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Laboratory Investigation
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Brief Methods

Ki-Mcm6, a New Monoclonal Antibody Specific to Mcm6: Comparison of the Distribution Profile of Mcm6 and the Ki-67 Antigen

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Genomic DNA replication must be completed prior to mitosis and should occur only once during the cell cycle. To date, several proteins have been identified that are involved in the initiation of DNA replication, including the origin recognition complex and minichromosome maintenance proteins (Mcm) (Kearsey and Labib, 1998; Labib et al, 2000). A new monoclonal antibody (mab), named Ki-Mcm6 (IgG1), was obtained after immunizing Balb/c mice with nuclear lysates of 3 days mitogen stimulated peripheral blood lymphocytes. In Western blots, this antibody recognizes a nuclear antigen with an apparent molecular mass of 105 kd (Fig. 1), which was identified by protein sequencing as the minichromosome maintenance protein 6 (mcm6). Immunohistochemical examination of cryostat and paraffin sections of nearly all human tissue types showed that the distribution of this nuclear antigen was restricted to sites harboring proliferating cells, such as germinal centers in lymphoid tissues. Human oocytes strongly expressed mcm6 (Fig. 1), whereas they were negative or only weakly positive for the Ki-67 antigen. A comparison of the mcm6 expression pattern with the profile of the Ki-67 antigen by flow cytometry analysis (FACS) showed that, at all measured intervals (24 hours, 48 hours, and 72 hours after mitogen stimulation), the number of Ki-Mcm6–expressing cells was slightly higher than that of Ki-67–expressing cells (Table 1). Cell cycle analysis revealed that this difference was mainly due to the larger numbers of positive cells in G_1 phase. This indicates that mcm6 is already expressed in the early G_1 phase, a cell cycle fraction that is not covered by antibodies specific to the Ki-67 antigen (Gerdes et al, 1984). Double immunofluorescence staining of mitogen-stimulated peripheral blood mononuclear cells revealed that all cells expressing the Ki-67 antigen also contain mcm6. Additionally, mcm6 is expressed in a small cell population that does not express the Ki-67 antigen. Confocal laser scanning microscopy showed that, during the G_1/S and G_2 phase, Ki-Mcm6 is diffusely distributed throughout the cell nucleus, sparing the nucleoli. During mitosis, mcm6 is localized in the cytoplasm and is also associated with the chromosomal scaffold. In the G_1/S and G_2 phase, the Ki-Mcm6 antigen was colocalized with the Ki-67 antigen around the nucleoli, whereas during mitosis both antigens were colocalized on the chromosomal scaffold (Fig. 2). To determine whether the detection of mcm6 as a marker protein of the entire G_1 phase might be of diagnostic relevance in low grade tumors, serial sections of 24 cases of follicular lymphoma Grade 1 (centroblastic-centrocytic lymphoma, updated Kiel classification) were stained with Ki-Mcm6 and Ki-S5. The mean proliferative activity was 20.5% (±7.5%) with Ki-Mcm6 and 14.6% (±6.6%) with Ki-S5. The median was 13.5% (5%–34%) and 18.1% (8%–45%), respectively. The correlation was significant at $p < 0.5$ ($n = 24$) with $r = 0.73$. The

