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Localization of ribonucleotide reductase in mammalian cells

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The results of immunocytochemical studies using two different monoclonal antibodies against the M1 subunit of ribonucleotide reductase show an exclusively cytoplasmic localization of this subunit both in cultured MDBK and mouse 3T6 cells, and in cells from various rat tissues. By fluorescent light microscopy, there is a diffuse staining of the cytoplasm, while by electron microscopy the immunoreactive material appears to be associated with ribosomes. In the rat tissues, only actively dividing cells show M1-specific immunofluorescence revealing a strong correlation between the presence of protein M1 and DNA synthesis. Therefore M1 immunofluorescence could be used to study cell proliferation in normal, inflammatory or neoplastic tissue. A lesser variation in M1 staining is observed between individual cells in tissue culture, where most cells are positive, but neither here nor in the tissues examined are any cells with nuclear staining detected. We interpret our results to mean that in mammalian cells ribonucleotide reduction takes place in the cytoplasm and from there the deoxyribonucleotides are transported into the nucleus to serve in DNA synthesis.

Key words: cytoplasmic localization/immunocytochemistry/M1 subunit/monoclonal antibodies/ribonucleotide reductase

Introduction

In all cells making DNA the required precursor deoxyribonucleotides are supplied by the enzyme ribonucleotide reductase which catalyzes the direct reduction of the corresponding ribonucleotides. In mammalian cells this highly regulated allosteric enzyme consists of two non-identical subunits, proteins M1 and M2 (Thelander and Reichard, 1979; Engström *et al.*, 1979). The active form of M1, purified to homogeneity from calf thymus, is a dimer of mol. wt. 170 000 capable of binding the allosteric effectors ATP, dATP, dTTP or dGTP (Thelander *et al.*, 1980). Protein M2 has a mol. wt. of ~110 000 and contains non-heme bound iron and a unique tyrosyl-free radical essential for activity (Thelander *et al.*, 1980, 1983; Gräslund *et al.*, 1982). The activity of ribonucleotide reductase in a cell is strongly correlated to the rate of DNA synthesis with very little activity detectable in G1 cells and maximal activity in S phase cells. Consequently, fast growing tissues with a large proportion of S phase cells have high activities of ribonucleotide reductase (Thelander and Reichard, 1979).

While DNA synthesis is unequivocally localized in the cell nucleus, it has been an open question as to where in the cell the deoxyribonucleotides are synthesized. Are they made in the cytoplasm and then transported into the nucleus or are they actually made in the nucleus close to the DNA synthesizing machinery? A cytoplasmic localization of ribonucleotide reductase was suggested by Larsson (1969) who found >80% of the enzyme activity localized to the cell sap after subcellular fractionation of regenerating rat liver. In contrast, measurements of the distribution of the four deoxyribonucleoside triphosphates (dNTP) between the nucleus and the cytoplasm in CHO cells after fractionation in non-aqueous media showed that, in S phase cells, all the dNTPs are concentrated in the nucleus, suggesting a nuclear localization of ribonucleotide reductase (Skoog and Bjursell, 1974).

Finally, according to Prem veer Reddy and Pardee (1980, 1982) ribonucleotide reductase, together with a number of other enzymes associated with DNA metabolism, is translocated from the cytoplasm to the nucleus when cells pass from the G1 to the S phase of growth. They suggest that the enzymes form a nuclear multienzyme complex termed 'replitase' and this event is believed to signal the initiation of the S phase of the cell cycle.

We recently succeeded in isolating a number of murine hybridomas that produce monoclonal antibodies against the M1 subunit of ribonucleotide reductase (Engström, 1982).

