Reed-Sternberg Cells in Hodgkin's Cell Lines HDLM, L-428, and KM-H2 Are not Actively Replicating: Lack of Bromodeoxyuridine Uptake by Multinuclear Cells in Culture

By Su-Ming Hsu, Xun Zhao, Subendu Chakraborty, Y-Fa Liu, Jacqueline Whang-Peng, Ming S. Lok, and Shiroh Fukuhara

We compared the proliferation of mononuclear and multinuclear cells in four Hodgkin's cell lines, HDLM-1, HDLM-1d, L-428, and KM-H2, by examining their capacity to incorporate bromodeoxyuridine (BrdUrd) into nuclei. Approximately 5% of all cells in HDLM-1 cultures had two or more nuclei, a characteristic of Reed-Sternberg (RS) cells. Unlike mononuclear Hodgkin's (H) cells, these RS cells exhibited no uptake, or only minimal uptake of BrdUrd, suggesting that they did not replicate actively. Cytogenetic study showed that 25% of the HDLM-1 cells contained a tetraploid (4X) set of chromosomes with a characteristic two-peak distribution. Following treatment of HDLM-1 cells with phorbol ester, the percentages of 4X cells and RS cells increased to 50% and 12%, respectively. This increase in RS cells was not likely to be due to cell fusion as shown by the absence of hybridization of BrdUrd-positive and-negative nuclei. Phorbol ester has a short-term effect of blocking the exit of cells from G1 into S phase, but no effect on the transition from S phase to G2/M phase. The block is more prominent in 2X cells than in 4X cells, which may explain the increase in percentage of 4X cells in phorbol ester-treated cultures. In addition, phorbol ester induced the differentiation of H-RS cells, which was accompanied by loss of the marker HeFi-1 from the cell surface. Approximately one third of the RS cells did not express HeFi-1, or expressed only minimal amounts. The findings led us to the following conclusions: (1) The 4X cells probably are formed from 2X H cells as a result of disturbed cytokinesis, but not a cell fusion. (2) A considerable number of 4X cells were H cells, because the number of 4X cells consistently exceeded that of RS cells. (3) Since mitotic figures are extremely rare in RS cells and these cells did not show active BrdUrd uptake, the increased number of RS cells must also be a consequence of disturbed cytokinesis of H cells or a result of nuclear transformation (twisting, convolution, or separation of the nucleus) in H cells. (4) Most RS cells lose their proliferating capacity and some RS cells may undergo further differentiation. Uptake of BrdUrd and phorbol ester induction were also studied on the other three H-RS cell lines, HDLM-1d, L-428, and KM-H2, with results similar to those for HDLM-1.

From the Department of Pathology, University of Texas Health Science Center at Houston; Cytogenetic Oncology Section, Medicine Branch, National Cancer Institute, Bethesda, MD; Hematology-Oncology Service, Veterans Administration Hospital, Leavenworth, KS; and First Division of Internal Medicine, Faculty of Medicine, Kyoto University, Japan.


Address reprint requests to Su-Ming Hsu, MD, Department of Pathology, University of Texas Health Science Center at Houston, PO Box 20708, Houston, TX 77225.

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cytokinesis, as has been suggested in previous studies,6 by twisting and convoluting of the nucleus.

MATERIALS AND METHODS

Tissue culture of H-RS cells. HDLM is a series of four cell lines derived from a patient with HD. All of these cell lines (HDLM-1, -2, -1d, -2d) have similar, if not identical, phenotypes and cytogenetic markers.2-5 The HDLM-1d and -2d cells had been cultured in the presence of phorbol ester (TPA) for more than 1 year; they were then cultured in a TPA-free medium. We used HDLM-1 and HDLM-1d cells in the present study because, in our laboratory, these cells grew more rapidly than did HDLM-2 and -2d cells. In addition, we used two H-RS cell lines, L-428 and KM-H2.10,16 These cells show a phenotypic expression (ie, they react with MoAbs Ki-1, IRac, 2H9, and HeFi-1) similar to that of HDLM cells. All cells were grown at 4 x 10^6 to 2 x 10^6 cells/mL in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 50 mmol/L 2-mercaptoethanol, and 50 μg/mL gentamicin at 37°C in a humidified, 5% CO2 atmosphere. The medium was changed every two to three days. The viability of these cells has generally been maintained at 95%. Cell viability was determined by Trypan Blue dye exclusion test.

