Ki-67 detects a nuclear matrix-associated proliferation-related antigen

I. Intracellular localization during interphase

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Summary

Ki-67 is a commercially available mouse monoclonal antibody, which reacts with a nuclear antigen in proliferating cells. The antibody can be used to determine the growth fraction of human tumours in situ and has been shown to be of prognostic importance.

In this study it is shown that in interphase cells Ki-67 reacts with an antigen, mainly present in the nucleoli. Confocal scanning laser microscopy and immunoelectron microscopy on human MR65 monolayer cells revealed that this nucleolar antigen is predominantly localized in the nucleolar cortex and in the dense fibrillar components.

The Ki-67 antigen appeared to be preserved in nuclear matrix preparations obtained after in situ fractionation of MR65 cells. Despite many efforts, we could not identify the antigen in immunoblotting or immunoprecipitation assays.

Testing of cell cultures of different species by means of indirect immunofluorescence revealed that the antibody reacted with human cells and with the Rhesus monkey kidney-derived cell line LLC-MK2.

Key words: nucleolar antigen, confocal scanning laser microscopy, immunocytochemistry.

Introduction

In 1983 Gerdes and co-workers described the production of a mouse monoclonal antibody, Ki-67, which recognized a nuclear antigen expressed in proliferating cells, but not in resting cells. Ki-67 was obtained from a fusion of mouse myelomas with lymphocytes from a mouse injected with crude nuclear fractions of L428 cells, a Hodgkin's disease-derived cell line (Gerdes et al. 1983). It was found that normal peripheral blood lymphocytes were negative for Ki-67, while stimulation of these cells with phytohaemagglutinin (PHA) resulted in a positive nuclear reaction pattern. On the contrary, when HL-60 cells (acute promyelocytic leukemia) were stimulated to differentiate into mature resting macrophages by treatment with the phorbol ester TPA, the Ki-67 antigen expression disappeared.

A more detailed analysis of the cell cycle performed by Gerdes et al. (1984a) revealed that expression of this antigen is consistently detectable throughout the S, G2 and M phases of continuously cycling cells but not in G0 cells. However, the results concerning antigen expression in G1 phase varied. In these early studies it was also noted that Ki-67 reacted with an antigen associated with chromosomes (Gerdes et al. 1984a). Expression of the Ki-67 antigen could not be demonstrated in peripheral blood leucocytes during the early events of PHA-triggered transition from G0 to G1, whereas continuously cycling cells were permanently positive in G1 phase. These observations led to the conclusion that Ki-67 could be a useful tool in determining the growth fraction of a given human cell population and especially in assessing the proportion of proliferating cells in immunostained tissue sections of neoplasms (Gerdes et al. 1983, 1984a).

This latter aspect has been explicitly examined for non-Hodgkin's lymphomas (Gerdes et al. 1984b), the colonic epithelium in ulcerative colitis (Franklin et al. 1985), and for tumours of breast (Gerdes et al. 1986; Barnard et al. 1987; Franklin et al. 1987), lung (Gatter et al. 1986) and brain (Giangaspero et al. 1987). Barnard et al. (1987) have determined a Ki-67 score (positive cells/total tumour cells) for a number of primary breast carcinomas and investigated the possible relationship between this proliferative index and a number of clinical and
pathological parameters. These investigators concluded that the Ki-67 score may prove to be an objective indicator of biological behaviour of breast carcinomas and thus may be of clinical significance. Ki-67 has also been shown to be of great value in assessing the proliferative capacities of the lymphoid cells in cutaneous infiltrates (Ralfkiaer et al. 1986) and in determining the growth fraction of tumour cells in tissues affected by Hodgkin's disease (Gerdes et al. 1987).

Recently, Schwaitz et al. (1986) have shown that the growth fraction of cell suspensions labelled with Ki-67 can be determined flow cytometrically. Furthermore, Franklin et al. (1987) have shown the usefulness of an image analysis system in quantifying the immunoperoxidase Ki-67 labelling in tissue sections of breast carcinomas.