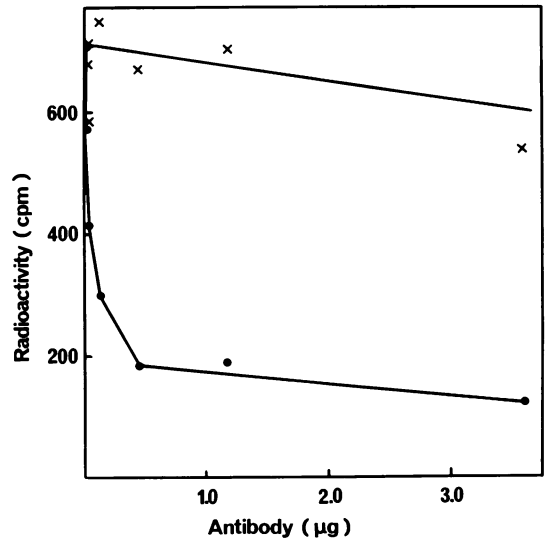

Fig. 1. Non-competitive binding of antibodies AD203 and AC1 to protein M1. The wells of plastic microtiter plates were coated with calf thymus protein M1 by incubating 50 μl per well of a solution containing 4 μg of protein/ml under gentle shaking for 3 h at 20°C. After rinsing with a solution containing 10 mg/ml of bovine serum albumin (BSA) and 0.1 mg/ml of thimerosal in PBS the wells were post-coated with the same solution for 16 h at 4°C. The solution was removed and 0-4 μg of unlabelled AD203 or AC1 antibodies in PBS containing 10 mg/ml of BSA were added in separate series of cells. Then 0.7 μg of ^{125}I-labelled AD203 antibody [labelled by the method of Bolton and Hunter (1973) to a specific activity of 14 000 c.p.m./μg] diluted in the same solution was added to each well. After 4 h gentle shaking at 20°C the wells were carefully rinsed with PBS and counted in a gamma counter. Unlabelled AC1 antibody (×——×), unlabelled AD203 antibody (●——●).

