Patterns of Cell Proliferation and Cell Migration in the Sezary Syndrome

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The patterns of cell proliferation and cell migration were studied in three patients with the Sezary syndrome using autoradiographic techniques. Cell labeling patterns following pulse labeling with tritiated thymidine in vivo indicated that Sezary cells proliferate actively in skin and in lymph nodes but that few if any Sezary cells proliferate in the peripheral blood. In two of the patients serial samples were obtained. Label dilution patterns in skin and blood over time suggested that circulating Sezary cells originated in extracutaneous sites where cells were proliferating more rapidly than in the skin. Cells labeled in extracutaneous sites of proliferation appear rapidly in the blood, and their transit time through the peripheral blood compartment is short. Circulating Sezary cells may then be deposited in the skin where they resume proliferation at a low rate. Thus, while Sezary cells proliferate in both cutaneous and extracutaneous sites, proliferation appears to be more rapid in extracutaneous sites such as lymph nodes. This suggests that trials of systemic therapeutic approaches should be undertaken.

THE SEZARY syndrome, first described in 1938 by Sezary and Bouvrain,1 is characterized by generalized erythroderma with atypical lymphocytes infiltrating the dermis and epidermis and circulating in the peripheral blood.2 4 The circulating Sezary cell has been shown to have the membrane characteristics of thymus-derived lymphocytes and may exhibit functional properties of normal T cells, including migration inhibition factor production and the ability to "help" in vitro immunoglobulin synthesis.7 10 The Sezary syndrome is part of a spectrum of cutaneous T-cell lymphomas, which include mycosis fungoides and lymphomatoid papulosis.11,12

Clinical observations suggest that Sezary cells migrate between the blood and skin.13 Tritiated thymidine (3HTdR) studies in vitro and studies using local intradermal injections of 3HTdR in vivo have shown that Sezary cells have the capacity to synthesize DNA in the skin but not in the peripheral blood.14 16 On the basis of these observations, it has been suggested that Sezary cells migrate from their sites of production in the skin into the peripheral blood. It is commonly held that lymph nodes and visceral organs are seeded by the hematogenous route in late stages of disease. More recently, Miller et al. have provided evidence that the major flow of Sezary cells is from the blood into the skin and that cells entering the skin do not return to the circulation.17

Materials and Methods

Two patients were given a single intravenous bolus injection of sterile, pyrogen-free 3HTdR (6.7 Ci/mmole, New England Nuclear Corp., Boston, Mass.) at a dosage of 0.2 mCi/kg. Serial 3-mm skin punch biopsies and peripheral blood samples were obtained at intervals. Skin punch biopsies were fixed in 10% buffered formalin and embedded in paraffin. Serial 5-μ sagittal sections through the entire specimen were mounted on acid-cleaned microscope slides and stained by a modified Feulgen method. Heparinized peripheral blood specimens were centrifuged at 600 g for 20 min. Cells from the buffy coat were then smeared on acid-cleaned microscope slides and fixed in methanol. In one patient, Sezary cells were simultaneously obtained from peripheral blood, lymph node, and skin and labeled in vitro with 3HTdR. Lymph node and skin biopsy specimens were mechanically disaggregated by mincing, passage through a 25-gauge needle. The peripheral blood sample was subjected to a Ficoll-Hypaque separation, and mononuclear cells were obtained from the interface layer. All three samples were incubated with 0.2 mCi/ml of 3HTdR (6.7 Ci/mmole, New England Nuclear Corp.) for 45 min.

For autoradiographic processing, the slides were dip-coated with a mechanical dipper in Kodak NTB2 liquid emulsion (Rochester, N.Y.), diluted 1:1 with distilled water. Slides were air dried, exposed to 3% H2O2 vapors for 3 hr,18 sealed in light-tight boxes containing Drierite, and stored in the dark at 5°C. The emulsion exposure time was 10 wk for slides labeled in vivo and 1 wk for slides labeled in vitro. The autoradiographs were developed in Kodak D19 developer (Rochester, N.Y.) for 5 min at 15°C. The Feulgen-stained...
skin sections were counter-stained with 1% aqueous light green for 1 min, dehydrated, and cover slipped. Lymph node and peripheral blood samples were stained with Giesma for 7 min, air dried, and cover slipped.

