hank’s solution.

Autoradiographic Procedure

For kinetic studies an in vitro double-label autoradiography technique was applied. The cells ($10^7$) were labeled with $^3$H-thymidine (specific activity, 50-60 Ci/mmol; 1 µCi/ml of cell suspension) for 1 hr and with $^3$H-thymidine (specific activity, 2 Ci/mmol; 10 µCi/ml of cell suspension) for another. Incubations were done at 37°C with 2.2 atm of pure oxygen. Following the labeling procedure, cytospin smears were prepared, covered with

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Fig. 1. See legend facing page.
Ilford emulsion in gel form (Type G-5), and exposed at -20°C for 5 days. After developing, the preparations were stained with Mayers Himalaun and microscopically evaluated. During the autoradiographic process 14C- and 1H-thymidine had been added successively to the cell suspension. The time interval between the two isotope applications (1 hr) allowed part of the cells to leave the S phase and to stay purely labeled with 14C. On the other hand, additional cells entered the S phase during the second incubation period with 1H-thymidine, and they show the pure 1H label. By the end of the experiment most of the cells, however, were double-labeled with 14C and 1H. According to Klein et al., the ratio of nuclei containing the 14C label to purely 1H-labeled nuclei was used as a direct measure of the length of the DNA synthesizing phase of the cell cycle (Ts). The labeling index (LI) was determined by counting 5000 nonerythroid mononuclear cells, on an average. The background count was between less than 1 and 5 grains per nucleus. Cells that were considered labeled had 10 and more grains overlaying the nucleus. Counting of LI was done on 2-3 autoradiographic slides from the same experiment. Variation of LI between the samples was found to be 1%-2% at most. The length of the entire turnover time of the cells (Tc) was calculated as the ratio of Ts to LI, assuming a simple steady-state growth pattern of the cell population. Furthermore, the percentage of mitotic figures (MI) was counted. The length of mitosis (Tm) was calculated as a function of MI and Tc. To determine the length of the G2 phase, the method of labeled mitoses might be used. Normally, the length of the entire generation time (Tc) by far exceeds that of G2. Therefore, uncertainty about the value for G2 is only of minimal influence on the kinetic results.

Lennartz and Maurer reported relatively constant lengths of G2 ranging between 1 and 4 hr. The general assumption of 2 hr for the length of G2 in the kinetic study presented, therefore, seemed to be appropriate. Finally, the duration of the G1 phase was calculated by subtracting the S, G2, and M phases from Tc.

**Determination of Cyclic Adenosine 3',5'-Monophosphate (cAMP)**

The concentration of cAMP in mononuclear cells was determined by the protein binding assay according to Gilman. Details of purification procedures for the cyclic nucleotide have been reported elsewhere.

**Leukapheresis Procedure**

Leukapheresis was carried out using the AMINCO continuous flow celltrifuge. One single run was performed that lasted 4 hr. At a flow rate of 50 ml/min, 12 liters of blood were centrifuged. Plasma-steril (500 ml; 6% hydroxyethyl starch in 0.9% sodium chloride) was infused as plasma expander. Coagulation was prevented by collecting the cell suspensions in BIOPACK P ACD-Adenin (sodium citrate 22.0 g/liter; citric acid 7.3 g/liter; glucose H2O 24.5 g/liter; adenine 5.8 mg/liter).

**CASE REPORT**

Z.E., a 74-yr-old female was first admitted to the First Department of Dermatology, University of Vienna Medical School, in July 1977. Four months before, the patient had begun to develop generalized erythroderma. Physical examination showed exfoliative erythroderma with several small infiltrated plaques in the upper chest region. There were no enlarged lymph nodes nor hepatosplenomegaly. The skin biopsy revealed Pautrier's microabscesses, containing mononuclear cells with deeply clefted nuclei within the epidermis. The histologic diagnosis of erythrodermic mycosis fungoides was confirmed by electron microscopy. Laboratory findings showed normal white blood cell count (WBC) and peripheral blood differentials. Treatment with photochemotherapy and with topical corticosteroids resulted in some relief from itching, whereas the erythroderma did not respond. In March 1978, pruritus and skin infiltration with plaque formation markedly increased, lymphadenopathy and modest hepatosplenomegaly developed, and the WBC increased to 18,000/µl. At this time, the patient was admitted to the Hematology Service, First Medical Department, University of Vienna Medical School. Histologic and laboratory findings revealed the presence of Sézary cells (Sc) in peripheral blood so that Sézary syndrome was diagnosed. The most relevant clinical data are listed below.*

- **Peripheral blood:** Hb 13.7 g/dl, Hct 42%, WBC 18,000/µl with 75% lymphocytes (more than 30% were typical Sc with cerebriform nuclei).
- **Bone marrow:** no evidence of infiltration with Sc
- **Cytochemistry of Sc from peripheral blood:** 80% of the cells revealed increased activity of β-glucuronidase and acid phosphatase (focal pattern).
- **Serum immunoglobulins:** IgG 980 mg/dl, IgA 200 mg/dl, IgM 100 mg/dl.
- **Serum LDH:** 399 mU.

