Induction of Nuclear Contour Irregularity During T-Cell Activation Via the T-Cell Receptor/CD3 Complex and CD2 Antigens in the Presence of Phorbol Esters

By Uwe Reinhold, Martin Herpertz, Sylvia Kukel, Iris Ottermann, Manfred Uhrlach, and Hans-Wilhelm Kreysel

To determine whether specific T-cell activation pathways could produce nuclear contour irregularity in normal human lymphocytes, purified T cells were stimulated in vitro and subsequently analyzed by electron microscopy. The degree of nuclear contour irregularity was determined with the use of a computerized planimeter. Stimulation via the T-cell receptor (TCR)/CD3 complex using anti-CD3 monoclonal antibodies induced Sézary-like morphology (nuclear contour indices > 6.5) in a significant portion (9% to 28%) of T cells derived from different normal donors. T-cell activation via CD2 antigens showed induction of Sézary-like nuclear morphology in a lower percentage of cells in comparison with stimulation via the TCR/CD3 complex. In contrast, mitogenic stimulation in vitro did not induce alterations of nuclear morphology in T cells. Immunoelectron microscopy showed that nuclear contour irregularity induced in vitro did not correlate with surface-antigen expression of T-cell subpopulations. The results indicate that Sézary-like morphology is associated with cell activation in normal human T cells.

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SÉZARY SYNDROME is a cutaneous T-cell lymphoma (CTCL) characterized by a generalized exfoliative erythroderma with lymphadenopathy. The most characteristic feature of the disease is the presence of Sézary cells in the peripheral blood (PB) and skin. The Sézary cell, first described in 1968 by Lutzner and Jordan, is a structurally distinctive lymphoid cell that has a highly convoluted cerebriform nucleus, a high nucleus-to-cytoplasm ratio, and condensed chromatin along the nuclear membrane. Immunophenotypic analyses of malignant Sézary cells showed a phenotype indistinguishable from that of mature T cells with the exception of CD7 expression. Sézary cells predominantly lack CD7 expression. Furthermore, cutaneous infiltrates in CTCL lesions have been found to contain a high number of CD7⁻ T cells. Recently, we identified a subset of CD7⁻ T cells in the normal human blood that may represent the circulating counterpart of CD7⁻ T cells found in benign and malignant skin lesions.

The cerebriform morphology of T cells in CTCL is poorly understood. Several investigators have emphasized the non-specificity of Sézary-like morphology because similar cells have been described in benign inflammatory-skin diseases, dermopathic lymphadenopathy, and a small percentage of normal PB lymphocytes. These data indicate that the Sézary-like cell per se is not a malignant cell, but a reactive cell that may be associated with skin inflammation. Investigators emphasizing the non-specificity of the Sézary cell often quote the study from Yeekey and others, who reported that Sézary-like cell morphology could be induced in normal PB lymphocytes by stimulation with mitogens. However, other groups could not reproduce this finding and, until now, there was no study confirming the induction of Sézary-like morphology in normal human lymphocytes by cell activation. We describe the differential induction of nuclear contour irregularity in normal lymphocytes during T-cell activation via specific T-cell surface structures that participate in signal transduction. Our results indicate that Sézary-like morphology is associated with cell activation in normal human T cells.

**MATERIALS AND METHODS**

**Cell preparation.** PB mononuclear cells (PBMC) were isolated from whole blood of different normal healthy donors by Ficoll-Hy-paque density centrifugation (Seromed, Berlin, Germany). T-cell-enriched populations were purified from PBMC by passage through a nylon-wool column (Wako Chem, Neuss, Germany). The proportion of residual CD14⁺ monocytes and CD19⁺ B cells was in every case less than 1%. Purified T cells were distributed at 1 × 10⁶/mL in 250-mL culture flasks in RPMI 1640 + 10% fetal calf serum. They were cocultured with titrated amounts of anti-CD3 monoclonal antibody (MoAb) (OKT3; 250, 25, 2.5, 0.25, 0.025 ng/mL), 12-O-tetradecanoyl-phorbol 13-acetate (TPA; 10 ng/mL), anti-CD2 (T1.1; 250, T1.2; 200 ng/mL), anti-CD28 (1 µg/mL), phytohemagglutinin (PHA) (5 µg/mL), pokeweed mitogen (PWM) (5 µg/mL), concanavalin A (ConA) (5 µg/mL), or combinations of these for different periods of time (24, 48, 120 hours). OKT3 was obtained from Ortho Pharmaceutical (Heidelberg, Germany); T1.1/T1.2 from Dianova (Hamburg, Germany); anti-CD2 from Janssen Biochimica (Neuss, Germany); PHA, PWM and Con A from Seromed; and TPA from Sigma (Munich, Germany). Cells were harvested, washed, and prepared for electron microscopy.