In the in vitro studies, grain counts were performed on mitotic and interphase basal epidermal cells, on mitotic and interphase cutaneous Sezary cells, on circulating interphase Sezary cells, and on circulating neutrophilic granulocytes. At each time point, 500 interphase cells of each type were counted. In the in vitro studies, labeling indices were calculated by counting 1000 interphase Sezary cells in each sample. Cells with more than 5 grains overlying the nucleus were considered to be labeled. In all three patients, more than 90% of the circulating lymphocytes had the classical light and electron microscopic appearance of Sezary cells and had T-cell membrane properties, as previously reported. In addition, the dermal infiltrates in all three patients contained clusters of lymphocytes with highly convoluted nuclei that had the characteristic light microscopic appearance of Sezary cells. The labeling indices of the cutaneous Sezary cells were determined in these cells. For normal cells in the epidermis, all nucleated epithelial cells were included in the labeling index calculation. At least 50 and usually 100 mitoses of each type were counted at each time point. A local background correction was made for grain counts over each mitotic figure and interphase nucleus as described previously. The mitotic index was determined in basal epidermal and cutaneous Sezary cells by counting the number of mitoses per 10,000 interphase cells.

Circulating lymphocytes from patients J.N. and P.S. were prepared by Ficoll-Hypaque separation for flow cytometry. The cells were fixed in 10% formalin and stained with acriflavine-Feulgen or stained directly with hypotonic propidium iodide. The DNA contents of 100,000 cells were measured on a Los Alamos cell sorter or a Coulter TPS-1 cell sorter (Coulter Electronics, Hialeah, Fla.). The fraction of cells in the S phase was determined by the method of Jett.

The depth of the dermal Sezary cell's infiltrate was measured microscopically with a micrometer in each skin biopsy at each sample point, and an average value was estimated for each patient. The number of cells was counted in each of ten 50 × 50 μm areas in each skin biopsy. Crude estimates of average Sezary cell population densities (cell/volume) were obtained assuming that all sections were cut evenly at a thickness of 5 μm. No attempt was made to correct for specimen shrinkage during fixation and processing.

Average cell cycle times in the basal epidermal cells, cutaneous Sezary cells, and circulating Sezary cells were estimated from mean grain count, halving times in interphase cells obtained by a least squares fit using the MLAB computer modeling program. The following formulas were used to arrive at estimates of the body burden of circulating and cutaneous Sezary cells in each patient:

\[
\text{Estimated blood volume (EBV) (liter)} = 2.65 \times \frac{\text{Total body surface area (BSA) (sq m)}}{10}
\]

Circulating Sezary cell pool = Circulating Sezary cell concentration (cells/liter) × EBV (liter)

Cutaneous Sezary cell pool = BSA (sq m) × Average depth of cutaneous Sezary cell infiltrate (m) × Sezary cell population density (cells/cm³)

Patients

Three patients with histologically confirmed diagnoses of the Sezary syndrome were studied. Patient M.V. was a 52-yr-old white female who was diagnosed 3 yr prior to study. Involvement of skin, peripheral blood, and lymph nodes had been demonstrated histologically. Prior treatment consisted of topical and systemic corticosteroids, which were discontinued 1 mo prior to study. During the 2 wk of study, her WBC count was 9000–10,000/cu mm, with 30% circulating lymphocytes of which 90 were classical Sezary cells on light and electron microscopy.