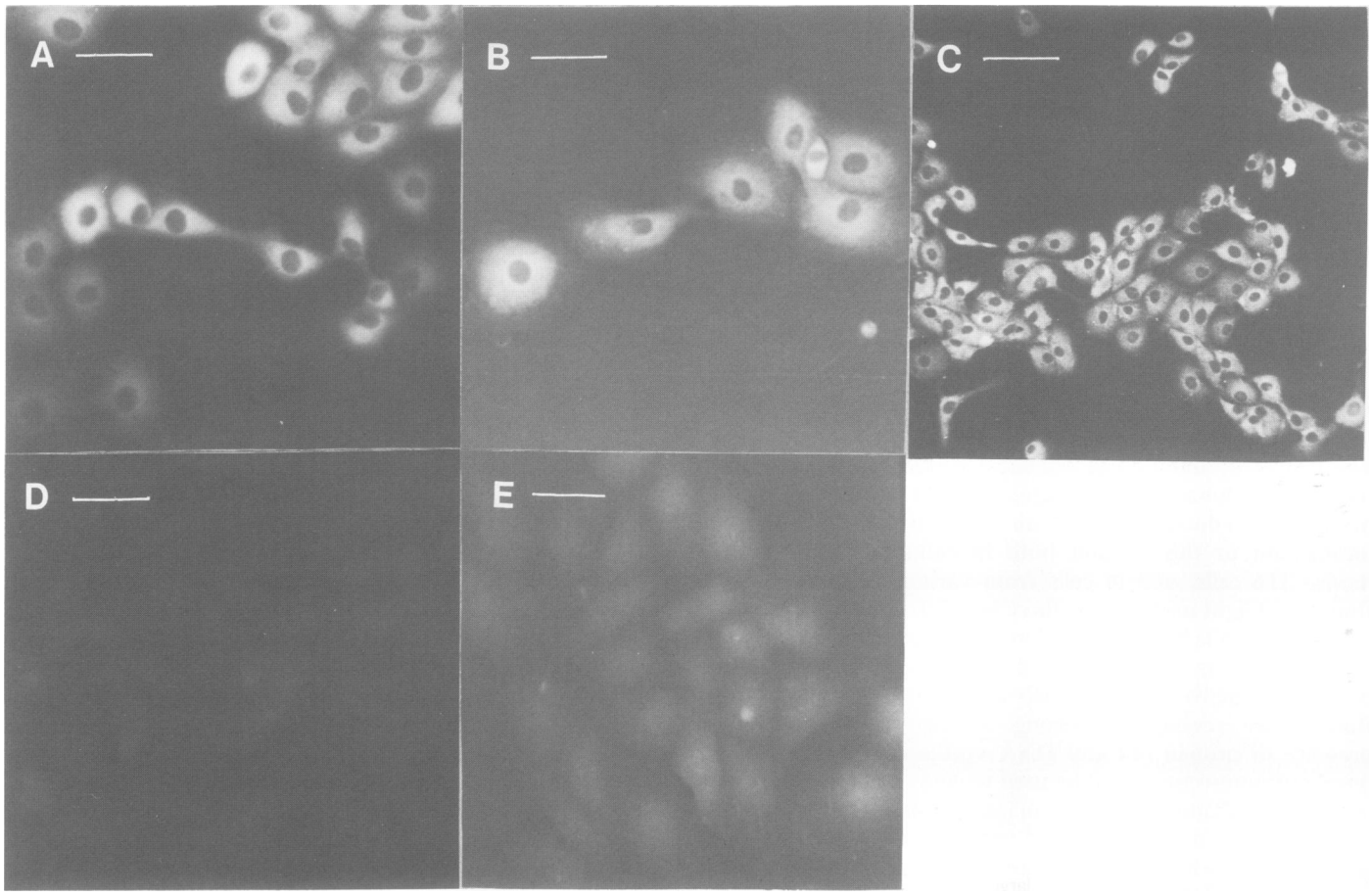

Fig. 2. Localization of the M1 subunit of ribonucleotide reductase in MDBK cells. **(A)** Fixation in 3.7% paraformaldehyde, permeabilization in Triton X-100. Antibody AD203 (18 μg per ml) 15 s exposure, Bar: 50 μm. **(B)** Fixation in 3.7% paraformaldehyde, permeabilization in acetone. Antibody AD203 (7.1 μg/ml), 30 s exposure, Bar: 50 μm. **(C)** Acetone fixation. Antibody AD203 plus AC1 (both 18 μg/ml), 30 s exposure, Bar: 100 μm. **(D)** Same as in **A** but using normal mouse immunoglobulin (18 μg/ml). 15 s exposure (negative control), Bar: 50 μm. **(E)** Same as in **B** but using antibody AD203 pre-adsorbed with protein M1 (7.1 μg/ml). 30 s exposure, Bar: 50 μm. For experimental details, see Materials and methods.

With these homogeneous reagents of unambiguous specificity we have tried to resolve the question of ribonucleotide reductase localization. Here we report the results of immunocytochemical experiments demonstrating the exclusive cytoplasmic localization of ribonucleotide reductase.

Results

Characterization of the anti-M1 monoclonal antibodies

The AD203 and AC1 monoclonal anti-M1 antibodies showed about the same ability to neutralize the activity of calf thymus ribonucleotide reductase (Engström, 1982). However, the AC1 antibodies were about two orders of magnitude less active than the AD203 antibodies in neutralizing the activity of ribonucleotide reductase from mouse or rat cells, suggesting binding to different antigenic determinants on protein M1 (data not shown). The binding to protein M1 was further investigated, using the following competitive binding assay. After immobilization of calf thymus protein M1 to plastic microtiter plates, the binding of a constant amount of ^{125}I-labelled AD203 antibody was measured in the absence or presence of increasing amounts of added unlabelled AC1 or AD203 antibodies (Figure 1). Addition of AC1 did not influence the binding of AD203 to protein M1, while in the control experiment unlabelled AD203 clearly competed with the labelled antibody. We therefore conclude that the AD203 and AC1 antibodies bind to different epitopes on protein M1.