Cyto genetics: Culturing of peripheral mononuclear cells in order to determine the cell karyotype was unsuccessful.

The patient was treated with 3 cycles of adriamycin (20 mg), vinblastine (10 mg), and bleomycin (15 mg) and achieved partial hematologic (10% of residual Sc) and clinical remission. She was kept under maintenance therapy with chlorambucil and prednisone and showed good clinical response.

In April 1979, WBC began to rise within a few weeks from normal values to 28,000/µl. At the same time, a dramatic increase in lymph node size, massive enlargement of liver and spleen, and numerous skin infiltrates occurred.

Using the modified trypsin banding method according to Wang-Peng, the cyogenetic examination revealed 2 malignant cell clones. Mitoses from unstimulated peripheral mononuclear cells, cultured for 24-72 hr, showed a normal karyotype; after phytohagemagglutinin stimulation, karyotypes with 67-69 chromosomes were observed with numerous chromosomal aberrations including 5q-, 6q-, 12q-, and 14q+.

Despite intensive chemotherapy, the patient died within 3 wk. The autopsy revealed a lymphoma of high-grade malignancy (immunoblastic lymphoma according to the Kiel classification). Figure 1 shows the lymph node biopsies obtained at the time of diagnosis of Sézary syndrome (Fig. 1A) and after death (Fig. 1B).

**RESULTS**

**Kinetic Measurements**

Mononuclear cells from both the peripheral blood and cutaneous infiltrates were subjected to double-label autoradiographic examination in order to determine the mean length of single cell cycle phases. The results are summarized in Table 1. When proliferation

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*Immunologic data to this case were reported elsewhere (Acta Med Austriaca 6:237, 1979).
kinetics were first analyzed, the labeling index (LI) for Sc from the skin markedly exceeded that for peripheral blood. While 5.2% of the cutaneous cells were labeled with thymidine, only 0.2% of the mononuclear blood cells showed the label. The different proliferative situation in the two tissues was also reflected by the mitotic indices (MI): the MI for skin cells amounted to 0.1%, whereas the MI for blood cells was as low as 0.001%. Although the length of the DNA synthesizing phase (Ts) was quite comparable in both cell populations (12 and 16 hr, respectively), the mean turnover times differed considerably. The Tc of the cutaneous cells was 239 hr versus 7571 hr measured for the peripheral cells. This indicates that the turnover rate of mononuclear cells was much higher in the cutaneous lesions than in the peripheral blood.

Occasionally, promising therapeutic effects of leukapheresis in SS have been suggested.28 This prompted us to study the proliferative behavior of the mononuclear blood cells from our patient before and after cell separation. The question was whether leukapheresis could mobilize cells from some storage sites (skin? lymph node?) and, perhaps, stimulate quiescent cells to proliferate. Cell kinetic measurements performed 96 hr after leukapheresis revealed that by that time the cells removed from the blood (10^11 cells) had been replaced by an equal number of cells which, in their proliferative behavior, did not differ from the cell population observed before (LI: 0.24%; MI: 0.001%; Tc: 7542 hr). These kinetic results are summarized in Table 2.

After the disease had undergone transition into lymphoma of high-grade malignancy (immunoblastic lymphoma), autoradiographic reexamination was performed. The kinetic analyses had to be limited to peripheral blood cells, since the poor condition of the patient ruled out repeat skin or lymph node biopsies. As shown in Table 1, the immunoblastic T cells isolated from the peripheral blood exhibited a relatively short turnover time (Tc 238 hr), a high percentage of labeled cells (LI 2.21%), and a considerable number of cells in mitosis (MI 0.1%). These kinetic data corresponded to those observed in cutaneous Sc, except for the markedly shorter duration of the S phase (Ts 5.3 hr).

**cAMP Measurements**

In order to further characterize cell cycle parameters, the concentration of cAMP in the mononuclear peripheral blood cells was determined. The intracellular cAMP content amounted to 7.9 pmole/10^7 cells. Similar cAMP levels have been measured in peripheral lymphocytes from chronic lymphocytic leukemia,29,30 suggesting comparable blood cell kinetics in these two diseases.

The cAMP level of mononuclear cells appearing in the peripheral blood after leukapheresis was 8.3 pmole/10^7 cells. This finding and the autoradiographic data described before indicated that the proliferative state of the cell population after leukapheresis was identical to that of the cells present before.

**DISCUSSION**

We studied the incorporation of ^3H- and ^14C-thymidine into the neoplastic cells obtained from blood and skin infiltrates of a patient with Sézary syndrome to get further insight into the proliferative behavior of Sézary cells (Sc). The autoradiographic analysis revealed a markedly longer turnover time for peripheral Sc than for those from the skin. It should be considered, though, that the mean generation time determined by double-label autoradiography refers to the total cell number of a cell population. It does not reflect the contribution of few rapidly proliferating cells likely to exist within a large mass of slowly or nonproliferating (G0) cells. Some indication of the size of this more active compartment is provided by the labeling index (LI) of the entire cell population.11 In our case, the low LI (0.2%) measured for peripheral

### Table 1. Proliferation Kinetics

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<tr>
<th>Sort of Mononuclear Cells</th>
<th>Kinetic Results</th>
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<tr>
<td></td>
<td>LI (%)</td>
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<td>Sézary Cells</td>
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<tr>
<td>Peripheral blood</td>
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<tr>
<td>Skin</td>
<td>5.19</td>
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<tr>
<td>Immunoblastic T cells</td>
<td>2.21</td>
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LI, labeling index; MI, mitosis index; Ts, length of the S phase in hours; T61, length of the G1 phase in hours; Tc, turnover time in hours; cAMP, concentration of cyclic adenosine 3',5'-monophosphate.

