New antiserum against Ki-67 antigen suitable for double immunostaining of paraffin wax sections

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Abstract

Aims—To prepare a rabbit antiserum equivalent to MIB 1 to permit the simultaneous assessment of cell proliferation and other markers of interest using double labelling studies.

Methods—Rabbits were immunised with a synthetic peptide deduced from the cDNA sequence coding for the Ki-67 antigen. Serum samples were tested for immunoreactivity using different immunobiochemical methods.

Results—A polyclonal antiserum was derived which detects the native as well as recombinant parts of the Ki-67 antigen in different test systems. Furthermore, the antiserum stains the Ki-67 antigen in routinely processed, paraffin wax embedded material.

Conclusions—After antigen unmasking by microwave treatment the antiserum described here represents a powerful tool for the determination of growth fractions even in archival material. It is especially suitable for double staining experiments in combination with monoclonal antibodies.

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The proliferation of tumour cells is an important prognostic indicator for a variety of different malignancies. In recent years the estimation of the growth fraction by immunocytochemical staining with antibodies against proliferation-associated antigens has been applied ever more widely. One of the antibodies frequently used for this purpose is Ki-67.¹ This antibody reacts with a nuclear antigen present in G₁, S, G₂ phases, and mitosis, but is absent in G₀.² Ki-67 turned out to be excellent for the estimation of the growth fraction in a wide variety of normal and malignant human tissues.³ An increasing number of studies have emphasised that the Ki-67 index is of important prognostic value for the individual clinical course in different neoplasias.⁴⁻⁹

The antigen determined by Ki-67 could be detected in immunoblots as a double band with apparent molecular weights of 345 and 395 kilodaltons, respectively. It was found only in proliferating cells and was absent in quiescent cells.¹⁰ Parts of the cDNA encoding for the Ki-67 antigen were cloned recently,¹⁰ and a synthetic peptide deduced from this was used in this study to immunise rabbits.

The main drawback of Ki-67—its restriction to frozen tissue material—was recently overcome by a new monoclonal antibody (MIB 1).^{11,12} After antigen retrieval by microwave irradiation MIB 1 detects the Ki-67 antigen in formalin fixed, paraffin wax embedded material. The aim of this study was to prepare a polyclonal antiserum that would also be suitable for detecting the Ki-67 antigen in routinely processed paraffin wax sections to facilitate double labelling studies with mouse monoclonal antibodies without the need to modify chemically one of the antibodies.

Methods

A 20mer peptide

(NH₂-AGGDEKDIKAFMGTPVQKLD-COOH) deduced from a 62 base pair DNA region and presumed to encode for the Ki-67 epitope¹⁰ was synthesised by Kem-En-Tec A/S, Denmark.

For immunisation purposes the synthetic peptide was used without coupling to a carrier protein. The peptide, in a final concentration of 500 µg/ml, was mixed with equal amounts of Freund's incomplete adjuvant, and five rabbits were immunised subcutaneously with 0.1 ml per immunisation, according to the immunisation schedule described.¹³

IM-9 cell line cells (3 × 10⁷) were harvested by centrifugation (10 minutes at 400 × g) and suspended in 250 µl phosphate buffered saline (PBS) containing 1 mM phenylmethylsulfonyl-fluoride (PMSF). The cell suspension was snap-frozen in liquid nitrogen and homogenised in a mortar cooled in liquid nitrogen. The resulting powder was suspended in 300 µl double concentrated Laemmli-SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis)¹⁴ sample buffer containing 100 mM DTT, boiled for 5 minutes in a water bath, and sonicated to destroy contaminating DNA. Samples were then subjected to SDS-PAGE.

The preparation of a 1002 base pair derived, bacterially expressed, fusion protein was performed as described.¹⁵

Immunohistochemical staining was carried out according to the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique, as described,¹⁶ or by the Duet streptavidin biotin complex-horse radish peroxidase (ABC/HRP) technique (Dako, Copenhagen) according to the manufacturer's guidelines. For double labelling, the

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Figure 1 Immunoblot analysis of lysates obtained from IM-9 cell line cells. Western blots were stained with Ki-67 and the new polyclonal antiserum. Both reagents react with identical bands with apparent molecular weights of 395 and 345 kilodaltons.

new antiserum was first targeted using the Duet system, followed by the detection of the monoclonal antibody using the APAAP technique. The alkaline phosphatase development was performed by visual control under the microscope.

Before staining, paraffin wax sections were treated according to the method described by Shi *et al.*¹⁷ as modified by Gerdes *et al.*¹² Briefly, sections were dewaxed, rehydrated, and boiled two to five times for 5 minutes in 10 mM citrate buffer, pH 6, in a household microwave oven (power setting: 720 W). During microwave treatment care must be taken to ensure that slides do not dry out. After cooling to room temperature, the slides were rinsed in TRIS-buffered saline (TBS).