Localization of the M1 subunit of ribonucleotide reductase in cultured cells by immunofluorescence

The results from immunofluorescence studies on cultured MDBK cells, using the monoclonal antibodies AC1 and AD203 directed against protein M1, are illustrated in Figure 2. In all experiments, protein M1 showed cytoplasmic localization without any detectable nuclear staining. Both antibodies gave the same localization, although the stain was more intense with AD203. When the two antibodies were combined, an additive effect was observed, resulting in brighter fluorescence than that observed using either antibody alone (Figure 2C). To exclude artifacts depending on the method of fixation and permeabilization, a number of different procedures described in Materials and methods were used. In Figure 2A–C the results of three different fixation procedures are illustrated, clearly showing exclusive cytoplasmic staining in all cases. Control experiments, using normal mouse immunoglobulin, always resulted in a very low background staining (cf. Figure 2D and 2A). Furthermore, pre-incubation of antibody AD203 with calf thymus protein M1 led to a pronounced decrease in fluorescence (Figure 2E) compared with the bright cytoplasmic stain observed with non-adsorbed antibody (Figure 2B).

To check our technique, positive controls of antibodies directed against antigens of known localization were used. Anti-tubulin and anti-vimentin antibodies gave the expected

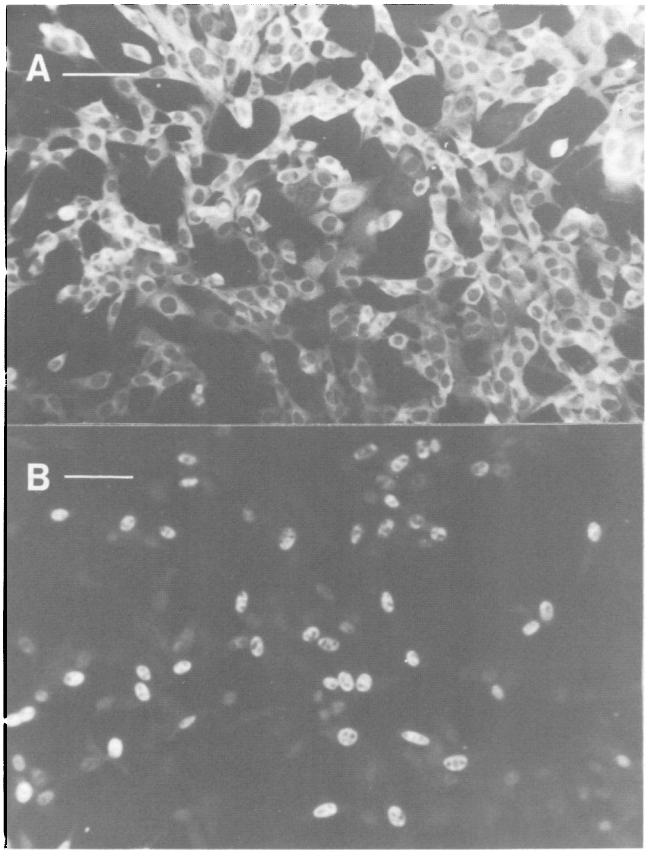

Fig. 3. Localization of protein M1 and large T-antigen in polyoma virus-infected 3T6 cells. 3T6 cells were grown, infected with polyoma virus, fixated 26 h post-infection in 3.7% paraformaldehyde and permeabilized in Triton X-100 as described in Materials and methods. **(A)** Monoclonal antibody AD203 (18 μg/ml), Bar: 100 μm. **(B)** Monoclonal antibody αPyLT1, Bar: 100 μm.

pattern with microtubules and intermediate filaments, respectively (data not shown). We also tested an antibody directed against a nuclear antigen, namely the monoclonal antibody αPyLT1 directed against polyoma virus large tumor antigen. In Figure 3 the staining patterns of polyoma virus-infected mouse 3T6 fibroblast cells with antibody αPyLT1 and antibody AD203 are compared. Again, a cytoplasmic staining was observed with the AD203 antibody, while the αPyLT1 antibody gave the expected nuclear staining showing that our experimental conditions allowed penetration of antibodies into the cell nucleus. Uninfected cells did not stain with the αPyLT1 antibody, while the result with the AD203 antibody was the same in uninfected 3T6 cells.