Ultrastructural study of HDLM-1 cells. HDLM-1 cells were fixed in 3% glutaraldehyde, embedded in Epon, and processed for electron-microscopic examination of the nuclear structure.

MoAbs and immunoperoxidase reagents. To compare the phenotypes of mononuclear H cells and RS cells, we used the MoAbs HeFi-1, 2H9, and anti-IRac for immunostaining on cytopsin smears. The specificities of these MoAbs and the staining procedure have been described in detail previously.1,5,12,13 The labeling reagents used were biotin-labeled horse anti-mouse Ig and avidin-biotin-peroxi-
dase complex (ABC), both obtained from Vector Laboratories (Burlingame, CA). In addition, we also examined the expression of a nuclear antigen, Ki-67, in these cells. Ki-67 has been reported to be an antigen for proliferating cells.21

TPA induction of cells. To study the effect of TPA on the formation of RS cells, we used a protocol for TPA induction of HDLM-1 cells that has been described previously.11,22 Briefly, TPA dissolved in DMSO (14 μg/mL) was added at a final concentration of 2 ng/mL to cultures of H-RS cells. Every second day, two-thirds of the medium was replaced with fresh medium containing TPA. The induction was carried out for three to four days. Cytospin smears were prepared during the course of induction and were evaluated for the percentage of RS cells. The increase in the number of RS cells was also determined with right-angle light scatter, which we used as a detection parameter in flow cytometry.23

Effect of TPA on cell cycle. To determine the effects of TPA on cell proliferation, we examined the DNA cycle of HDLM-1 and HDLM-1d cells by using propidium-iodide staining and flow cytometry.13,22 Briefly, the cells were cultured in medium with or without TPA for two days. The cells were washed in RPMI 1640 medium, and then 1 mL aliquots of suspensions containing 2 x 10^6 cells were incubated with 95% ethanol (2.5 mL) for 24 hours at 2 to 8°C. After being washed in phosphate buffered saline (PBS), the cells were incubated with 100 μL RNase A (1,000 U/100 μL, Worthington Diagnostic, NJ) for one minute. The samples were then stained with propidium iodide (50 μg/mL) for two minutes and vortexed gently. Finally, the cells were analyzed by flow cytometry (Ortho Cytofluoi-
ogruf system 50H). The phase of TPA-treated cells in the DNA cycle was compared with that of control cells.

BrdUrd uptake. HDLM-1, HDLM-1d, L-428, and KM-H2 cells were cultured in the presence of BrdUrd (1 x 10^-6 mol/L) for various periods (one to 72 hours). The cells were washed with Tris buffered saline (TBS) (0.1 mol/L, pH 7.6), prepared as cytopsin smears, and examined for uptake of BrdUrd by the nuclei. We used the ABC immunoperoxidase method with an anti-BrdUrd MoAb (Becton Dickinson, Sunnyvale, CA) to detect BrdUrd uptake in the nuclei.21,26 Briefly, smears were treated with 1 mol/L HCl in normal saline for 30 minutes and then washed with TBS for ten minutes. Next, the cells were stained with anti-BrdUrd MoAb (1:40), biotin-labeled horse anti-mouse IgG, and ABC as described previously.21,26 Cells that take up BrdUrd exhibit dark granular staining in the nuclei. At various intervals, the percentage of BrdUrd uptake by RS cells was compared with that by mononuclear cells.

To test for possible fusion of mononuclear cells, we took cells previously treated with BrdUrd for 36 hours and cultured them with control, untreated cells for one to two days. For fusion, the percentage of hybrid nuclei by BrdUrd-positive and BrdUrd-negative nuclei was analyzed by cytofluorography.

Cytogenetic study. We studied the chromosome distribution in both TPA-treated and control HDLM-1 cells. Both TPA-induced and control cells were treated with colcemide (0.1 μg/mL, 90 minutes) and hypotonic solution and were examined for the number of chromosomes that they contained. The colcemide treatment did not affect the number of RS cells in culture. A total of 215 cells were included.

