High proliferative activity of Reed Sternberg associated antigen Ki-1 positive cells in normal lymphoid tissue

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SUMMARY An improved immunoenzymatic double labelling method was developed, which simultaneously shows the Ki-1 membrane antigen and the nuclear proliferation associated antigen, defined by monoclonal antibody Ki-67. This new approach permits in situ discrimination of cells that are proliferating or not with a particular membrane antigen. Most Ki-1 positive cells in normal lymphoid tissue also express the Ki-67 nuclear antigen and thus appear to be proliferating. As proliferating cells are more susceptible to malignant transformation than quiescent cells, it is possible that the small number of normal Ki-1 positive cells might, none the less, cause a large proportion of all lymphomas in man.

The origin and identity of the tumour cells in Hodgkin's disease—that is, Hodgkin Reed Sternberg (HSR) cells—remain controversial. The most recent theories have suggested that HSR cells are related to B cells,^{1–5} macrophages,^{6–8} follicular dendritic reticulum cells,⁹ interdigitating reticulum cells,^{10,11} myelomonocytic precursor cells,¹² or dendritic cells of Steinman type.¹³

The antigen detected by monoclonal antibody Ki-1, prepared by our group,¹⁴ is consistently expressed on HSR cells in Hodgkin's disease.^{15,16} We have previously shown that Ki-1 antibody also reacts with a small population of large cells in reactive lymphoid tissue cells, which are to be found at the rim of B cell follicles.^{15,16} As it has been reported that the perifollicular region is where the earliest signs of activity are seen in Hodgkin's disease,¹⁷ we suggested that Ki-1 positive cells in normal lymphoid tissue may represent the physiological counterpart of HSR cells.^{16,18}

If so, how can such a small population of cells give rise to such a common disorder as Hodgkin's disease, which comprises one third of all malignant lymphomas? One possible explanation could be that normal Ki-1 positive cells have a high proliferative activity and thus might be especially susceptible to malignant transformation.

To explore this possibility we developed an improved simultaneous double immunolabelling method for Ki-1 antigen and the cell proliferation associated nuclear antigen detected by antibody Ki-67.^{14,19} This new approach permits in situ immunohistological determination of the proliferative activity of cells defined by membrane antigen.

Material and methods

CELLS AND SPECIMEN

Normal blood samples were obtained from healthy laboratory staff at the Klinikum Steglitz and five fresh human tonsils from five different donors were a gift from the ear, nose, and throat department. Mononuclear cell suspensions were prepared by standard techniques. Phytohaemagglutinin A (PHA) stimulation was carried out, as previously described.¹⁴

ANTIBODIES

Monoclonal antibodies Ki-1 and Ki-67 were prepared as described elsewhere.^{15,19} The monoclonal antibody Tü-69 was a generous gift from Dr A Ziegler, Tübingen. Tü-69 is a monoclonal antibody against the interleukin 2 receptor (IL-2R). Alkaline phosphatase-antialkaline phosphatase (APAAP) complexes were prepared, according to the methods of Cordell *et al.*²⁰ Unlabelled and peroxidase conjugated rabbit

Fig 1 Double immunostaining of peripheral blood lymphocytes after three days in culture with PHA with monoclonal antibodies Ki-1 and Ki-67. Ki-1 staining appears red (performed according to APAAP method). In contrast, Ki-67 nuclear antigen is brownish. (Three step immunoperoxidase method; haemalum counterstain.) Note that all Ki-1 positive cells also reacted with Ki-67.

Fig 2 Double immunostaining of frozen section of human tonsillar tissue with monoclonal antibodies Ki-1 and Ki-67 (carried out as described in fig 1). Most cells that reacted with Ki-1 antibody were also positive for Ki-67 antigen in their nuclei. Inset shows higher magnification of Ki-1 and Ki-67 positive cell.

Proliferating Ki-1 cells in normal human tissue

antimouse IgG and peroxidase conjugated antirabbit IgG were purchased from Dianova, Hamburg, West Germany.

IMMUNOSTAINING

Immunostaining of cytocentrifuged slides was performed using the APAAP method, as described by Cordell *et al.*²⁰ The alkaline phosphatase reaction was carried out, as described by Stein *et al.*²¹

Double immunostaining of frozen sections was performed as follows: Frozen sections were fixed in acetone for 15 minutes followed by chloroform for 15 minutes. The slides were then incubated with 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 serum for 30 minutes and again after a further washing with peroxidase conjugated antirabbit serum. Peroxidase reaction was then carried out according to the principles described by Graham and Karnovsky.²² After five washes the slides were labelled with monoclonal antibody Ki-1 using the APAAP method.²⁰ Briefly, slides were incubated with Ki-1 for 30 minutes and subsequently incubated with rabbit antimouse serum for 30 minutes. The sections were then treated with APAAP complexes for 30 minutes. The incubation with rabbit antimouse serum and APAAP was repeated once, and thereafter, the alkaline-phosphatase developing reaction was performed with the modified New Fuchsin method.²¹ This reaction was permanently controlled under the microscope. Finally, slides were counterstained with haemalum and mounted.