According to flow cytometric analysis, 43% of the MDBK cells used in the experiments illustrated in Figure 2 were in the G1 phase of the cell cycle, 34% in the S phase and 23% in the G2 + M phase. Considering that we never observed any cells showing nuclear staining with the anti-M1 antibodies, this strongly argues against a cell cycle-dependent localization of ribonucleotide reductase.

Localization of protein M1 in cultured MDBK cells by electron microscopy

Electron microscopic analysis of thin sections revealed that the immunoreactive material is exclusively localized in the cytoplasmic compartments, i.e., the cytoplasmic matrix. Higher magnification revealed that the marker apparently

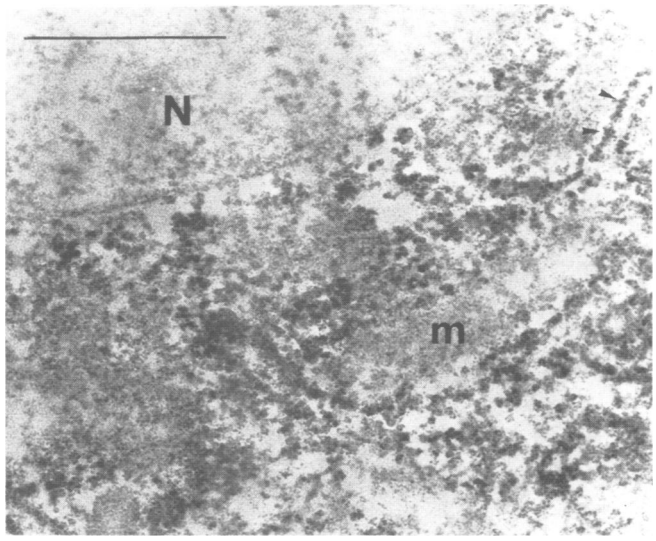

Fig. 4. Electron micrograph of an MDBK cell processed for demonstration of protein M1. The nucleus (N) and the mitochondria (m) show no reactivity. The ribosomes (arrow) along the endoplasmic reticulum are strongly positive, Bar: 1 μm.

was associated with ribosomes (Figure 4). There was no activity related to other cell organelles, nor to the nucleus.

Localization of ribonucleotide reductase in the rat testis

Fluorescence microscopic analysis of the testis revealed a characteristic pattern of distribution of ribonucleotide reductase (Figure 5). The spermatogonia arranged in a single row close to the basement membrane were strongly reactive while all other cells in the tubules remained negative. Rarely, positive connective tissue cells could be observed, but generally the interstitial cells were negative. As observed earlier all positive cells showed only cytoplasmic immunoreactivity. Similar results were obtained for ovary, small intestine, spleen, thymus, lymph nodes as well as for developing tissue such as the cerebellum (data not shown).

Discussion

Our data clearly show an exclusively cytoplasmic localization of the M1 subunit of ribonucleotide reductase, using a number of different fixation protocols to exclude the possibility of antigen leakage or deformation of antigenic determinants. The same results were obtained using cultured cells or cells in tissues, two monoclonal antibodies directed against different antigenic determinants and both fluorescent light microscopy and electron microscopy. This strongly indicates that, in mammalian cells, ribonucleotide reduction takes place in the cytoplasm and from there the deoxyribonucleotides are transported into the nucleus to serve as precursors for DNA synthesis.

In the cultured cells, the intensity of the M1-specific fluorescence varied to some extent from cell to cell but most cells were positive suggesting the persistence of protein M1 throughout the mitotic cycle. In contrast, in the testis tissue only actively dividing cells gave M1-positive staining showing a strong correlation between the presence of protein M1 and DNA synthesis.