Sorting of cells with high DNA content. We sorted HDLM-1d cells according to their DNA content and determined the percentages of RS cells in populations with high and in those with low DNA content. The sorting procedure was carried out with an Ortho Cytofluorograf 50H controlled by a 21 50 computer system that was attached to the machine. Cells with high (highest 25%) and low DNA content were deflected into separate containers, centrifuged onto slides, and stained with Diff-Quik. The percentage of RS cells in each sample was then determined as described in a previous section (Tissue culture of H-RS cells).

Single-cell culture. The uptake of nucleic acid into cell nuclei does not necessarily indicate that these cells can successfully complete the replication cycle. We used a limiting-dilution technique to culture single cells in 96-well plates to determine the replication capacity of the cells. We examined each well under a phase contrast microscope to determine the number of cells and the size of cell. Cells with multiple nuclei generally had a size three to four times that of mononuclear cells. Frequently, the number of nuclei in the large cell can be determined by the presence of two or more refractile nuclei. Wells that contained more than one cell and those in which the number of nuclei per cell could not be ascertained were excluded from the study. The plates were cultured for ten to 14 days and examined for the formation of colonies in each well.

RESULTS

Number of RS cells in culture and their morphologic characteristics. The percentages of RS cells varied among the four cell lines studied. From 7% to 11% of HDLM-1d and L-428 cells were RS cells, followed by 4% to 6% for HDLM-1, and 3% to 5% for KM-H2 cells, respectively (Fig 1). The
percentage of RS cells remained constant in control cultures throughout the 18-month study period.

Most of the RS cells contained two nuclei that were either widely separated or in contact. Often, the two nuclei were connected to one another by a thin, thread-like substance. A highly twisted nucleus was frequently observed in H cells; these cells presumably became RS cells after going through several stages of transformation (Fig 2). The twisting and deep convolution of nuclei were apparent on electron microscopy (Fig 3). Phenotype of RS cells v H cells. The phenotypic expression of RS cells was similar, if not identical, to that of H cells. Both expressed HeFi-1, 2H9, and IRac. The staining intensity of the H cells was fairly uniform; however, the expression of HeFi-1 in RS cells was quite variable. The intensity of HeFi-1 staining in 20% to 35% of the RS cells was very weak or negative (Fig 4), but, in 10% to 20% of these cells, it appeared to be more intense than that in H cells. The TPA-induced RS cells and H cells both had diminished expression of HeFi-1 and 2H9, but remained IRac-positive.

The expression of Ki-67 was highly variable among cell lines (Table 1). In L-428, most H as well as RS cells showed variable staining. In HDLM, intense staining was observed in most H cells, but a considerable number of RS cells were not stained or stained only weakly (Fig 5). The staining of KM-H2 cells was uniformly weak. Treatment with TPA reduced the staining intensity in all of the H-RS cell lines.

Effect of TPA on the formation of RS cells and on their phase in the cell cycle. Induction with TPA had a dramatic effect on the formation of RS cells. In all cultures, the percentage of RS cells increased during a three-day induction period (Figs 1 and 6). For example, the percentage of RS cells in HDLM-1 cultures increased from 4% to 6% to 11% to 15%. The cell viabilities were slightly lower in TPA-treated cultures than in control cultures.

The TPA induction also affected the cell replication cycle. The precise percentage of cells in each phase is difficult to determine because the aneuploidy of chromosome. However, we estimated that, in control cultures, approximately 25% of HDLM-1 (2X) cells were in G0/G1 phase (region 1, Fig 7) and 20% in S phase (region 2). In TPA-treated cultures, 46%
were positively stained by Ki-67 (arrows). Few small binucleated RS cells compared with only 30% of the nuclei of RS cells, moderately positive; + 25% chromosomes. Approximately nuclei of most (>90%) H cells expressed Ki-67, shown). The distribution was similar in more chromosomes (tetraploid, numbers modal 30 and 42 (hypodiploid) with most HDLM-l cells had chromosomes. Most HDLM-l cells were illustrated in Table 2 and in the histogram result of cell fusion. The uptake of BrdUrd by HDLM-l cells are in HDLM-l and HDLM-ld cells, even though the HDLM-ld cells had been treated continuously with TPA for more than 1 year. TPA treatment apparently increased the numbers of 4X cells (50%) over those in untreated (control) cultures (25%).