In summary, the estimation of the Ki-67 positive cell fraction of tumours is being introduced more and more into routine pathology and in future may have important prognostic and therapeutic implications. On the other hand, virtually nothing is known about the nature and biochemical characteristics of the antigen recognized by Ki-67. The present study was performed to examine the intracellular localization of the Ki-67 antigen. In this respect it is shown that during interphase the strongest immunohistochemical staining reactions with Ki-67 are obtained in the nucleoli and that the antigen is preserved in nuclear matrix preparations of MR65 cells.

Materials and methods

Antibodies

Monoclonal antibody Ki-67 (IgG1; trade-name DAKO-PC, code no. M722) was purchased from DAKOpatts (Glostrup, Denmark) as tissue culture supernatant that had been dialysed against 0-05 M-Tris·HCl, pH 7-2 and 15 mM-sodium azide.

Ascites fluid obtained after intraperitoneal injection of Ki-67 hybridoma cells in Balb/c mice was kindly provided by Dr J. Gerdes (Borstel, FRG).

Other monoclonal antibodies used in this study include 2.73, 41CC4 and RKSE 60. Antibody 2.73, directed against the 70K protein is not expressed in MR65 cells, RKSE 60 was used as a negative control antibody in these cells.

Cell cultures

Tissue-culture media and calf sera were purchased from Flow Laboratories Ltd, Irvine, UK.

Monolayer cells were grown on coverslips in Eagle's modified Minimum Essential Medium supplemented with 10% newborn calf serum until about 50% confluency was reached. These included: HeLa S3 (human cervix carcinoma), MR65 (human lung carcinoma; Brosers et al. 1987), T24 (human bladder transitional-cell carcinoma), HEP 2 (human epithemoid larynx carcinoma), Clio I (human melanoma cell culture), cultured human fibroblasts, PtK2 (Potomus tricida kidney), BHK-21 (baby Syrian hamster kidney cells), VERO (African green monkey kidney cells), LLC-MK2 derivative (Rhesus monkey kidney cells), a primary culture of dog endothelial cells, a hamster lens cell culture (Bloemendal et al. 1980) and a bovine lens cell culture (Ramakers et al. 1980).

Molt-4 (human acute T lymphoblastic leukaemia) and mouse myeloma SP2/0-Ag14 cells were grown in suspension at 37°C at densities of approximately 0·3 x 10^6 cells ml^-1 in RPMI 1640 (Dutch modification) supplemented with 15% fetal calf serum. Drosophila melanogaster mei-218 cells were cultured at 25°C at about 75% confluency in Schneider's Drosophila medium (Gibco, UK) supplemented with 15% heat-inactivated foetal calf serum.

Immunohistochemical staining procedures

The indirect immunofluorescence assay was performed essentially as described by Verheijen et al. (1986). Culture supernatant of the mouse monoclonal antibody Ki-67 was used in a 1:25 (v/v) dilution. As second antibody FITC-conjugated rabbit anti-mouse IgG (heavy and light chains; Nordic, Tilburg, The Netherlands) was used in a 1:25 (v/v) dilution. All dilutions were made in phosphate-buffered saline (PBS). DNA was then stained by incubating the cells for 15 min with Hoechst 33258 (0·1 µg ml^-1 in 22 mM-citric acid, 56 mM-disodium hydrogen phosphate).

Immunoelectron microscopy

For immunoelectron microscopy, MR65 cells were grown on Meline foil (ICI, Hertz, UK) until about 75% confluency was reached. The cells were washed in PBS and fixed in methanol (−20°C, 5 s), followed by dipping in acetone at room temperature (3 times, 5 s). Subsequently, the cells were incubated with 1:20 (v/v) diluted culture supernatant of Ki-67 for 60 min at room temperature, washed with three changes of PBS for 10 min each, incubated with 1:50 (v/v) diluted peroxidase-conjugated rabbit anti-mouse Ig antibodies (DAKOpatts, Glostrup, Denmark) and again washed in PBS (3 times, 10 min). After detection of the peroxidase activity with 3,3′-diaminobenzidine (DAB; Sigma Chemical Co., Munich, FRG) the slides were processed for electron microscopy as described by van Duinen et al. (1984).