Interestingly enough, the positively stained spermatogonia in each tubule displayed about the same fluorescence intensity, but there was a marked variation between adjacent tubules

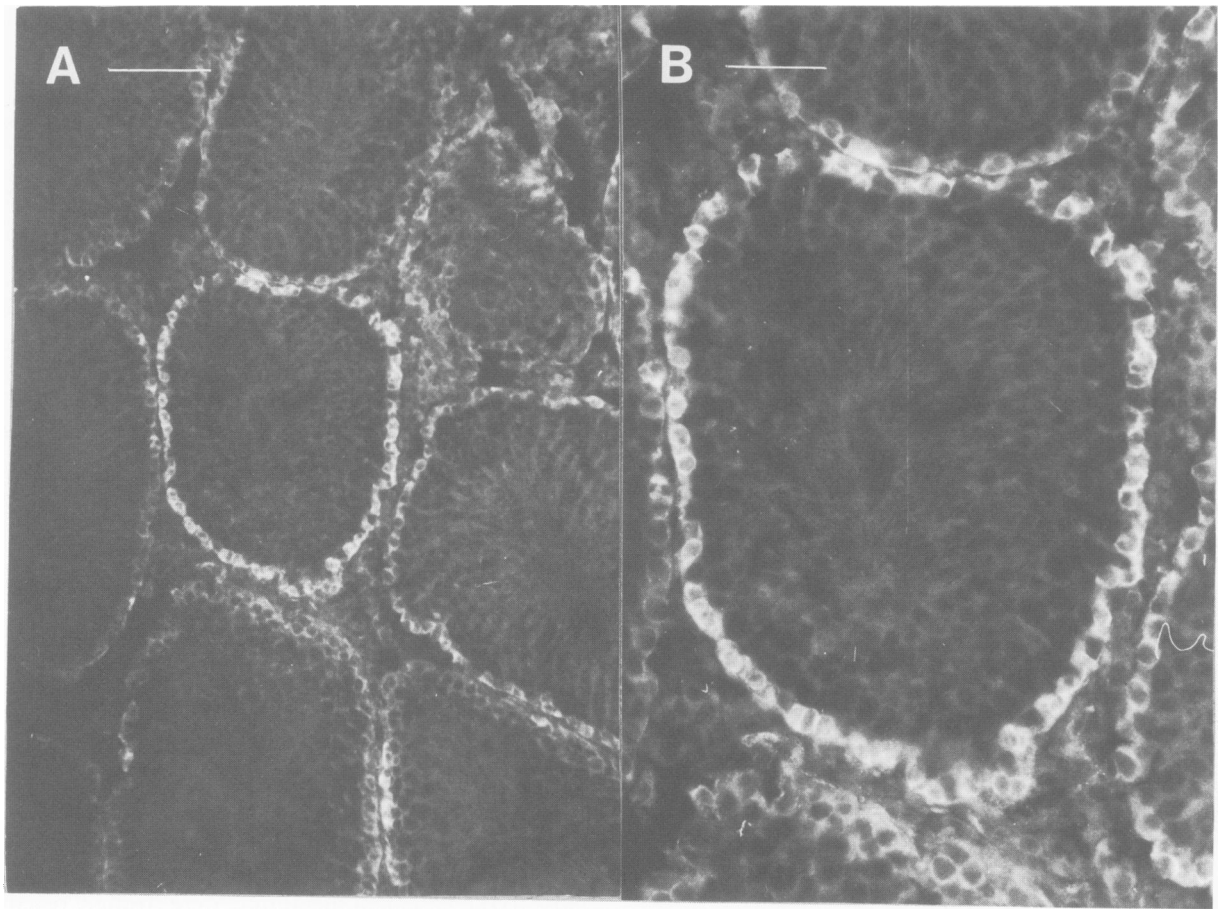

Fig. 5. Fluorescence micrographs of rat testis. In **A** several tubules are seen with the spermatogonia strongly positive along the periphery while all other cell types are negative. Note the variation in intensity between adjacent tubules. At higher magnification the exclusive cytoplasmic localization of protein M1 is evident, **A**. Bar: 100 μm. **B**. Bar: 50 μm.

(Figure 5A and B). Since the spermatogonia in each segment of a tubule in the rat testis divide in a fairly synchronous manner (Clermont, 1972) this indicates a correlation between the staining intensity of protein M1 and the cell cycle. However, in spite of these cell cycle-dependent variations in the M1 levels, we never observed nuclear staining in cultured cells or in the various tissues examined. This was not due to inability of the antibodies to penetrate the nucleus under our conditions of fixation, as demonstrated in the control experiment by the nuclear reactivity of the antibodies against the polyoma large tumor antigen. Also, Bensch *et al.* (1982) recently demonstrated the exclusively intranuclear localization of DNA polymerase α in cultured human cells using monoclonal antibodies and fixation methods very similar to the ones we have used.