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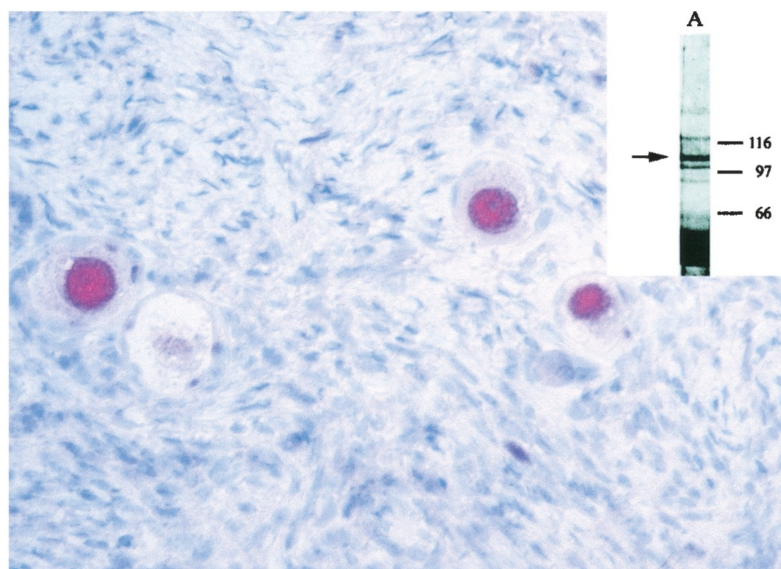

Figure 1.
Ki-Mcm6 staining of a paraffin section of a formalin-fixed human ovary. Minichromosome maintenance protein 6 (mcm6) is strongly expressed in oocytes (×250). A, Coomassie blue staining of the immunopurified 105 kd protein (*arrow*). The 105 kd protein purified in this experiment was excised and digested with Lys C. Sequencing of three of the derived peptides revealed that the 105 kd protein is mcm6. Molecular weight markers are shown on the right (in kilodaltons).

Table 1. Cell Cycle-Specific Staining of Cells with Ki-S5 or Ki-Mcm6[a]

	Time (h)					
	24	24	48	48	72	72
Monoclonal antibody	% pos	% pos in G_0/G_1	% pos	% pos in G_0/G_1	% pos	% pos in G_0/G_1
Ki-Mcm6	7.3	7.2	50.2	21.4	67.3	46.0
Ki-S5	3.6	3.4	43.0	16.9	64.0	43.5

[a] Peripheral blood mononuclear cells (PBMC) were stimulated with 10 μg/ml PHA for the indicated times, stained with Ki-Mcm6 or Ki-S5, and their cell cycle distribution was analyzed. The percentage of positive cells (overall or specifically G_0/G_1 phase cells) is indicated. The table shows representative results from one of three experiments.

differences in the mean were significant at $p < 0.5$ ($n = 24$). Consequently, Ki-Mcm6 may prove to be a unique marker of the complete cell cycle. It might thus qualify as a prognostic marker in cancer management.

For all experiments, crude supernatant of Ki-Mcm6 was used. Tissue samples (provided by Department of Hematopathology and Lymph Node Registry, University of Kiel, Germany) were fixed in 5% to 10% formaldehyde for various periods of time and routinely processed. Antigen retrieval was achieved by boiling the slides in 0.01 M citric acid (pH 6.0) for 2 minutes in a pressure cooker. The immunoreaction was visualized by means of the alkaline phosphatase-antialkaline phosphatase complex (APAAP) method. For double immunofluorescence stainings of mitogen-stimulated peripheral blood mononuclear cells (PBMC), cytospin preparations were fixed at first for 10 minutes in 2% paraformaldehyde buffered in PBS and then for 10 minutes in acetone. Extended fixation in acetone abolishes the Ki-Mcm6 staining of mitotic cells. The immunofluorescence staining was performed with the monoclonal antibody Ki-Mcm6 and a goat anti-mouse polyclonal antibody labeled with Alexa 594 and with MiB1 (specific to the Ki-67 antigen) labeled with Alexa 488 (Molecular Probes, Leiden, The Netherlands). DNA staining was performed with 2 μM TOTO-3. Confocal laser scanning microscopy was carried out using a Zeiss LSM laser scanning microscope (Carl Zeiss, Jena, Germany). Double staining images represent optical slices of 0.5 μm taken from the middle of the cells. Lysates of L428 cells or PBMC were separated by SDS-PAGE on a gel gradient of 7.5% to 15% and then transferred to nitrocellulose membranes overnight (Heidebrecht et al, 1996, 1997). After blocking with 3% bovine serum albumin, the membranes were incubated with supernatant containing Ki-Mcm6. Visualization was done with 4-chloro 1-naphthol after the membranes had been incubated with peroxidase-conjugated rabbit anti-mouse IgG. For protein sequencing experiments a lysate preparation of 1×10^9 L428 cells was used. Immunoprecipitation was performed with Ki-Mcm6 coupled to protein A Sepharose