Results

DOUBLE STAINING OF PHA BLASTS FOR Ki-1 AND Ki-67

Untreated mononucleated peripheral blood lymphocytes obtained from 10 different healthy donors showed that less than 0.1% expressed the Ki-1 antigen. In contrast, after three days in culture in the presence of PHA 9% to 28% of these cells expressed the Ki-1 antigen, while 98% of the cells exhibited interleukin 2 receptors, as measured by immunostaining with Tü 69; and 79% of these cells were proliferating, as determined by immunostaining with Ki-67. Fig 1 shows the double immunostaining of peripheral blood lymphocytes after three days in culture in the presence of PHA with Ki-1 and Ki-67.

IMMUNOHISTOLOGICAL DOUBLE STAINING OF Ki-1 POSITIVE CELLS IN NORMAL LYMPHOID TISSUE

Fig 2 shows the double immunostaining of a human tonsil with monoclonal antibodies Ki-1 and Ki-67.

Only some scattered cells at the rim of the B cell follicles were Ki-1 positive, whereas all other cell types, including germinal centre cells and T blasts, were negative. Nearly all cells that exhibit a red membrane staining for Ki-1 were also stained in their nuclei for Ki-67. By evaluating five tonsils we found that 87% (range 74% to 95%) of Ki-1 positive normal cells were also positive with Ki-67.

Discussion

To find out whether the few Ki-1 positive cells found in normal human lymphoid tissue around B cell follicles are proliferating cells we developed an immunoenzymatic doublestaining method, using Ki-67 as second antibody, which reliably stains the nucleus of all proliferating human cells.^{14 19} The membrane antigens were visualised with the Ki-1 antibody using the APAAP technique, whereas the cell proliferation associated nuclear antigen was shown with Ki-67 by applying the three step indirect immunoperoxidase method. This is the first time that an in situ discrimination of whether a cell is proliferating or not with a certain membrane antigen has become possible. Furthermore, the double staining method we have described has the advantage that the slides can be counterstained and morphological details precisely evaluated. The use of Ki-67 antibody in this new approach should be of diagnostic value where pathological cells cannot be readily identified by morphological criteria or single immunostainings—as may arise in malignant lymphomas composed of a complex mixture of neoplastic and reactive cells.

The double staining experiments we have described clearly show that most of the scattered Ki-1 positive cells found in normal human lymphoid tissue are also labelled by Ki-67. This indicates that the perifollicular Ki-1 positive cells are rapidly proliferating cells and could explain why this cell population might undergo malignant transformation much more often than other cells in lymphoid tissue. This finding also poses the question whether the expression of the Ki-1 antigen is simply related to the cell cycle rather than to a certain stage of differentiation.

Our recent observation that the expression of Ki-1 antigen can be induced on peripheral blood T or B cells after exposure to PHA, *Staphylococcus aureus*, HTLV I and II, or Epstein-Barr virus²¹ might also be taken as evidence for such an assumption. There is evidence, however, to the contrary:

1 Proliferating T and B cell precursor cells are consistently Ki-1 negative, as shown by immunostaining fetal organs that contain such cells (liver, bone marrow, and thymic cortex).²¹

2 Germinal centre cells, which are highly proliferative^{14 19} and thus represent a part of the B

cell renewal system, are Ki-1 negative.^{15 16 18 21}

3 In Hodgkin's disease there are cells that express the Ki-1 antigen but not the proliferation associated antigen Ki-67 (unpublished observations).

We conclude that the expression of Ki-1 is not strictly linked to the cell cycle but rather defines a certain stage of lymphoid cell differentiation, often designated by immunologists as "activated," and which is usually but not always associated with proliferation. Thus the Ki-1 antigen recalls interleukin 2 receptor (IL-2R),²³ a structure that is also present on activated T and B cells but absent from precursor T and B cells and germinal centre cells.^{23 24} Despite these similarities the two structures are different for four reasons. First, IL-2R are expressed on macrophages,²⁴ whereas the Ki-1 antigen is not^{15 16 18 21}; secondly IL-2R are present on 95–100% of PHA blasts²⁴ and Ki-1 only on 10–30%²¹; thirdly, IL-2R are found on HSR cells in only 60% of patients with Hodgkin's disease while Ki-1 is expressed in all cases²¹; and fourthly immunoprecipitates prepared with Ki-1 and SDS gel electrophoresis show a double band of 105 and 120 kD molecular weight, whereas the IL-2R is represented by a single polypeptide chain of about 55 kD.

It is evident, therefore, that Ki-1 antigen represents a new activation associated molecule of the lymphoid cell lineage. It is important because:

(1) It shows a greater lymphoid cell lineage specificity than any other activation associated antigen so far described—IL-2R, GP 240, and HLA-DR all are also expressed on macrophages.

(2) It defines a previously unidentified small population of rapidly proliferating cells in normal lymphoid tissue, which most resemble HSR cells in morphology and distribution.

(3) It permits the identification of a group of so far poorly characterised large lymphoid cell lymphomas that have been regarded in the past as neoplasms of the macrophage histiocytic system and therefore have been called malignant histiocytoses.²¹

In conclusion, it seems reasonable to assume that the Ki-1 positive lymphomas, including Hodgkin's disease, are neoplasms of activated lymphoid cells that might be derived from or related to the perfollicular Ki-1 positive cell population of normal lymphoid tissue. The findings reported here, that nearly all normal Ki-1 positive cells are proliferating, make it at least conceivable that these cells, although comprising less than 3% of normal lymphoid tissue, may give rise to more than one third of all malignant lymphomas.

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