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RAPID COMMUNICATION

Tumor Cell Growth Fraction in Hodgkin's Disease

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The growth fraction of tumor cells was studied in 45 cases of Hodgkin's disease by means of a recently developed double immunostaining technique using monoclonal antibody Ki-1, which reacts selectively with Hodgkin and Reed–Sternberg cells in tissues affected by Hodgkin's disease, and antibody Ki-67, which recognizes a cell proliferation-associated nuclear antigen. The medians of the growth fractions of the tumor cells in all histologic subtypes of Hodgkin's disease varied between 78% and 83%. In none of the cases investigated did we find a growth fraction below 50%. Furthermore, mononucleated Hodgkin cells as well as multinucleated Reed–Sternberg cells showed a similar Ki-67 labeling index, indicating that both tumor cell types belong to the proliferating pool of this malignancy. (Am J Pathol 1987, 129:390–393)

CELL KINETIC studies in Hodgkin's disease (HD) have been hampered in the past by a number of practical problems: 1) ^{3}H-thymidine labeling had to be carried out in cell suspension, a method that does not provide representative results, at least in cases with a high content of fibers like nodular sclerosis; 2) there were no markers specific for Hodgkin and Reed–Sternberg (H&RS) cells; and 3) there were no sufficiently sensitive *in situ* immunohistologic detection systems. Thus, cytokinetic data on H&RS cells are restricted to some early ^{3}H-thymidine incorporation studies with a small number of patients investigated.[1-3] To our knowledge, the investigation by Peckham and Cooper[1] with 10 patients is the most comprehensive.

The limitations of the earlier determination of the growth fraction (GF) in HD could now be overcome by introducing the monoclonal antibodies Ki-1[4] and Ki-67[5] in a modified *in situ* immunoenzymatic double labeling technique.[6] For the identification of H&RS cells the Ki-1 antibody was used, because this reagent recognizes selectively H&RS cells in tissues affected by HD.[7-10] The monoclonal antibody Ki-67 was applied for identifying those Ki-1 antigen-positive tumor cells that are in the active parts of the cell cycle. Ki-67 is suitable for this purpose, because it reacts with all nuclei of human cells in G_1, S, and G_2 and in mitosis, but not with G_0 cells.[11]

Using this approach in a series of 45 cases of HD, we found that in all instances the tumor cell GF was larger than 50% and that thus, from the cell kinetic point of view, HD resembles non-Hodgkin's lymphomas of high-grade malignancy, rather than those of low-grade malignancy.[12]

Materials and Methods

Twenty-five cases were chosen at random. Twenty cases were selected because of their richness in H&RS cells, because these tissue samples were additionally used for DNA studies.

Biopsies of HD were typed according to the Rye classification.[13] Double immunostaining of cases of HD with Ki-1 and Ki-67 was performed as detailed recently.[6] Briefly, frozen sections were fixed in acetone for 15 minutes, followed by chloroform for 15 minutes. The slides were then incubated with the monoclonal antibody Ki-67 for 30 minutes at room temperature. After three brief washes in Tris-buffered saline, the sections were incubated with peroxidase-conjugated antimouse immunoglobulin (Ig) serum

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(Dakopatts, Copenhagen, Denmark) for 30 minutes and again after a further washing step with peroxidase-conjugated anti-rabbit IgG serum (Dianova, Hamburg, FRG). Peroxidase reaction was then carried out following the principles described by Graham and Karnowsky.[14] After five washes, the slides were labeled with monoclonal antibody Ki-1 by the alkaline phosphatase/anti-alkaline phosphatase (APAAP) method.[15] For this purpose, slides were incubated with Ki-1 for 30 minutes and subsequently incubated with rabbit anti-mouse serum for 30 minutes. The sections were then treated with APAAP complexes for 30 minutes. The incubation with rabbit anti-mouse Ig serum and APAAP was repeated once, and thereafter the alkaline-phosphatase was visualized with the modified new fuchsin method.[16] This reaction was permanently monitored under the microscope. Finally, slides were counterstained with hemalum and mounted.