**Electron microscopy.** Cells were fixed in 2.5% glutaraldehyde, postfixed in 2% osmium tetroxide, dehydrated, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM9 electron microscope (Zeiss, New York, NY). The degree of nuclear indentation was calculated by a nuclear contour index (NCl = nuclear perimeter/area²). The nuclear perimeter and area were measured by means of a graphic tablet interfaced with a computer using an in-house program (courtesy of W. Schneichel, University of Cologne, Cologne, Germany, unpublished). Cells in which the plane of section grazed the nucleus were not analyzed. Morphometrically, a lymphocyte with NCl greater than 6.5 is defined to be a Sézary-like cell. In each preparation a minimum of 100 cells was analyzed. For

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Submitted April 28, 1993; accepted September 9, 1993.

Supported by the Deutsche Forschungsgemeinschaft DFG (Grant No. Re-690-3-1).

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Table 1. Effect of Various Stimuli on Nuclear Morphology in T Lymphocytes

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Mean NCI ± SD</th>
<th>NCI Max.</th>
<th>% Sézary-Like Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>4.42 ± 0.76</td>
<td>6.60</td>
<td>1</td>
</tr>
<tr>
<td>TPA</td>
<td>4.33 ± 0.64</td>
<td>6.01</td>
<td>0</td>
</tr>
<tr>
<td>Anti-CD3</td>
<td>4.18 ± 0.50</td>
<td>6.14</td>
<td>0</td>
</tr>
<tr>
<td>Anti-CD2</td>
<td>4.26 ± 0.64</td>
<td>8.33</td>
<td>1</td>
</tr>
<tr>
<td>Anti-CD28</td>
<td>4.18 ± 0.48</td>
<td>6.18</td>
<td>0</td>
</tr>
<tr>
<td>Anti-CD3 + TPA</td>
<td>5.46 ± 1.48*</td>
<td>10.32</td>
<td>22</td>
</tr>
<tr>
<td>Anti-CD2 + TPA</td>
<td>4.84 ± 0.86*</td>
<td>8.62</td>
<td>9</td>
</tr>
<tr>
<td>Anti-CD28 + TPA</td>
<td>4.49 ± 0.71</td>
<td>6.77</td>
<td>1</td>
</tr>
<tr>
<td>Anti-CD3 + CD2 + TPA</td>
<td>5.17 ± 1.14*</td>
<td>8.55</td>
<td>13</td>
</tr>
<tr>
<td>Anti-CD2 + CD28 + TPA</td>
<td>4.78 ± 0.83*</td>
<td>8.05</td>
<td>9</td>
</tr>
<tr>
<td>Anti-CD3 + CD2 + CD28 + TPA</td>
<td>5.30 ± 1.13*</td>
<td>8.94</td>
<td>13</td>
</tr>
<tr>
<td>PHA</td>
<td>4.42 ± 0.76</td>
<td>6.60</td>
<td>1</td>
</tr>
<tr>
<td>PWM</td>
<td>4.53 ± 0.74</td>
<td>7.69</td>
<td>2</td>
</tr>
<tr>
<td>ConA</td>
<td>4.35 ± 0.64</td>
<td>6.80</td>
<td>1</td>
</tr>
</tbody>
</table>

* Purified T cells were stimulated for 5 days with various cell-activating agents and subsequently analyzed by electron microscopy (see Materials and Methods). Anti-CD3 was used at 2.5 ng/mL.