**Correlation of DNA content with number of nuclei.** We sorted the cells with the highest DNA content (highest 25%) and compared the number of nuclei in these cells with that of the remaining cells, which had a low DNA content. The percentage of RS cells (7% to 10%) in the high-DNA-content population was similar to that for low DNA content and that for presorted cell samples. This result indicates that cells with high DNA content (4X cells ?) did not necessarily contain multiple nuclei.

**Capacity of RS cells to divide.** A total of 563 single-cell cultures were evaluated for their capacity to proliferate. Among 476 cultured small cells (H cells), 125 (25%) multiplied successfully. However, among 87 cultured large cells (RS cells), only five cells multiplied. It appeared that most large RS cells with widely separated nuclei did not proliferate in single-cell culture.

**DISCUSSION**

By using H-RS cell lines, we have shown that RS cells do not participate actively in cell replication, and that these are likely to be end-stage cells, as had been suggested by Peckham and Cooper. This is confirmed by the lack of BrdUrd uptake in the majority of RS cells during three days of incubation, and by the lack of proliferation of large RS cells in single-cell culture. Furthermore, we have provided evidence that cell fusion is not responsible for the formation of RS cells by showing that no cells containing hybrid BrdUrd-positive and -negative nuclei were present. Disturbed cytokinesis may contribute to the formation of 4X cells, and probably of RS cells as well. Other possible mechanisms of RS cell formation include convolution, twisting, and separation of nuclei.

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**Table 1. Ki-67 Expression and BrdUrd Uptake in H-RS Cells**

<table>
<thead>
<tr>
<th></th>
<th>HDLM-1</th>
<th>L428</th>
<th>KM-H2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67</td>
<td>&gt;95% (3+)</td>
<td>30-40% (2+/3+)</td>
<td>60-70% (2+/3+)</td>
</tr>
<tr>
<td>BrdUrd</td>
<td>&gt;90% (3+)</td>
<td>&lt;30% (+)</td>
<td>&gt;90% (3+)</td>
</tr>
</tbody>
</table>

The staining was performed on cells in stationary phase (Ki-67) or on cells incubated with BrdUrd for three days. 3+. Strongly positive; 2+, moderately positive; +, weakly positive; −, negative.

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Fig 5. Ki-67 staining of HDLM-1 cells. The nuclei of most (>90%) H cells expressed Ki-67, compared with only 30% of the nuclei of RS cells (arrow heads). Few small binucleated RS cells were positively stained by Ki-67 (arrows).
Fig 8. BrdUrd uptake by HDLM-1 cells. The nuclei of most H cells contained BrdUrd after 24 hours of incubation, compared with only 15% of the nuclei of RS cells. The numbers of RS cells containing BrdUrd increased as the incubation period increased. Since RS cells are likely to be derived from H cells, the increase in the number of BrdUrd-positive RS cells may result from a transformation of BrdUrd-positive H cells into RS cells.

Fig 6. Increase in number of RS cells after TPA induction. This effect of TPA was observed best with HDLM-1d cells. The increased number of RS cells can be measured by the increase on right-angle scatter on flow cytometry (A. control HDLM-1d cells; B. TPA-treated HDLM-1d cells), as indicated by the shift to the right in B. (C) Some of the TPA-induced HDLM-1d cells that contained two or more nuclei (arrows).

We observed BrdUrd uptake in virtually all H cells, but in only 25% of RS cells, after 36 hours of culture. To reach 50% uptake, H cells had to be cultured with BrdUrd for more than 12 hours. The extent of nucleic-acid incorporation in H cells in this study was similar to that in a previous study in which 36.5% of cells were labeled after incubation with ³H-thymidine (1 μCi/mL) for 17 to 18 hours. However, Kadin and Asbury⁶ reported a similar degree of uptake (24% to 43%) by H cells when they used the same amount of ³H-thymidine, but only one hour of incubation. In both studies,⁶,⁹ multinuclear (RS ?) cells made up between 1% and 2% of the total number of cells, and 20.7% to 44% of these were labeled. The difference in the source of cells and the conditions of culture could account for the difference in results in their studies and ours.