Although we cannot exclude the existence of a minor fraction of the M1 protein of a cell forming a nuclear multiprotein complex with hidden antigenic determinants, our results argue against the replitase model of Prem veer Reddy and Pardee (1980, 1982) which states that most of protein M1 in a cell should be located in the nucleus during the S phase. Furthermore, one of the major arguments for the existence of such a multiprotein complex, namely the experimental evidence for its channeling of ribonucleotides into DNA, has recently been seriously questioned (Spyrou and Reichard, 1983).

The present study demonstrates that there is a good correlation between the presence of M1 in cells and cell proliferation. The monoclonal antibodies against M1 should therefore be suitable to use for demonstration of cell proliferation in specimens with inflammatory reactions or in tumors.

Materials and methods

Cell lines

The MDBK cell line (NBL-1) was obtained from the American Type Culture Collection. Mouse fibroblast 3T6 cells were supplied by Dr. P. Reichard, Karolinska Institute.

Cell cultures

Monolayer cultures were maintained in Dulbecco's modification of Eagle's medium plus 10% heat-inactivated fetal calf-serum (MDBK) or 10% heat-inactivated horse serum (3T6).

Antibodies

The establishment of four different hybridoma cell lines, producing monoclonal antibodies against subunit M1 of calf thymus ribonucleotide reductase, was described earlier (Engström, 1982). Antibodies from lines AD203 and AC1 were used and they were produced by incubating 5 x 10^5 cells/ml of medium without serum in roller bottles. After 7 days incubation at 37°C, the medium was collected and cells were removed by centrifugation. The antibodies were precipitated by the addition of 0.313 g ammonium sulfate/ml and after centrifugation, the precipitates were dissolved in a small volume of 50 mM Tris-Cl pH 7.6, 0.1 M KCl. Finally, the solutions were dialysed against the same buffer or passed through Sephadex G-25 columns to remove the ammonium sulfate. Control immunoglobulins were prepared from non-immune mouse serum by ammonium sulfate fractionation as described above.

Preparation of antigen-adsorbed antibody AD203 was made by mixing 36 μg of calf thymus protein M1 with 7.1 μg of antibody in a total volume of 25 μl of phosphate-buffered saline (PBS), pH 7.3. After incubation on ice for 2 h the sample was diluted in the same way as the non-adsorbed antibody.

Monoclonal antibodies against polyoma virus large T-antigen (αPyLT1) in the form of ascites fluid were a generous gift from Dr. B. Griffin, Imperial Cancer Research, London, UK (Dilworth and Griffin, 1982). Monoclonal antibodies against tubulin were obtained from Amersham International, UK, and against vimentin from Lab. System Oy, Helsinki, Finland.

Preparation of cells for immunofluorescence microscopy

Cells were grown as monolayers to 40–50% confluence either on glass cover slips (washed in acetone, 95% ethanol and flamed), placed on the bottom of Petri dishes or grown in tissue culture chamber slides (Lab-Tek Products, Miles Lab. Inc., IL). Before fixation, the cells were rinsed in medium without serum for 2 x 5 min at 37°C. Then one of the following schemes for fixation was used. (i) Freshly prepared 3.7% (w/v) paraformaldehyde (Merck AG, Darmstadt, FRG) solution in PBS or in isotonic Hepes buffer, pH 7.6 [0.04 M 4-(2-hydroxyethyl)-1-piperazine sulfonic acid buffer, 0.137 M NaCl, 0.0027 M KCl] for 15, 30 or 60 min at 20°C. (ii) 4% (w/v) paraformaldehyde as in (i). (iii) 4% (w/v) paraformaldehyde plus 0.1% (w/v) glutaraldehyde in PBS or isotonic Hepes buffer for 15 or 30 min at 20°C. (iv) 1% (w/v) glutaraldehyde in PBS or isotonic Hepes buffer for 15 or 30 min at 20°C. (v) Acetone for 5 min at −20°C.