J.N. was a 54-yr-old white male in whom the diagnosis of Sezary syndrome was made 6 yr prior to study. He had been previously treated with topical nitrogen mustard, topical and systemic corticosteroids, electron beam radiation therapy, chlorambucil, a 3-drug combination of cyclophosphamide, vincristine, and prednisone, and leukapheresis. No chemotherapy or radiation therapy had been administered for more than 8 mo prior to study. The patient underwent leukapheresis 2 mo prior to study and received no further cytoreductive therapy of any kind until after the study was completed. Involvement of skin, peripheral blood, and lymph nodes had been confirmed histologically prior to the time of study. During the 2 wk of study, the WBC count was 21,000–24,000/cu mm with 75%–80% lymphocytes, of which 95% had the classic light and electron microscopic appearance of Sezary cells.

P.S. was a 61-yr-old white male in whom the diagnosis was made 6 mo prior to study. He had previously been treated with systemic hyperthermia and subcutaneous injections of BCG. Involvement of lymph nodes and liver was confirmed histologically, while the bone marrow was histologically negative. At the time of study, the WBC count was 23,900/cu mm with 52% circulating lymphocytes, 90% of which was classical Sezary cells by light and electron microscopy, as were the results of leukapheresis in J.N. The Sezary cells in patients J.N. and M.V. were predominantly of the “small” cell type, while those in patient P.S. were predominantly “large” cell type. A written informed consent in compliance with DHEW guidelines was signed by each patient prior to study.

RESULTS

Mitotic Indices, Pool Sizes, and Transit Times

Mitotic indices and Sezary cell pool sizes for patients M.V. and J.N. are given in Table 1. In both patients, Sezary cell mitotic indices were lower than the mitotic indices in the basal epidermal cells, indicating lower rates of cell production in the cutaneous Sezary cell populations of both patients.

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<thead>
<tr>
<th>Table 1. Mitotic Indices and Estimated Pool Sizes</th>
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<tr>
<td>Patient</td>
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<tr>
<td>Mitotic index, basal epidermal cells</td>
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<td>Mitotic index, cutaneous Sezary cells</td>
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<td>BSA (sq m)</td>
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<td>Estimated blood volume (liter)</td>
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<td>Circulating Sezary cell pool size</td>
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<td>Average depth of cutaneous Sezary cell infiltrate (m)</td>
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<td>Sezary cell population density (cells/sq m)</td>
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<td>Cutaneous Sezary cell pool size</td>
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Fig. 1. Radioautograph of skin biopsy in patient J.N. 2 hr after intravenous ³HTdR administration. Epithelium is seen in upper left, and dermal infiltrate of Sezary cells in center and lower right. Stained with Feulgen and aqueous light green (x 400). (A). Labeled basal cell (arrow) x 1000. (B). Labeled Sezary cell (arrow) x 1000.
In both patients the cutaneous body burden of tumor cells was of the order of 2–5 kg (assuming 10^{12} cells/kg). The magnitude of the cutaneous Sezary cell pool exceeded that of the circulating cell pool by almost 50-fold in patient J.N. and by over 200-fold in patient M.V.

Two hours after pulse ³HTdR injection, epidermal cell labeling was confined to the basal layer. By day 8, labeled cells had migrated from the basal layer into the stratum malpighii in both patients. In J.N., labeled cells were seen in the stratum granulosum by day 14. An example of basal epidermal labeling at 2 hr is shown in Fig. 1. Labeled cutaneous Sezary cells in the dermis are also apparent in the figure. Labeled Sezary cells were also observed in the epidermis of both patients.

**Interphase Cell Labeling Patterns as a Function of Time After Pulse ³HTdR Exposure**

Thresholded labeling indices as a function of time after pulse ³HTdR administration for the basal epidermal cells and for the cutaneous Sezary cells are shown in Fig. 2. In both patients, basal epidermal cells were labeled within 2 hr of ³HTdR exposure. The fraction of heavily labeled cells decreased with time (Figs. 2A1 and 2B1). At 2 hr, the cutaneous Sezary cells in J.N. were also labeled, and the fraction of labeled cutaneous Sezary cells was higher than the fraction of labeled basal epidermal cells (compare Figs. 2B1 and 2B2). The labeling was also more intense. In contrast, in patient M.V., the fraction of labeled cutaneous Sezary cells was initially low; it rose progressively to a maximum at 24 hr and then declined (Fig. 2A2). In M.V., the peak fraction of heavily labeled cutaneous Sezary cells was smaller than the fraction of heavily labeled basal epidermal cells.