In our study, we used BrdUrd, a pyrimidine analogue of thymidine, to examine the DNA synthesis of the cells.²⁴-²⁶ By using a MoAb to BrdUrd, one can measure the incorporation of this thymidine analogue into the DNA of H-RS cells exposed in vitro, without the need for a radioactive isotope. The method is very sensitive requiring only 10⁻⁵ or 10⁻⁶ mol/L BrdUrd.²⁴-²⁶ Thus, the lack of uptake by RS cells cannot be attributed to the sensitivity of the method used.

The uptake of BrdUrd by RS cells, if it occurs, is far less than that by H cells. The staining in RS cells usually consists of a few granules and specks, unlike the intense, diffuse

Fig 7. Cell cycles of TPA-treated HDLM-1 cells (A, left) and control cells (B, right). The precise percentage of cells in each phase of cell cycle cannot be determined because the aneu-ploidy of cells. The cell cycle can be separated into five regions. Region 1 corresponds to the G₀/G₁ of 2X cells; region 2, S phase of 2X cells; region 3, G₂/M phase of 2X cells plus G₀/G₁ phase of 4X cells; region 4, S phase of 4X cells; region 5, G₂/M phase of 4X cells. Note that there is a significant decrease of percentage of cells in region 2, but not in region 4 following TPA treatment. Regions 4 and 5 consisted of approximately 27% of total cells in both treated and control cultures.
staining seen in most H cells. In limiting-dilution cultures, we rarely observed any proliferation of RS cells. It is apparent that, even though we observed BrdUrd uptake in a few (<25%) RS cells, the probability that these will complete the cellular replication cycle is small because of their minimal uptake of nucleic acid.

There was a considerable discrepancy between the number of cells positive for BrdUrd and those positive for Ki-67. Ki-67 is a nuclear antigen for proliferating cells. It has been reported that H-RS cells in tissues express Ki-67,21 but whether the expression alone can be used to indicate the replicating capacity of cells is not known. In this study, the staining by Ki-67 in the H-RS cells used yielded some confusing results. For example, most (>95%) mononuclear HDLM-1 cells were Ki-67-positive even though at least 25% of them were in G0/G1 phase. In KM-H2 cells, the staining was uniformly weak and did not vary among cells. A successful cell division is a result of multiple, integrated biochemical processes, and one cannot deduce the entire replicating function of cells by detecting a single protein, especially when the cells are neoplastic and have biochemical derangements.

Unlike most human neoplastic cell lines, HDLM-1 cells are characterized by a two-peak (2X and 4X) distribution of the number of chromosomes; this is seen also for other H-RS cell lines, including L-428, L-439, L-591, and possibly, KM-H2.10,11 The precise percentage of 4X cells in culture, however, cannot be determined. On cytogenetic cycle analysis, we estimate that the percentage of 4X HDLM-1 cells was in the vicinity of 25%.

Since the number of 4X cells exceeded the number of RS cells, it is quite clear that a considerable number of 4X cells have a single nucleus. This conclusion is also supported by the following observations: (1) The cells with high DNA content included a large number (90%) of H cells. (2) Most RS cells had absent or minimal BrdUrd uptake and thus were not likely to enter the mitotic cycle. It was clear that most mitotic cells in prophase, which we examined, had only one nucleus. Since RS cells are not likely to undergo cell division, it is impossible to determine the number of chromosome in RS cells.

The formation of 4X cells is probably attributable to disturbed cytokinesis of 2X cells, rather than to cell fusion. The 4X sibling cells may contain two nuclei that are widely separated (as in some RS cells). The RS cells lose the capacity to replicate because the two nuclei are incapable of synchronization in the cell cycle. A large number of 4X cells may have a single nucleus because of incomplete separation during mitosis. These cells can undergo mitosis and thus can be detected on cytogenetic study. Some of the single-nucleated cell may become RS cells as a result of convolution, twisting, and subsequent separation of nuclei. We suspect that mitosis without cytokinesis is a property of some 2X cells, but generally not of 4X cells, because there were only rare cells with four times (8X) the normal number of chromosomes (Fig 11).