In situ preparation of nuclear matrices from MR65 cells

Cell fractionations were performed in the presence of 0·5 mM-phenylmethylsulphonyl chloride (PMSC) and 5 mM-N-ethylmaleimide (MalNEt) to reduce proteolytic degradation and disulphide bridge formation, respectively. These agents were added from freshly prepared stocks. Ribonuclease A (RNase A) (Sigma Chemical Co., Munich, FRG) was pre-incubated for 5 min at 100°C to reduce possible protease activity.

MR65 cells were grown on coverslips under appropriate culture conditions (Verheijen et al. 1986) until about 75% confluency. The entire fractionation procedure was carried out under continuous shaking of the slides in six-well plates.

The procedure that we have established for the isolation of nuclear matrices in situ, carried out at 0−4°C, was as follows: cells were first washed in PBS for 5 min followed by three washes in NMK buffer (130 mM-NaCl, 5 mM-KCl, 1·5 mM-MgCl₂), 5 min each. Each of the following steps in the procedure was preceded by washing the slides twice with reticulocyte suspension buffer (RSB) (10 mM-NaCl, 10 mM-Tris·HCl, pH 7·4, 1·5 mM-MgCl₂). Subsequently, the slides were subjected to a hypotonic buffer (RSB with 0·5 M-sucrose) and incubated for 10 min after addition of 0·05 vol. 10% Triton X-100 in RSB. Thereafter, the slides were incubated for 10 min in a freshly prepared mixture of 0·5% sodium deoxycholate (DOC)/1·0% Tween-40 in RSB, followed by a nucleic acid
digested for 20 min at 20°C in a mixture of 1 mg ml⁻¹ deoxyribonuclease I (DNase I) (DPFF quality; Cooper Bio-medical, Malvern, USA) and 50 μg ml⁻¹ RNase A (Sigma) in RSB110 (110 mM-NaCl, 10 mM-Tris-HCl, pH 7.4, 1.5 mM-MgCl₂). During this digestion step, MalNEt was omitted, but immediately after the incubation it was added again to a final concentration of 5 mM. The DNA-depleted nuclei were extracted for 10 min with a high-salt buffer containing 0.4 M-(NH₄)₂SO₄, 50 mM-Tris-HCl, pH 7.4, 1.5 mM-MgCl₂. The obtained nuclear matrices were prepared for immunofluorescence microscopy as described (Verheijen et al. 1986a).

Analysis of proteins and RNA
SDS-polyacrylamide gel electrophoresis and immunoblotting were performed as described (Verheijen et al. 1986a), while analysis of immunoprecipitated proteins and RNA using culture supernatant of Ki-67 as well as ascites fluid were done as documented by Mimori et al. (1984).

Confocal scanning laser microscopy (CSLM)
The confocal scanning laser microscopy technique has been described in detail by Brakenhoff et al. (1985, 1988) and Van der Voort et al. (1988). Usually, 16 optical sections of 256x256 pixels each were made per three-dimensional image. Fluorescein-stained specimens were excited at 476 nm using a krypton ion laser. Typical optical resolutions were 0.2 μm and 0.8 μm, perpendicular and parallel to the optical axis, respectively. The signal-to-noise ratio in the images of the optical sections was improved by using a two- or three-dimensional median filter (Brakenhoff et al. 1988; Van der Voort et al. 1988). Furthermore, the contrast range in each series of sections was optimized with respect to the sensitivity of the photographic film.

Actinomycin D treatment
After reaching a confluency of about 50%, various coverslips with MR65 cell monolayers were placed in fresh culture medium containing 1-0, 2.5 and 10 μg ml⁻¹ actinomycin D (Sharpe and Dohme International, New Jersey, USA), respectively. Each culture was permitted to grow for another 1, 2 or 4 h, after which the cells were prepared for indirect immunofluorescence.

Results
Subcellular localization of the Ki-67 antigen in MR65 cells
To study the intracellular localization of the Ki-67 antigen in human cells, we have used MR65, a human lung carcinoma monolayer culture in which the cells contain large nuclei and remain relatively flat during mitosis. In such cultures the Ki-67 antigen appeared to be exclusively located in the nuclei of virtually all individual cells (Fig. 1). However, there was considerable variability of the staining intensities between cells. Besides a weak staining of the nucleoplasm, the highest fluorescence intensity was found in the nucleoli. The nucleoplasmic reactivity was represented by variable numbers of small discrete structures, with the nucleolus staining patterns appearing to be very heterogeneous. In some cells fluorescence could be seen to be more intense in certain regions at the nucleolar periphery.