The thresholded labeling indices as a function of time after pulse ³HTdR exposure for the circulating Sezary cells and granulocytes are shown in Fig. 3. In both patients, initial labeling and labeling intensity were low. At the >3 grain/cell threshold, the fraction of labeled circulating Sezary cells was less than 0.01 2 hr after pulse. In both patients, the fraction of labeled circulating Sezary cells rose progressively to a maximum at 24 hr and then declined gradually. Sezary cells with grain counts exceeding 100 grains/cell were found exclusively in the blood and not in the skin in both patients. This could be due to differing grain detection efficiencies in smears and tissue sections.

The rise in the labeled fraction of circulating mature granulocytes was delayed by 3–4 days, with a peak at 8 days in both patients (Figs. 3A1 and 3B1). While the peak labeling indices of the mature granulocytes were higher than those of the circulating Sezary cells, peak granulocyte cell labeling intensities were lower in both patients.

Mean cell grain count patterns as a function of time after pulse ³HTdR exposure are shown in Fig. 4. In the basal epidermal cells of both patients, the mean grain count was maximal at 2 hr and declined slowly (data not shown). In the cutaneous Sezary cells of patient J.N. the mean grain count was also maximal at 2 hr and declined slowly (Fig. 4B1). In contrast, in patient M.V., the mean grain count of the cutaneous Sezary cells was low initially, increased progressively to 24 hr, and then decreased slowly with time (Fig. 4A1).

In both patients, the mean grain count of the circulating Sezary cells was low initially, rose progressively during the first 24 hr, and then decreased with time (Figs. 4A2 and 4B2). Grain count halving times, calculated from the curves shown in Fig. 4 from day 2 onward, were 8 and 4 days for the circulating Sezary cells in M.V. and J.N., respectively, and 13 and 17 days for the cutaneous Sezary cells. The grain count halving times of circulating Sezary cells and epidermal cells may be taken as crude estimates of the average cell cycle times in these tissues.

**Percent Labeled Mitosis (PLM) Curves**

Multithresholded PLM curves for the epidermal basal cells and cutaneous Sezary cells in each patient are shown in Fig. 5. All four PLM curves were qualitatively similar. The labeled mitoses peaked at 70%–80% labeling at 12–24 hr; a trough was discernible at 3 days. The second wave was broad, low, and poorly defined at low grain count thresholds. At higher thresholds, a more distinct second wave was apparent at 4–6 days.

**Simultaneous In Vitro Labeling Indices**

The labeling indices from the peripheral blood, lymph node, and skin of patient P.S. determined after in vitro labeling of the Sezary cells are shown in Table 2. The labeling index was highest in the lymph node and lowest in the peripheral blood.

**Flow Cytometry**

The fraction of cells in S phase from the peripheral blood mononuclear cells of both patients J.N. and P.S. was calculated as less than 0.01. The Sezary cells in J.N. had a diploid DNA content, while those of P.S. were hyperdiploid with a DNA content 1.2 times greater than diploid cells.

**DISCUSSION**

The data presented in this article suggest that (1) Sezary cells proliferate actively in both the skin and lymph nodes, but that Sezary cells circulating in the
Fig. 2. Thresholded labeling indices in the epidermal basal cells and in the cutaneous Sazary cells as a function of time after pulse H'dr administration in patients M.V. and J.N.
Fig. 3. Thresholded labeling indices in circulating granulocytes and in the circulating Sezary cells as a function of time after pulse H1-DR administration in patients M.V. and J.N.
peripheral blood are not actively proliferating. (2) The rate of Sezary cell production in lymph nodes is greater than in the skin. (3) The majority of circulating Sezary cells originate in extracutaneous sites of cell production. (4) There is cell migration among the extracutaneous, circulating, and cutaneous Sezary cell compartments. (5) In the presence of a relatively high rate of cell proliferation, the relatively indolent clinical course of Sezary syndrome suggests a high degree of cell loss. These data suggest that the Sezary syndrome is usually systemic in nature, with considerable extracutaneous cell proliferation. Thus, systemic therapeutic approaches, including systemic chemotherapy and/or radiotherapy, should be considered in this disease.