COMPETITION ASSAY BY ELISA

Polystyrene microtitre plates were incubated overnight at 4°C with 0.5 µg/well of lysates of induced *Escherichia coli* JM109 pAX X2/1, expressing a 1002 base pair insert¹⁵ in a total volume of 50 µl PBS. After 30 minutes of

Figure 2 Immunoblot analysis of recombinant parts of the Ki-67 antigen. Bacteria were transformed with a vector containing a 1002 cDNA fragment. Ki-67 and the new antiserum react with identical bands representing the expression product of the DNA insert.

Figure 3 Two synthetic peptides were tested in a competition ELISA for their ability to compete for the binding of the new polyclonal antiserum to the solid phase-bound recombinant part of the Ki-67 antigen. The antiserum is displaced by the peptide ALC; TCC has no effect.

incubation at room temperature with the synthetic peptides at different concentrations, the monoclonal antibodies were subjected to precoated ELISA (enzyme linked immunosorbent assay) plates. Unspecific binding was blocked by 2% gelatin in TBS (TBS-Gel). The plates were incubated for 1 hour at 37°C. After three washes with TBS all wells were incubated for 1 hour at 37°C with 50 µl of a goat anti-mouse POD conjugate (Bio-Rad, Munich, Germany, 170-6516), diluted 1 in 5000 in TBS-Gel, followed by three washes with TBS and an incubation with 50 µl/well peroxidase substrate (63 µg/ml 3,3',5,5'-tetramethylbenzidine and $2.5 \times 10^{-3}\%$ H_2O_2 in 0.1 M acetate-citrate buffer, pH 6) at room temperature. The reaction was stopped by the addition of 50 µl/well 1 M H_2SO_4 . Optical density was evaluated at 450 nm on a Flow immunoplate reader (Flow Laboratories, Meckenheim, Germany).

SDS-PAGE

SDS gel electrophoresis was performed according to the method of Laemmli¹⁴ using 3.5% stacking gels and 5% and 7.5% separation gels, respectively. Sample buffer contained 50 mM TRIS-HCl, pH 6.8, 4% SDS, 20% glycerol and 100 mM DTT. Samples were boiled for five minutes before electrophoresis. Molecular weight markers were obtained from Pharmacia/LKB (Freiburg, Germany) or Sigma (Munich, Germany).

IMMUNOBLOTTING

Proteins were transferred to nitrocellulose membranes (SM 11306, Sartorius, Göttingen, Germany) using the western blot technique.¹⁸ The transfer was performed at 50 mA for 16 hours in a water cooled device. Unoccupied binding sites were blocked by incubation for 1 hour with 1% bovine serum albumin or gelatine in TBS. After one hour of incubation with primary antibody and thorough washing with TBS, the bound antibody was first targeted with mouse anti-rabbit monoclonal antibody (Dako M 737, diluted 1 in 100 in TBS-Gel) and in a second step with goat anti-mouse alkaline phosphatase

conjugate (Dianova, Hamburg, Germany), diluted 1 in 10 000 in blocking solution. The first step was omitted in case of Ki-67 detection. The phosphatase activity was visualised using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrates.¹⁹

FACS ANALYSIS

Cell culture

Lymphocytes were isolated from the peripheral blood of a normal healthy donor by Ficoll-hypaque density gradient centrifugation. Half of the cells were kept unstimulated, while the other half were stimulated with 3 µg/ml phytohemagglutinin (PHA) for 72 hours. Cells were kept at 1×10^6 cells/ml in culture bottles with RPMI 1640 containing 10% fetal calf serum in a CO₂ incubator. After three days the cells were stained either with antiserum or with Ki-67 using an indirect staining method. Raj1 cells were held in exponential growth in RPMI 1640 medium with 10% fetal calf serum under standard conditions.

Cell fixation

The cell membrane was stripped off and the nuclear membrane was penetrated by using the membrane shedding solution (MSS), as described by Larsen.²⁰

Fluorescence staining

Cells ($0.6 - 1.0 \times 10^6$) were washed in PBS and 0.2 ml of MSS were added to the cell pellet and mixed thoroughly. After 15 minutes at room temperature the primary antibody was added. After another 15 minutes cells were incubated either with an affinity isolated swine anti-rabbit IgG, fluorescein isothiocyanate (FITC) conjugated (Dako, Copenhagen, Denmark), or an affinity isolated rabbit anti-mouse immunoglobulin, FITC labelled F(ab)₂ fragment (Dako, Copenhagen, Denmark). For DNA staining, the samples contained 50 µg/ml propidium iodide (Sigma, Germany) and 5 Kunitz units/ml RNase (Sigma). After 15 minutes the samples were subjected to analysis using a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany).

Results

Serum samples from three of five immunised rabbits gave immunostaining patterns in frozen sections of human tonsils comparable with those obtained with Ki-67. All positive sera reacted with proliferating cells in paraffin wax sections after antigen unmasking by microwave irradiation (fig 5A).

Positive sera were pooled and the immunoglobulin fraction was isolated.¹³ This fraction was used for all experiments detailed in the following and is referred to as "antiserum" so that it is clearly distinguished from monoclonal antibody.