The antibiotic actinomycin D preferentially blocks RNA synthesis, resulting in a gradual nucleolar segregation, depending on concentration and exposure time. In order to follow the Ki-67 antigen localization during this segregation process, we exposed MR65 cell monolayers for several hours to 1-0, 2.5 and 10 μg ml⁻¹ of the drug. The nucleoli appeared to be completely fragmented after 2 h at a concentration of 2.5 μg ml⁻¹ actinomycin D or after 1 h at a concentration of 10 μg ml⁻¹ as concluded from phase-contrast microscopic observations (not shown). The micrographs in Fig. 2 show the Ki-67 antigen distribution patterns at several stages of actinomycin D treatment. As the nucleoli gradually dispersed into a great number of small fragments, the distribution of the Ki-67 antigen simultaneously changed from a distinct nucleolar localization (Fig. 2A) via a speckled pattern (Fig. 2B,C) to a diffuse distribution throughout the entire nucleoplasm (Fig. 2D). Fig. 2B represents the situation in which the fibrillar and granular components of the nucleoli have been segregated and in which the antigen seems to be associated with one of these two developed subcompartments.

Confocal scanning laser microscopy (CSLM) was applied on MR65 cells to extend our indirect immunofluorescence data and to obtain more information about the precise, spatial localization of the Ki-67 antigen in the nucleolus. Fig. 3 shows the image obtained from a CSLM analysis after superposition of 16 optical planes (all 0.85 μm apart) from two nuclei of MR65 cells in pseudocolour to extend the contrast range in the pictures. The colour sequence purple, blue, orange, red and white represents regions of increasing Ki-67 fluorescence. These images emphasize the heterogeneous distribution of the Ki-67 antigen in the nucleolus. The antigen appears to be localized predominantly in small areas in
Fig. 2. Immunofluorescence micrographs of actinomycin D-treated MR6S cells. After reaching a confluency of about 50%, various coverslips with MR6S cell monolayers were placed in fresh culture medium containing 1.0 (A), 2.5 (B) and 10 μg ml⁻¹ (C,D) actinomycin D, respectively. Each culture was permitted to grow for another 1 (A,B,C), 2 or 4 h (D), after which the cells were prepared for indirect immunofluorescence with Ki-67. ×1150.

the cortex of the nucleoli. Furthermore, most of these strong positive areas contain centres with a low fluorescence intensity.

The results obtained with immunoelectron microscopy on MR65 cells confirmed our immunofluorescence data (Fig. 4). It should be noted that the periphery of many intersected nucleoli was positive with Ki-67. Also a heterogeneous internal nucleolar staining pattern was observed, with some parts stained as strongly as the nucleolar rim, while other areas showed a significantly lower staining intensity. The nucleolar interstices, the fibrillar centres and the granular components seem to be negative, while the structures surrounding the fibrillar centres, probably the dense fibrillar components, were strongly stained. In nucleoli of a human stomach adenocarcinoma a similar localization of the Ki-67 antigen was seen at the ultrastructural level (Fig. 4F).

After in situ extraction of MR65 cells with Triton X-100, a DOC/Tween mixture, DNase I, RNase A and high-salt solution (0.4 M ammonium sulphate), the obtained nuclear matrices were essentially negative with Hoechst 33258, consistent with the removal of most of the nuclear DNA in these structures (Fig. 5E'). When comparing the staining patterns of the nuclear matrix (associated) 70K protein and the lamins (Verheijen et al. 1986b) in untreated and extracted cells, it became obvious that these proteins remained present in the nuclear matrix preparations (see Fig. 5B,C). The Ki-67 antigen remained associated with the nucleolar residue under these circumstances (Fig. 5A). The micrographs in Fig. 5 clearly indicate structural rearrangements of the various antigens during extraction and enzyme treatment. The Ki-67 fluorescence in the nuclear matrices appears to have accumulated more at the nucleolar cortex compared with its staining pattern in untreated cells.

**Distribution of the Ki-67 antigen in different cell lines**

The species cross-reactivity of the antibody was tested on a series of cell cultures of different origins, ranging from man to *Drosophila*.