### Table 2. Effect of Leukapheresis on Cell Cycle Kinetics and cAMP Content of Peripheral Sc

<table>
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<tr>
<th></th>
<th>Kinetic Results</th>
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<tr>
<td></td>
<td>LI (%)</td>
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<tr>
<td>Before leukapheresis</td>
<td>0.21</td>
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<tr>
<td>After leukapheresis</td>
<td>0.24</td>
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LI, labeling index; MI, mitosis index; Ts, length of the S phase in hours; T61, length of the G1 phase in hours; Tc, turnover time in hours; cAMP, concentration of cyclic adenosine 3',5'-monophosphate.

*Cells studied 96 hr after leukapheresis.*

The cAMP level of peripheral Sézary cells (Sc) suggests the existence of an infinitesimally small fraction of short-lived cells side by side with a large pool of long-lived ones. Comparable results were obtained with peripheral lymphocytes from patients with chronic lymphocytic leukemia (CLL), where a low LI suggested accumulation of nonproliferating cells in the blood.2 Double-label autoradiography in vitro3 as well as long-term infusions with 3H-thymidine in CLL patients4 revealed that two populations of peripheral lymphocytes exist in this disease: a very small fraction with a high turnover rate and a large one with an extremely prolonged turnover time. Data obtained by Shackney et al.5 with pulse doses of 3H-thymidine may be compatible with the view that the pool of peripheral Sc also consists of two cell compartments. The authors found that the majority of peripheral Sc were in a nonproliferating state. The appearance of rather rapidly proliferating cells in the blood 18-24 hr after 3H-thymidine application suggested influx from some extravascular site that could not have been the skin, since cutaneous Sc showed different kinetic behaviour.

The LI of cutaneous Sc of our patient considerably exceeded that of peripheral Sc (5.2 compared to 0.2%); therefore, the portion of rapidly proliferating cells in the skin was obviously greater. In both populations, the length of the DNA synthesizing phase was quite comparable, indicating that the different duration of the mean generation time essentially had to be due to differently long G1 phases. The much longer G1 phases of circulating Sc do not exclude, however, that these cells could possibly originate from the skin. If so, the depletion of peripheral Sc by leukapheresis might result in migration and at least in a transitory occurrence of rapidly proliferating cutaneous Sc in the blood. Despite extensive leukapheresis, however, we were unable to detect any change in cell cycle parameters of Sc in the peripheral blood. In accordance with that, the cAMP concentration of Sc in the blood did not differ before and after the procedure. Since the cAMP level in a cell fluctuates during the various cell cycle phases,6-7 changes in the proliferative state of the cell should be reflected by the nucleotide concentration. The lack of influence of leukapheresis on the cAMP level of peripheral Sc implies that cell cycle kinetics did not change with leukapheresis. It seems very unlikely, therefore, that cell separation induced rapidly proliferating cells from the skin to migrate towards the blood.

Miller et al.8 recently reported on the migratory pattern of Sc labeled with 111In. They concluded that Sc accumulate in the blood and from there migrate into the skin. Our experiments do not object to such a cell flux. It is difficult, however, to relate Miller’s data to clinical observations in other cutaneous lymphomas. In mycosis fungoides, for instance, which usually precedes SS, cutaneous infiltration with neoplastic T cells occurs despite complete sparing of the peripheral blood.

The neoplastic cells in cutaneous lesions as well as in peripheral blood of SS probably originate in lymph nodes, as suggested by several authors.8,9 Our observation of blastic transformation of SS and the follow-up of the cell cycle kinetics of the immunoblastic cells may provide further support of this view. In the terminal stage of SS in our patient, a sudden increase in the size of all lymph nodes, enlargement of liver and spleen, dramatic increase in skin infiltrates, and the appearance of large immunoblastic T cells occurred. At this time, the autoradiographic analysis of peripheral tumor cells demonstrated a much higher LI and a shorter turnover time than observed before. The great similarity between the turnover time of the immunoblastic T cells and that of cutaneous Sc indicated their common origin. This was substantiated by the post-mortem finding that the Sc had been completely replaced by immunoblastic T cells and that these cells were found in lymph nodes, the skin, as well as in the peripheral blood. To the question whether this transition to an immunoblastic lymphoma should be considered as a new malignancy or the mere change from differentiated to undifferentiated lymphoma, histologic findings in our patient favor the latter. Previous reports on lymphomatous expansion of the original malignant cell clone in mycosis fungoides10 and SS11 confirm our conclusion.

REFERENCES

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