The presence of labeled Sezary cells in the skin 2 hr after pulse intravenous $^3$HTdR and after in vitro $^3$HTdR labeling and the multithreshold PLM curves demonstrate that Sezary cells proliferate in the skin. This is consistent with the data of others demonstrating in vitro uptake of $^3$HTdR by cutaneous Sezary cells. The PLM curves of the cutaneous Sezary...
Fig. 5. Multithreshold percent labelled mitoses in the epidermal basal cells and cutaneous Sézary cells as a function of time after pulse H3TDR administration in patients M.V. and J.N.
circulating S-phase cells by flow cytometry in patients cells at 18-24 hr after pulse could have been due only dividing fraction detected in PLM curve analysis (vide infra).

In contrast, there is no evidence for active proliferation of circulating Sezary cells. In both patients who were given \(^3\)HTdR in vivo, the labeling indices and mean grain counts of the Sezary cells circulating in the peripheral blood rose from initially low values to maximum values at 24 hr. \(^3\)HTdR administered as a pulse dose is available for incorporation into DNA for 1-2 hr at most.\(^{26}\) Hence, the increase in labeling index and labeling intensity observed in circulating Sezary cells at 18-24 hr after pulse could have been due only to the migration into the blood of cells that had acquired their heavy label at some site other than the blood at the time of initial \(^3\)HTdR exposure. The absence of Sezary cell proliferation in the blood is supported by the absence of significant numbers of circulating S-phase cells by flow cytometry in patients J.N. and P.S. and by the low initial labeling and labeling intensity in all patients. In addition, others have shown that circulating Sezary cells do not incorporate \(^3\)HTdR in vitro to any significant degree.\(^{14,16}\)

The fraction of circulating Sezary cells in S phase has also been shown to be low (average 1%) in other flow cytometry studies.\(^{27,28}\)

In contrast with the circulating Sezary cells, there is a delay of several days in the appearance of labeled circulating neutrophilic granulocytes. Proliferating granulocyte precursors are known to undergo several maturational divisions in the bone marrow before being released into the blood. It would appear that Sezary cells that are proliferating at their primary site of production are under no such constraints.

Since circulating Sezary cell counts did not change appreciably over the course of the study, cell influx into the peripheral blood must have been balanced by cell outflow from this compartment. Thus, rapid changes in labeling index and particularly in cell labeling intensity must reflect rapid transit of Sezary cells through the peripheral blood compartment. This is consistent with the data of Miller et al., who demonstrated significant cutaneous radioactivity 24 hr after the intravenous injection of radiolabeled Sezary cells.\(^{17}\)

Given that Sezary cells do not proliferate in the peripheral blood, the fall in mean grain count that was observed in circulating Sezary cells (Fig. 4) after 24 hr can be attributed only to the progressive replacement of the more heavily labeled circulating Sezary cells by progressively more lightly labeled Sezary cells migrating from their primary site of cell production. That is, the grain count halving time of the circulating Sezary cells must mirror the grain count halving time of Sezary cells at the primary site of cell production. Thus, the grain count halving time of Sezary cells at the primary site of production must be 4-8 days or less.

The grain count halving times of the cutaneous Sezary cells of patient M.V. was 13 days, and that of patient J.N. was 17 days. These grain count halving times are much longer than that expected for the site of origin of the circulating Sezary cells. Thus, the data suggest that there must be an extracutaneous cell production site where the rate of cell proliferation is higher than in the skin and which serves as the major source of circulating Sezary cells. It should be noted that this conclusion is based only on comparisons of observed grain count halving patterns in skin and blood, and does not depend on any specific cell kinetic model.