IMMUNOREACTIVITY

With native Ki-67 antigen

IM-9 cells were lysed, subjected to SDS-PAGE, and western blots were stained with Ki-67 and the new antiserum, respectively. Figure 1 shows that both reagents reacted with two polypeptide chains with apparent molecular weights of 345 and 395 kilodaltons.

With recombinant parts of the Ki-67 antigen

A 1002 base pair fragment of the Ki-67 antigen cDNA was expressed in bacteria and the lysate was subjected to SDS-PAGE. Subsequently, immunoblots were stained with Ki-67 and the new polyclonal antiserum. Figure 2 shows the similar reaction pattern of Ki-67 and the antiserum with the blotted bacterial lysates.

COMPETITION ASSAY BY ELISA

To substantiate further our findings, the antiserum was tested in a competition assay by ELISA with a fusion protein expressed in bacteria as the solid phase-bound antigen and synthetic peptides as competitors. Figure 3 clearly shows that only the peptide ALC, which was used as immunogen, was able to

Figure 4 (A) Dot plot from PHA-stimulated peripheral blood lymphocytes stained with Ki-67; (B) same as in (A), but cells were stained with the new antiserum; (C) double labelling study with Raj1 cells stained with Ki-67 and the new antiserum.

Figure 5 (a) Immunostaining of a paraffin wax embedded tonsil section with the new antiserum visualised by the Duet ABC/HRP system; (b) (c) immunohistochemical double staining of paraffin wax sections of human tumours with the new antiserum (Ki-67 antigen) and the monoclonal antibody KL 1 (cytokeratin). (b) Breast tumour; (c) lung tumour. The antiserum was detected by the peroxidase based Duet system (Dako) (brown nuclei); the monoclonal antibody KL 1 was targeted with the APAAP technique (red staining).

compete for the binding of the antiserum; the peptide TCC, which is adjacent to ALC on the Ki-67 antigen, had no effect.

FACS ANALYSIS

Figures 4A and B are dot plots from PHA stimulated cells immunostained either with Ki-67 (A) or the antiserum (B). They show an identical staining pattern, indicating the ability of this method to expose the fixation resistant epitope without further treatment. Fluorescence microscopy confirmed the similarity of the staining pattern, although fluorescence intensity was lower for the antiserum than for Ki-67 on PHA stimulated cells. Unstimulated lymphocytes, used as controls, showed no staining.

Figure 4C shows double staining using the peptide derived antiserum in combination with Ki-67. It is evident from the diagonal arrangement of the dots that all Raj1 cells were positive for both reagents, indicating that they react with the same nuclear antigen. Visual controls by fluorescence microscopy showed similar staining patterns for both the antiserum and Ki-67.

IMMUNOHISTOCHEMICAL DOUBLE STAINING WITH THE ANTISERUM AND A MONOCLONAL ANTI-CYTOKERATIN ANTIBODY

Figures 5b and c show the result of a double

labelling study of formalin fixed, paraffin wax embedded, and microwave irradiated sections of a mammary carcinoma (b) and a lung carcinoma (c). The reactivity of the antiserum can be clearly distinguished from the positivity of the monoclonal antibody. This new reagent therefore facilitates access to the parallel assessment of tumour cell characteristic membrane antigen and the growth fraction of the tumour.

Discussion

These results show that a synthetic peptide deduced from the cDNA coding for the Ki-67 antigen¹⁰ can elicit an antiserum in rabbits which reacts with the Ki-67 antigen in different test systems. The immunoblot experiment with lysates of proliferating human cells (fig 1) clearly showed that the serum is directed against the same polypeptide chains as Ki-67. This finding was further substantiated by immunoblot studies with part of the Ki-67 antigen expressed in a procaryotic expression system (fig 2) and a competition assay by ELISA with the fusion protein as solid phase-bound antigen and synthetic peptides as competitors. The synthetic peptide completely replaced the antiserum, whereas an unrelated peptide had no effect (fig 3). These results further confirm

the specificity and efficiency of the new serum. Flow cytometric analyses with nuclei double labelled with Ki-67 and the new antiserum (fig 4) also indicate that both reagents react with the same antigen. Although the antiserum detects an epitope which is distinct from that recognised by Ki-67 (Key *et al*, unpublished observations), it can be regarded as equivalent to Ki-67 with respect to its identical behaviour in all test systems using the Ki-67 antigen.

After antigen retrieval by microwave treatment the antiserum detects the Ki-67 antigen in paraffin wax sections of human tissues. The immunostaining of the antiserum is identical with that from Ki-67 and thus substantiates the immunobiochemical data, suggesting the equal value of the new antiserum and Ki-67 or MIB 1, respectively. The main advantage of this new reagent, however, is its usefulness for immunodouble labelling studies (fig 5), permitting the simultaneous assessment of the growth fraction and of tumour or tissue markers detected by mouse monoclonal antibodies.

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