The increases in the percentage of 4X cells in metaphase and in the percentage of RS cells following TPA induction are also of interest. TPA can produce a G1 block and delay entry into S phase, whereas cells in S phase can proceed to G2/M phase. The effect of TPA on the cell cycle of H-RS cells is similar to that on human peripheral-blood lymphocytes, HeLa cells, and the cells of the mouse fibroblast line C3H/10T1/2.27,38 Since the increased number of 4X cells can be detected as early as two days after TPA induction, and since the cell doubling time is approximately 60 hours, it

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**Table 2. Distribution of Chromosome Number in HDLM-1 Cells**

<table>
<thead>
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<th>Total No. of Cells Examined</th>
<th>&lt;35</th>
<th>36</th>
<th>37</th>
<th>38</th>
<th>39</th>
<th>40</th>
<th>41</th>
<th>42</th>
<th>43-69</th>
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<th>76</th>
<th>77</th>
<th>78</th>
<th>79-92</th>
<th>100-150</th>
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<tbody>
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<td>7</td>
<td>10</td>
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<td>2</td>
<td>11</td>
<td>3</td>
<td>7</td>
<td>6</td>
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<td>2</td>
</tr>
<tr>
<td>HDLM-1</td>
<td>11</td>
<td>1</td>
<td>18</td>
<td>16</td>
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is difficult to attribute the increase to the inhibition of cytokinesis by TPA, as such an effect would require two cell cycles to become evident. It appears that the block exerted by TPA is more prominent on 2X cells than on 4X cells, which causes a relative increase in the percentage of 4X cells detected on cytotgenetic study.

It is highly unlikely that the 4X and 2X cells represent two separate clones, because the chromosome distribution is not much different in HDLM-1 and HDLM-1d cells. (HDLM-1d cells were derived from HDLM-1 cells after incubation of the latter with TPA for more than 1 year.) Since TPA affects the mitosis of 4X cells to a lesser degree, one would expect a gradual replacement of 2X cells by 4X cells in HDLM-1d culture, if two different clones exist. Phenotypic study by multiple markers in HDLM-1 and HDLM-1d cells has not shown any significant heterogeneity of cells, or any other evidence in support of the two-clone theory. Furthermore, a considerable number of cells in other H-RS cell lines is also 4X. One cannot argue that all H-RS cell lines contain two distinct populations. Taken together, some of the 4X cells must be generated continuously from 2X cells as a result of distributed cytokinesis.

It has been known that TPA induces the differentiation of H-RS cells. Most H-RS cells express markers such as HeFi-1 and Ki-1, which are considered to be markers of immature or neoplastic cells because they are absent from normal lymphoreticular cells. Thus, it is not surprising that, after TPA induction, the H-RS cells become negative for these markers. The increased number of RS cells following TPA induction may be consistent with the status of cellular differentiation of H-RS cells, because a small portion of the RS cells did not express HeFi-1, or expressed it only minimally. However, cellular differentiation cannot totally explain the mechanism of RS-cell formation, because most of the RS cells still expressed amounts of HeFi-1 antigen comparable with those of H cells. The formation of RS cells following TPA induction may result from a decreased rate of cellular proliferation that allows the twisted nuclei in the cells to become separated.

In conclusion, the mechanism of RS-cell formation is probably a multifaceted rather than a simple process. The disturbed cytokinesis of H cells and the twisting, convolution, and separation of their nuclei lead to the formation of 4X cells and RS cells. RS cells do not proliferate to any extent, even though they take up a small amount of nucleic acid and express Ki-67 antigen. The capacity of RS cells to complete the cell division cycle is doubtful.
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Reed-Sternberg cells in Hodgkin’s cell lines HDLM, L-428, and KM-H2 are not actively replicating: lack of bromodeoxyuridine uptake by multinuclear cells in culture

SM Hsu, X Zhao, S Chakraborty, YF Liu, J Whang-Peng, MS Lok and S Fukuhara