In some experiments the fixation procedures (iii) and (iv) were followed by incubation 2 x 4 min at 20°C in PBS containing 0.5 mg/ml of sodium borohydride to reduce excess aldehyde reactivity.

After fixation, according to (i–iv), the cells were permeabilized by incubation in PBS or isotonic Hepes buffer, containing 0.2% (v/v) Triton X-100 for 3 min at 20°C or in acetone for 5 min at −20°C. Acetone-treated slides were air-dried after permeabilization, and then soaked in PBS or isotonic Hepes buffer before further processing. All other slides were rinsed in PBS or isotonic Hepes buffer 3 x 10 min at 20°C without allowing the slides to dry in any step.

Preparation of tissues for immunofluorescence studies

Sprague-Dawley rats weighing 150 g were anesthesized with Nembutal and fixed by transcardial perfusion at 100 mm/Hg with 4% (w/v) freshly prepared paraformaldehyde in PBS. After 20 min of perfusion the testis was dissected and further fixated by immersion for 15 min. After rinsing overnight in 7.5% sucrose in PBS at 4°C, the specimens were rapidly frozen in isopentane chilled with liquid nitrogen and sectioned on a cryostat at a temperature of about −20°C at a nominal thickness of 7 μm. The sections were allowed to dry onto glass slides.

Immunofluorescence microscopy

Slides with adhering cells or tissue were pre-incubated in normal rabbit serum (DAKO, Copenhagen, Denmark) diluted 1:50 in PBS or isotonic Hepes buffer for 30 min at 20°C. Excess serum was carefully removed without touching the cells or the tissue. Without drying, the slides were then immediately incubated with the different purified immunoglobulins, diluted to concentrations between 7 and 18 μg/ml in PBS or isotonic Hepes buffer, both containing 2% normal rabbit serum. After 16 h at 4°C or 3 h at 20°C the slides were rinsed 3 x 10 min in PBS or isotonic Hepes buffer, incubated with rabbit anti-mouse IgG, conjugated with FITC (DAKO or Amersham International, UK), (diluted 1:15 in PBS or in isotonic Hepes buffer) for 2 h at 20°C, rinsed and mountd in a solution containing 90% glycerol, 0.01 M Tris-Cl pH 8.6 and 1 mg/ml of *p*-phenylendiamine. The slides were observed in a Zeiss microscope equipped for epi-illumination and fluorescence microscopy.

Preparation of cells for electron microscopy

MDBK cells were grown on 3 cm dishes, rinsed and fixated according to method (ii) or (iii) for 60 min at 20°C and then rinsed in isotonic Hepes buffer overnight at 4°C. After permeabilization with Triton X-100 and rinsing as described earlier, the cells were pre-incubated in isotonic Hepes buffer containing 0.04% (v/v) Triton X-100 and 2% horse serum for 1 h at 20°C. The solution was then removed, and 0.5 ml per plate of the specific or control immunoglobulins (7 μg/ml) diluted in 2% horse serum solution was added. After 16 h incubation at 4°C, the plates were rinsed 3 x 15 min at 20°C with isotonic Hepes buffer. The first antibody was then detected using Vectastain ABC-kit (Vector Lab., Burlingame, USA), with horseradish peroxidase as a marker. After rinsing, the samples were incubated in isotonic Hepes buffer containing 0.5 mg/ml of 3,3-diaminobenzidine and 0.04% (v/v) hydrogen peroxide for 10 min at 20°C, followed by rinsing. Finally, the samples were post-fixated in 2% (w/v) buffered osmium tetroxide for 1 h at 20°C, dehydrated and embedded in EPON.

Electron microscopy

Thin sections of EPON-embedded cells were prepared on LKB Historange and Ultrotome microtomes. After staining, 1 μm sections were examined by light microscopy. Sections from selected areas with a thickness of 30–60 nm were then examined in a JEOL 100-CX Temscan electron microscope, operated at 60 kV.

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