First, epithelial as well as non-epithelial human cells in culture were examined for their presence of the Ki-67 antigen (Table 1). In MR65, HeLa 2 and Molt-4 (Fig. 6C) cultures virtually all cells were stained, while the cultures of HeLa S3 (Fig. 6A), T24 (Fig. 6B), Clo11 (Fig. 6F) and human fibroblasts (Fig. 6D) were only weakly positive (Table 1).

**Table 1. Detection of the Ki-67 antigen in cell cultures of various species using the indirect immunofluorescence technique**

<table>
<thead>
<tr>
<th>Species</th>
<th>Nuclear reaction with Ki-67</th>
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<tbody>
<tr>
<td>Human</td>
<td>+/— (30%)</td>
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<tr>
<td>Fibroblast</td>
<td>+/++ (100%)</td>
</tr>
<tr>
<td>Molt-4</td>
<td>+/++ (100%)</td>
</tr>
<tr>
<td>HEP 2</td>
<td>+/++ (100%)</td>
</tr>
<tr>
<td>MR65</td>
<td>+ (90%)</td>
</tr>
<tr>
<td>HeLa S3</td>
<td>+/++ (60%)</td>
</tr>
<tr>
<td>T24</td>
<td>+/++ (60%)</td>
</tr>
<tr>
<td>Clo11</td>
<td>+/++ (60%)</td>
</tr>
<tr>
<td>Monkey</td>
<td>—</td>
</tr>
<tr>
<td>LLC-MK2</td>
<td>—</td>
</tr>
<tr>
<td>VERO</td>
<td>—</td>
</tr>
<tr>
<td>Bovine</td>
<td>—</td>
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<tr>
<td>Lens cells</td>
<td>—</td>
</tr>
<tr>
<td>Dog</td>
<td>—</td>
</tr>
<tr>
<td>Primary endothelium culture</td>
<td>—</td>
</tr>
<tr>
<td>Hamster</td>
<td>—</td>
</tr>
<tr>
<td>BHK-21</td>
<td>—</td>
</tr>
<tr>
<td>Lens cells</td>
<td>—</td>
</tr>
<tr>
<td>Mouse</td>
<td>—</td>
</tr>
<tr>
<td>Myeloma SP2/0-Ag14</td>
<td>—</td>
</tr>
<tr>
<td>Rat kangaroo</td>
<td>—</td>
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<tr>
<td>PtK2</td>
<td>—</td>
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<tr>
<td><em>Drosophila</em></td>
<td>—</td>
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<tr>
<td>mei-218</td>
<td>—</td>
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</table>

−, All cells negative; +/−, weakly positive; +, positive; +++, strongly positive. Values in parenthesis indicate percentages of positive cells.

*For cell line characteristics see Materials and methods.*
Fig. 3. Pseudocolour representation of the superposition of the 16 optical sections of two nuclei of MR65 cells, immunostained with Ki-67, made by the CSLM technique. Grey levels have been transformed to colours to increase the number of fluorescence intensity levels that can be detected by the eye. The colour sequence purple, blue, orange, red and white represents increasing Ki-67 fluorescence intensity. ×1850.
partly positive. In all interphase cells the fluorescence was localized predominantly in the nucleoli and in some nucleoplasmic bodies (see Fig. 6). The presence of the antigen appeared not to be restricted to human cells, as about 60% of the cells of the Rhesus monkey kidney-derived cell line LLC-MK2 (Fig. 6H) gave a positive staining reaction as well (Table 1). In contrast, VERO (African green monkey kidney) cells appeared to be completely negative. Although the various positive cell types all gave similar staining patterns with Ki-67 compared with MR65 cells, a considerable variability of the staining intensities could be observed (see also Table 1). The highest fluorescence intensities were found in the positive fractions of CloII and LLC-MK2 (Fig. 6F,H, respectively). In cells with a moderately high fluorescence intensity such as HeLa S3, T24 and Molt-4 (Fig. 6A,B,C, respectively), again the brighter peripheral nucleolar fluorescence was observed. A weak staining reaction was seen in about one third of the cells in human fibroblast cultures. In none of the various cell cultures examined could cytoplasmic staining be detected.