Statistical analysis, Wilcoxon’s two-sample test was used. The level of significance was P < .05.

Indirect immunogold electron microscopy: Cells activated in vitro in the presence of OKT3 (2.5 ng/mL)/TPA (10 ng/mL) were harvested, prefixed with 4% paraformaldehyde for 30 minutes, and thereafter analyzed by indirect immunogold electron microscopy. Cells (1 × 10⁶) were incubated for 1 hour with MoAbs and washed twice in phosphate-buffered saline containing 1% bovine serum albumin and 0.01% sodium azide. The following antibodies were used: Leu-4 (CD3), Leu-3a (CD4), Leu-2a (CD8), from Becton Dickinson, Heidelberg, Germany; and 3A1 (CD7), from Biozol, Eching, Germany. Thereafter, cells were incubated with a goat antimouse IgG + IgM antiserum labeled with 12-nm colloidal gold (Dianova) for 30 minutes at 4°C, washed and fixed in 2.5% glutaraldehyde, postfixed in osmium tetroxide, dehydrated, and resin embedded. Control experiments were performed by incubating T cells with isotype-specific mouse Ig followed by the gold-labeled goat antimouse IgG + IgM. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by electron microscopy. A cell was considered to be reactive when several gold particles (at least three) were seen attached to the cell membrane. Cells in control experiments did not show gold labeling. In each sample at least 100 cells were analyzed.

RESULTS

The electron-microscopic preparations of purified T cells cultured in medium without stimulating agents showed a small number (~2%) of cells with NCI greater than 6.5. In contrast, preparations of T cells activated in vitro by OKT3/TPA contained a significant portion of cells that had acquired a nuclear contour irregularity, which would classify them as a Sézary-like cell (NCI > 6.5) (Table 1, Fig 1). Our results showed a wide variation in the number of Sézary-like T cells induced in vitro. Among five different donors tested, the number of Sézary-like cells after activation with OKT3/TPA was 9% to 28%. The highest NCI value observed was 11.28. Cell incubation with soluble OKT3 or TPA alone did not result in enhanced nuclear contour irregularity. On the other hand, cell activation by immobilized OKT3 showed similar induction of Sézary-like morphology in comparison with activation by soluble OKT3/TPA (not shown). Titration experiments showed that any concentration of OKT3 antibody sufficient to activate T cells in the presence of TPA was also sufficient to generate cells with Sézary-like morphology. Maximum of nuclear contour irregularity was noted with 2.5 ng/mL OKT3. Kinetic studies showed maximum of nuclear contour irregularity after 120 hours of culture. Increased numbers of Sézary-like cells were not observed before 72 hours of stimulation. Several other T-cell activating agents were tested for their capacity to induce nuclear contour irregularity in T cells (Table 1). Cell activation in vitro via the CD2 pathway also induced a significant number of Sézary-like cells. However, the percentage of Sézary-like cells induced by CD2 activation was lower in comparison with CD3 activation. Stimulation via CD28 did not show any effect. In addition, no alteration of nuclear morphology was observed after T-cell stimulation with mitogens. Also, no significant difference in nuclear morphology was detected when mitogenic activation was performed in the presence of TPA.

To evaluate the phenotype of Sézary-like cells induced by cell activation in vitro, indirect-immunogold electron microscopy was performed. Purified T cells were activated in vitro by OKT3/TPA and cells with NCI greater than 6.5 were subsequently analyzed for their expression of specific T-cell antigens. The results show that T cells exhibiting nuclear contour irregularity belong to both CD4⁺ and CD8⁺ subpopulations. The CD4⁺/CD8⁺ ratio (2.4) in T cells with NCI greater than 6.5 was in similar proportion to the total number of CD4⁺ and CD8⁺ T cells (2.6) in culture. Furthermore, the majority of Sézary-like cells expressed the CD7 antigen (>95%). Stimulation of purified CD7 T cells in vitro...
tro by anti-CD3 showed induction of nuclear contour irregularity in a similar portion of cells as compared with unseparated T cells.