The percentage of Ki-67 positive cells was evaluated by counting 100–200 Ki-1-positive cells with the formula:

$$\frac{\text{Ki-67-positive Ki-1-positive cells}}{\text{all Ki-1-positive cells}} \times 100$$

Results

To investigate the proliferative capacity of H&RS cells *in situ*, we immunostained 45 cases of HD using a double-labeling method by which the H&RS cells were identified with the antibody Ki-1, and the GF of these cells was assessed by demonstrating a nuclear proliferation-associated antigen with the monoclonal antibody Ki-67. Two biopsy collectives were used for this study. Twenty-five cases were chosen at random, whereas 20 cases were selected because of their richness in H&RS cells. The results of both collectives were identical.

Figure 1 illustrates a typical double-labeling result: most tumor cells, i.e., Ki-1-positive cells (red membrane staining), were also labeled with Ki-67 antibody (brownish nuclear staining) and are thus proliferating. The inset of Figure 1 demonstrates that all nuclei of a multinucleated SR cell were also labeled with Ki-67. It must be stressed that there was no significant difference in the degree of Ki-67 positivity in mononucleated or multinucleated Ki-1-positive cells.

Table 1 summarizes the results of this study. The medians of the growth fraction of the histologic subtypes of HD are in the range from 78% to 83%. Interestingly, in none of the cases investigated was the tumor cell GF below 53%.

Discussion

The present study shows that in all 45 cases of Hodgkin's disease more than 53% (with a median of about 80%) of the Ki-1-positive cells were also labeled for the proliferation-associated nuclear antigen Ki-67. In this context it is important to stress that previous investigations convincingly demonstrated that the Ki-1 antigen is selectively expressed on H&RS cells, i.e., the bizarre large cells in HD.[4,7–10] In contrast to labeling reactions with anti-interleukin-2-receptor antibodies, small activated lymphocytes or

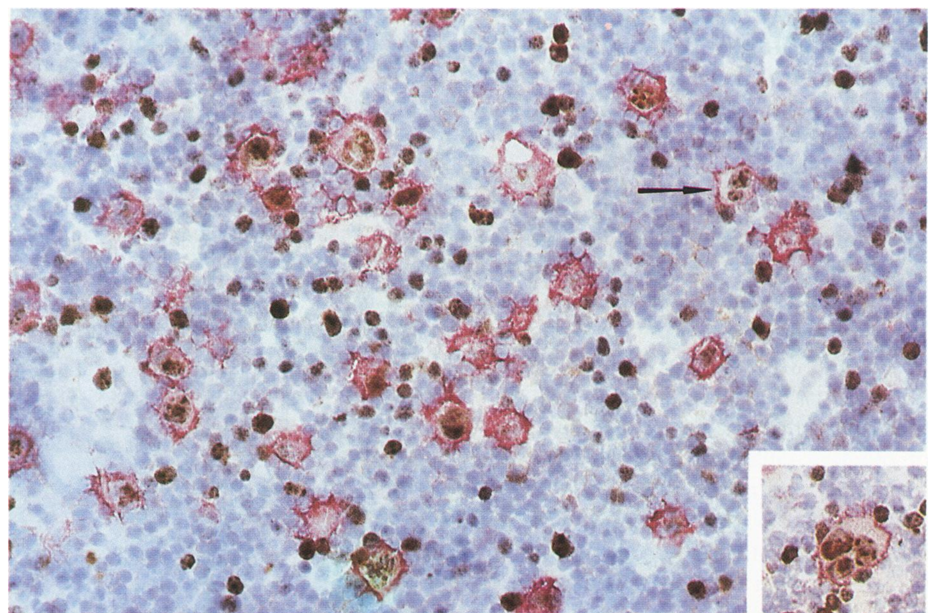

Figure 1—Double immunostaining of a frozen section of a case of Hodgkin's disease with monoclonal antibodies Ki-1 and Ki-67. The Ki-1 staining appears red (determined with the APAAP method) and Ki-67 labeling brownish (three-step immunoperoxidase method; hemalum counterstaining). A Ki-1-positive binucleated Reed–Sternberg cell is indicated by an *arrow*, and a Ki-1-positive trinucleated tumor cell is shown in the **inset**. In both cells all the nuclei are labeled with Ki-67.