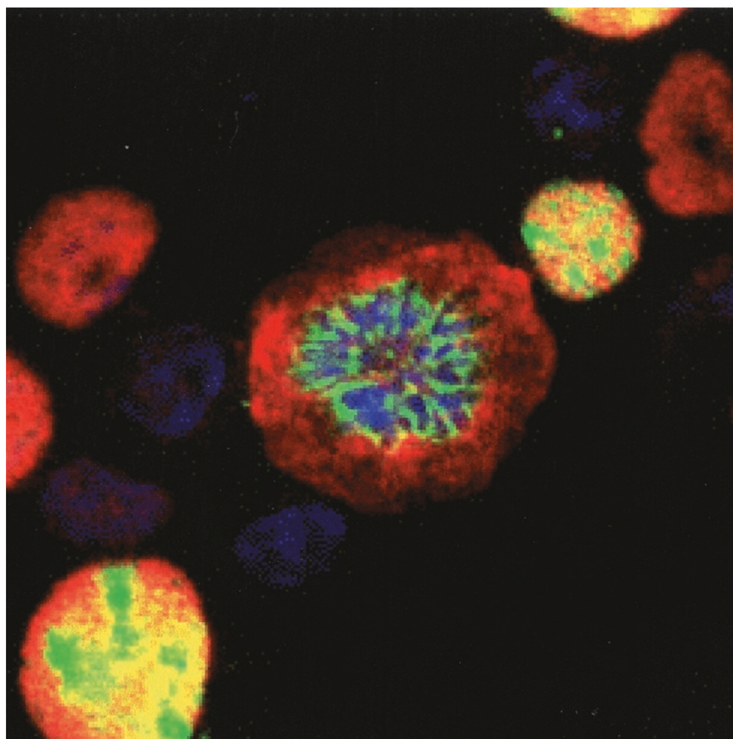

Figure 2.
Double immunofluorescence staining of mitogen-stimulated peripheral blood mononuclear cells (PBMC) with Ki-Mcm6 (red) and MIB-1 (specific for the Ki-67 antigen; green). DNA staining was performed with 2μM TOTO-3 (blue). During G1/S and G2 phases the antigen is distributed in a dotlike pattern in the cell nucleus. In mitotic cells the antigen is detectable in the cytoplasm and on the chromosomal scaffold. Note, some cells express mcm6 but not the Ki-67 antigen. Double staining images represent optical slices of 0.5 μm taken from the middle of the cell.

Cl-4B (Heidebrecht et al, 1997). The immunoprecipitates were separated by SDS-PAGE and stained with Coomassie blue. The Coomassie blue-stained antigen was excised and digested with Lys C, as described by Bauw et al (1988), and the proteolytic fragments were separated by narrow bore HPLC (130 Å, Applied Biosystems, Foster City, California) on a reverse phase column (Vydac C4, 300 Å pore size, 5 mm particle size, 2.1 × 125 mm; Vydac, Hesperia, California). Peptides were eluted with a linear mobile phase gradient (0%–80% B in 50 minutes; solvent A: water/0.1% trifluoroacetic acid (TFA); solvent B: 70% acetonitrile/0.09% TFA) at a flow rate of 200 ml/minute. Peptide-containing fractions detected at 214 nm were collected manually into siliconized Eppendorf tubes and frozen immediately. Protein sequences of six peptides were determined by standard Edman degradation on an automatic sequencer (473 A, Applied Biosystems).

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