**DISCUSSION**

In the present study, we have readdressed the question of nuclear morphology in T cells after activation in vitro. We show for the first time that T cells with Sézary-like morphology (NCI > 6.5) can be induced in vitro by incubation with antibodies to CD3 and CD2 antigens in the presence of phorbol esters. Antibodies to CD2 antigens were most effective in this respect. Soluble anti-CD3 without phorbol esters had no effect. On the other hand, nuclear contour irregularity could be induced by cross-linked anti-CD3. This indicates that induction of Sézary-like cells via T-cell receptor (TCR)/CD3 complex or CD2 antigens is dependent on secondary activation signals required for induction of proliferative responses in resting T cells. Stimulation with anti-CD3 in the presence of phorbol esters mimics the T-cell interaction with a complex of antigen and MHC-encoded protein and provides several intracellular signals including Ca²⁺-flux and protein kinase C activation. Several studies indicate that these intracellular signals may interact with certain cytoskeletal proteins that might, in turn, increase nuclear contour irregularity in T cells.

Consistent with the findings of other investigators, we did not find significant induction of Sézary morphology after stimulation with PHA, which might be caused by less potent mitogenicity of PHA or ConA in comparison with anti-CD3 reagents. Simultaneous triggering of CD28 molecules, shown to cooperate with both the TCR/CD3-directed pathway and the CD2-directed alternative pathway of T-cell activation, had no additional effect on the degree of morphologic changes induced in activated T cells. The observation that nuclear contour irregularity is inducible by T-cell-specific activation in vitro is consistent with the finding that Sézary-like cells have been found in a number of conditions associated with antigen-specific T-cell activation in vivo. These include contact dermatitis, reactive lymph nodes, and infiltrates of patch-test materials. Immunogold electron microscopy performed in this study showed that T cells exhibiting Sézary morphology after TCR/CD3 stimulation expressed both CD4 and CD8 phenotypes. Furthermore, cerebriform T cells were found in both CD7⁺ and CD7⁻ T-cell subsets. Sézary cells in malignant lymphomas express the helper T-cell phenotype and predominantly lack CD7 expression. Recently, we could identify a subset of helper T cells in normal humans that specifically lack CD7 expression and, therefore, may represent the physiologic counterpart of malignant Sézary cells. However, we found that normal CD7⁺ helper T cells do not exhibit nuclear contour irregularity. Our present study shows the lack of phenotypic specificity of nuclear contour irregularity and provides evidence that there is no obligatory morphologic association between the malignant Sézary cell and its possible physiologic counterpart.

The results of our study raise the question of possible discrimination between reactive Sézary-like cells and malignant Sézary cells. To define additional diagnostic criteria to differentiate CTCL from benign dermatoses a large number of ultrastructural studies were reported. Willemze et al. showed that most discriminating parameters for the differentiation between Sézary syndrome and benign forms of erythroderma were: (1) a mean NCI value of 5.5 or more; (2) more than 20% Sézary-like cells (lymphoid cells with a NCI > 6.5); or (3) the presence of cells with a NCI greater than 11.5. The fulfillment of one or more of these criteria was reported to be diagnostic for Sézary syndrome. Our results indicate that cell activation of normal T cells in vitro by TCR/CD3 stimulation may induce morphologic alteration corresponding to the criteria for malignant T cells in Sézary syndrome, with the exception of an NCI greater than 11.5. However, other investigators found that the highest NCI was not a good discriminating factor, because an NCI greater than 11.5 was found in only a subgroup of Sézary patients. On the other hand, lymphocytes with NCI greater than 11.5 have been observed in benign cases. Thus, the computer-assisted morphometric criteria for the differentiation between malignant and reactive T cells with nuclear contour irregularity must remain speculative. Further studies should clarify whether cell activation in vivo in conditions other than skin disease is also associated with the occurrence of Sézary-like morphology in T cells.

**ACKNOWLEDGMENT**

We thank Martina Impekoven for excellent technical assistance.

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Induction of nuclear contour irregularity during T-cell activation via the T-cell receptor/CD3 complex and CD2 antigens in the presence of phorbol esters

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