While our data do not exclude the migration of some cutaneous Sezary cells into the blood, they suggest that the major flux of Sezary cells is from the blood into the skin. In patient M.V., the labeling index in cutaneous Sezary cells was low at 2 hr and rose progressively up to 24 hr. In view of the short availability time of \(^3\)HTdR (see above), this implies that the cells were labeled elsewhere at the time of \(^3\)HTdR exposure and later migrated into the skin. This conclusion is supported by the recent findings of Miller et al.\(^{17}\) that labeled cells injected into the peripheral blood rapidly migrate into the skin and do not return to the peripheral blood.

The lymph nodes and other T-cell areas of the reticuloendothelial system would appear to be the most likely sites of extracutaneous Sezary cell production. All three of our patients had generalized lymphadenopathy with histologically confirmed lymph node involvement. Others have shown that patients with the Sezary syndrome invariably have lymphadenopathy with histologic evidence of lymph node involvement, even in early stages of disease.\(^{12,25}\) Bone marrow biopsies were histologically uninvolved in all 3 patients, and liver biopsies were positive only in patient P.S. These sites are not commonly involved in large series.\(^{25}\) The simultaneous labeling studies in patient P.S. demonstrated that the proliferative fraction in lymph nodes was considerably larger than in the skin. This is consistent with flow cytometric data.
showing high fractions of Sezary cells in S phase (average 10%) in lymph nodes.27

The present study suggests a pattern of cell production and cell migration in the Sezary syndrome that is diagrammed in Fig. 6. Rapidly dividing cells produced in extracutaneous sites migrate into the blood. These cells do not proliferate while in transit. From there they may migrate in the skin, where they resume proliferation at a lower overall rate. A small fraction of the cutaneous Sezary cells may migrate from the skin into the blood in some circumstances (e.g., after leukophoresis).11 Since the disease is indolent clinically, the high rates of cell production must be balanced by comparable high rates of cell loss. Cell loss may occur in any or all of the kinetic compartments shown in Fig. 6.

Each of our patients had advanced stages of disease. This could imply the model is valid only for Sezary patients with advanced stages of disease. However, advanced stages of disease are typical of all patients with the Sezary syndrome,25 and only one of our patients had visceral organ involvement and only one had received prior systemic chemotherapy.

There is a large discrepancy between the average cell cycle times estimated from the PLM curves (3–5 days) and the mean grain count halving times (13–17 days) in the cutaneous Sezary cell populations. This has been observed in other human tumors,29 and has been observed in computer simulation studies as well.30 It may be due to several factors: (A) the PLM curve estimate is biased in favor of the rapidly proliferating cells, which contribute the most cells to the mitotic cell pool, and (B) the PLM curve method is biased against the most slowly proliferating cells, the second waves of which may occur at times that exceed the duration of the observation period. Another reason for the discrepancy between average cell cycle times obtained from the PLM curves and the mean grain count halving times may be that the mean grain count halving time may be spuriously long because of heavily labeled cells that had undergone a lengthening of cell cycle time or had stopped proliferating during the course of the observation period. Tritiated thymidine reutilization may also be a factor. In view of the biases inherent in the PLM curve analysis, the grain count halving data would appear to be more indicative of the overall rates of cell production.

The present study has important therapeutic implications. Topical chemotherapy, electron beam radiation, and PUVA may decrease the overall body tumor burden of cells transiently, but will have no effect on the proliferation of Sezary cells at the primary site of cell production. The same holds true for leukaphoresis. In order to reduce or eliminate the tumor cells at the primary site of production, systemic chemotherapy and/or systemic radiotherapy should be considered in therapeutic regimens.

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REFERENCES

16. Bosman F, van Vloten WA: Sezary's syndrome: A cytoge-
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PA Jr Bunn, R Edelson, SS Ford and SE Shackney