Table 1—Percentage of Ki-67 Positive Tumor Cells in Hodgkin's Disease (HD)

Histologic type of HD	n	Median of % of Ki-67+ cells	Range of % of Ki-67+ cells
Lymphocyte predominance	3	78	76–80
Nodular sclerosis	33	83	53–95
Mixed cellularity	9	80	73–98

macrophages were not stained with the Ki-1 antibody. Thus, in HD, most tumor cells are in the active parts of the cell cycle. This finding is in conflict in a quantitative aspect with previous studies,[1-3] demonstrating the proliferating tumor cells by analysis of ^{3}H-thymidine uptake in cell suspensions and identifying tumor cells by morphology alone. In these studies it is reported that only a small or moderately large population of tumor cells proliferate in HD. Our results are also in conflict with these earlier data in a qualitative aspect. It has been described that only mononucleated H cells, but not multinucleated RS cells, incorporate ^{3}H-thymidine, which suggests that only the mononucleated tumor cells participate in the proliferation in HD.[1]

These discrepancies with our results are probably due to the differences in the methods employed: while our method allows a direct *in situ* correlation, the ^{3}H-thymidine incorporation studies are dependent on short-term cultures in cell suspensions. Thus, especially in cases of nodular sclerosis, the latter technique might provide results that are not representative of the whole neoplastic cell population.

Taken together, our results clearly indicate that in HD most of the tumor cells, the mononucleated variants as well as the multinucleated cells, are proliferating. Thus, from the cell kinetic point of view, HD appears to be a high-grade malignant lymphoma, rather than a low-grade malignant lymphoma. This finding might explain why HD responds so well to therapy, because cytostatic drugs affect cycling cells more efficiently than resting cells.[17]

The large size of the growth fraction of the tumor cell population in HD deserves comment, because it is not in keeping with the small proportion of tumor cells detectable in most cases of this disease. A high proliferative activity of the malignant cells implies that the tumor cells would highly exceed the number of reactive cells and that the resulting tumor mass would be composed of more or less pure sheets of tumor cells, as is seen, for example, in non-Hodgkin's lymphomas of high-grade malignancy. However, tumor-cell-rich HD cases are the exception. In most cases, there are only a few tumor cells separated from each other by broad sheets of various nonneoplastic cells. The following are possible explanations of this phenomenon: 1) prolonged duration of the cell cycle of H&RS cells results in a relatively inefficient amplification of the malignant cell population, and/or the lifetime of the tumor cells is short; 2) the H- and RS cells release factors, ie, lymphokines, which attract nonneoplastic cells to their vicinity, leading to a separation of H&RS cells from each other.

A recent comparison in HD and non-Hodgkin's lymphomas of the tumor cell GF assessed with Ki-67 and the percentage of neoplastic S-phase cells revealed by measurement of bromodesoxyuridine incorporation showed that the ratio between GF and the percentage of S-phase cells was significantly increased in HD. This suggests that the duration of the cell cycle of H&RS cells is, in fact, prolonged (data to be published).[21]

Support for the second hypothesis comes from *in vitro* data demonstrating that some HD-derived permanent cell lines, eg, L-428, release more than one lymphokine, such as those with interleukin-1-like activity,[18] granulocyte colony-stimulating activity,[19] and other activities.[20]

Because there is evidence for both hypotheses, it might be that both mechanisms work synergistically in HD. Of course we are aware of the fact that this concept prompts further investigation at an experimental level.

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ERRATUM

Following is a correction for the manuscript by Caddell et al: "Pulmonary Lesion Induced by Stress in Magnesium Deficient Rats. A Light- and Electron-Microscopic Study" (Am J Pathol 1987, 127:430–440). Under *Mg in Bone,* column 2, page 432, means ± SEM should be multiplied by a dilution factor of 15.