**Discussion**

Ki-67 is a mouse monoclonal antibody that reacts with proliferating cells and was recently introduced into histopathology to determine the growth fraction of human tumours in situ.

The present study was performed to examine the intracellular localization of the Ki-67 antigen in various cell types. Using immunohistochemical techniques we have shown that in cultured cells in interphase the most intense staining reaction with Ki-67 is seen in nucleoli, particularly at its periphery and in the dense fibrillar components. As several other anti-nucleolar antibodies appeared to react strongly with antigens localized in the interior of the nucleoli (data not shown; see Verheijen et al. 1986a), it is unlikely that the intense peripheral localization of Ki-67 is due to the presence of antigenic determinants localized in non-nucleolar structures.
Staining of Ki-67 is due to an accessibility artefact. In the human cell types tested, the nucleoplasmic appearance of the Ki-67 antigen varied from a distinct number of dot-like structures to an almost diffuse staining reaction.

In addition we have tested Ki-67 on cell cultures from various species. Our results indicate that expression of the antigen is not restricted to human cells and tissues, but that positive staining reactions can also be obtained in proliferating cells from Rhesus monkey.

Experiments using actinomycin D also revealed that in interphase cells the Ki-67 antigen behaved as a nucleolar component. This is concluded from the observations that during segregation of the nucleoli the Ki-67 antigen seems to be mainly located in one of the two developed subcompartments, while it shows a speckled distribution pattern in cells treated with high concentrations of actinomycin D, resulting in a total fragmentation of the original nucleoli.

For further identification of the nature of the Ki-67 antigen we examined its association with the nuclear matrix. This structure may be defined as the residual entity remaining after subsequent treatments of cells with detergents, nucleases and high-salt solutions. In nuclear matrix preparations of monolayer cell cultures, cytoskeletal elements remain intact and for this reason we use the term nuclear matrix–intermediate filament scaffold (NM-IF) according to Fey et al. (1984) for our MR65 in situ extraction preparations. It was demonstrated by means of immunofluorescence that the Ki-67 antigen was preserved in such MR65 NM-IF scaffolds. However, high-salt treatment causes shrinkage of nuclei and an apparent increase in the fluorescence intensity (see Fig. 5). Therefore, one has to be cautious about conclusions based on these fluorescence studies concerning the amount of Ki-67 antigen preserved in these scaffolds.

Despite many efforts we have not succeeded in determining the biochemical characteristics of the Ki-67 antigen. In situ extraction experiments showed that the antigen sustained all treatments to obtain NM-IF scaffolds (see Materials and methods) as the antibody still reacted with such structures in the immunofluorescence assay. However, the various Western blots on which the antibody was tested did not permit detection of any HeLa S3 nuclear protein specifically reacting with Ki-67. Also the zwittergent method (Mandrell & Zollinger, 1984), aiming at a partial renaturation of lost antigenic epitopes, gave no unambiguous results. From these data one could conclude that the procedure followed to identify the antigen on SDS-containing gels apparently alters the structure of the epitope in such a way that it is no longer recognized by the antibody. However, immunoprecipitation experiments in which no SDS was initially used also failed in precipitating any specific [35S]methionine-labelled proteins or any 32P-labelled RNA from HeLa S3 total cell lysates. We have no clear indication how these negative results can be explained.

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Fig. 5. Comparison of the immunofluorescence staining patterns in untreated MR6S cells (A–E) and in nuclear matrix–intermediate filament scaffolds of MR6S cells (A′–E′) with monoclonal antibodies Ki-67 (A), 2.73 (B), directed against the U1 RNP-associated 70K protein, 41CC4 (C), directed against the nuclear lamins and RKSE 60 (D), directed against cytokeratin 10. E. DNA staining with Hoechst 33258. ×1150.

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Fig. 6. Immunofluorescence localization of the Ki-67 antigen in different cell types: A, HeLa S3; B, T24; C, Molt-4; D, human fibroblasts; F, ColI1; H, LLC-MK2. E, G. DNA staining patterns with Hoechst 33258, of preparations D and F, respectively. Note the differences in the levels of Ki-67 fluorescence between the various cell cultures. × 1150.

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References


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