Monoclonal Antibodies Against Mammalian Ribonucleotide Reductase*

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Ribonucleotide reductase is present in all dividing cells, furnishing the cell with a balanced supply of the four deoxyribonucleotides needed for DNA synthesis. The reductase from calf thymus consists of two nonidentical subunits: protein M1, which has been purified to homogeneity, and protein M2. The substrate specificity and activity of the mammalian ribonucleotide reductase are controlled by nucleoside triphosphates acting as allosteric effectors and this regulation has been studied in detail.¹ In addition, the activity of the reductase is regulated during the cell cycle by some unknown mechanism, showing a strong positive correlation to the rate of DNA synthesis.

I have now used the hybridoma technique to produce monoclonal antibodies against mammalian ribonucleotide reductase to be able to simplify the purification of the enzyme, to study the localization of the enzyme in the cell and to study the control of enzyme synthesis during the cell cycle by measuring the amounts of protein M1 and M2 molecules in cell extracts using immunochemical technique.

Experimental. Female 6–8 weeks old BALB/c-mice were immunized with ribonucleotide reductase (material after affinity chromatography on dATP-Sepharose²), first subcutaneously using 100 µg of protein mixed with Complete Freund's Adjuvans then, after 4 weeks, intraperitoneally with 50–100 µg of protein mixed with Incomplete Freund's Adjuvans and, finally, after another 4 weeks, intraperitoneally with 100 µg of protein in phosphate buffered saline every day for four days. Fusion was made on the fifth day using spleen cells from one immunized mouse (10^8 cells) and 5×10^7 mouse myeloma cells, strain SP2/0, in the presence of 50% polyethylene glycol, m.w. 4000, as described elsewhere.³ The cell mixture was diluted into 250 ml of hypoxanthine – aminopterin – thymidine (HAT)-containing tissue culture medium (DMEM) supplemented with 15% fetal calf serum and spread in Costar trays to give 240 independent cultures

together with mouse peritoneal macrophages (10^5 cells/well) as a feeder layer. Cells were grown in HAT-medium for 10 days. Before changing to normal culture medium, the cells were grown in HT-medium without aminopterin for two passages. Hybridomas producing antibodies against ribonucleotide reductase were detected using the following modified ELISA-test.⁴ Plastic microtiter wells were coated with ribonucleotide reductase and remaining sites in the wells were blocked by careful washing with phosphate buffered saline containing 1% bovine serum albumin. Then cell supernatants were incubated in the wells and after washing with the same solution rabbit antimouse antibodies labelled with horse raddish peroxidase were added followed by addition of a solution containing 2 mM 2,2'-azino-di(3-ethyl-benzothiazolinsulfonate) – 2.5 mM H_2O_2 – 0.1 M sodium acetate – 0.05 M NaH_2PO_4 . Positive wells were detected by a green colour after incubation at 25 °C and the cells from such wells were frozen and kept in liquid nitrogen. Finally, cells from positive wells were cloned by limiting dilution in the presence of mouse spleen cells as feeders.

To produce antibodies, hybridoma cells (0.5×10^6 cells/ml) were incubated in DMEM tissue culture medium without serum for 7 days or 10^7 hybridoma cells were injected intraperitoneally into pristane treated BALB/c mice to form ascites tumors. The antibodies in the cells supernatants or ascites fluid were precipitated by the addition of 0.313 g of ammonium sulfate per ml solution. The precipitates were dissolved in a small volume of 0.1 M KCl – 50 mM TRIS – Cl pH 7.6, and then the ammonium sulfate was removed by dialysis or gel filtration on Sephadex G-25 columns.

To determine the specificity of the antibodies, a modified Western blot method was used.⁵ A crude preparation of ribonucleotide reductase from calf thymus (material after DEAE chromatography²) was analyzed by SDS-gel-electrophoresis and then the separated proteins were blotted from the gel to a diazobenzylxymethyl paper. The paper was cut in strips and each strip incubated with antibodies from a specific hybridoma clone followed by rabbit antimouse-Ig antibodies and finally ^{125}I -labelled protein A. Radioactive bands were detected by autoradiography.

Results and Discussion. Viable hybridoma cells were first seen 4 days after the fusion and at the 14th day hybridoma cells were found in 211 out of 237 possible wells. Of these 237 cultures, 31 were strongly positive and 201 slightly positive in the ELISA-test. A high unspecific background (measured in the same way but with 1% bovine serum albumin instead of ribonucleotide reductase as antigen) was found in 10 cultures from the first group and in 22 cultures from the second group. Eight of the strongly positive, low background

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Fig. 1. Inhibition of ribonucleotide reductase activity by the monoclonal antibodies was tested by incubating a series of tubes containing enzyme (15 µg) with increasing amounts of antibodies at 0 °C for 60 min. Then the normal ^3H -CDP assay mixture was added and enzyme activity was measured as described earlier.² Clone AH 1 (■), AG 8 (●), AD 203 (▲) and AC 1 (○).

cultures, were cloned and named AA–AH, and in Fig. 1 it is shown how 4 of these cloned lines (AC1, AD203, AG8 and AH1) could neutralize the activity of ribonucleotide reductase.

On SDS-polyacrylamide gel-electrophoresis of the same four antibodies, three of them, AC1, AD203 and AG8, gave one heavy chain band with a molecular weight around 55 000 and the fourth one, AH 1, gave two bands with molecular weights around 56 000 and 62 000, indicating that the AC 1, AD203 and AG8 antibodies belong to one of the heavy chain γ class and the AH 1 antibody to the heavy chain γ_{2b} class. The results were further confirmed by Ouchterlony double diffusion technique using subclass specific rabbit antimouse serum (Bionetics, Maryland). Now AD203 and AG8 were shown to belong to heavy chain class γ_1 , AC 1 to γ_{2a} and AH 1 to γ_{2b} . All antibodies were of the κ -light chain class. When antibodies from the same four clones were tested on Western blot strips containing an electrophoresed crude mammalian ribonucleotide reductase extract, all gave a single radioactive band at the position of protein M1 of ribonucleotide reductase, while no other bands were detected. This demonstrated in a convincing way the anti-M1 specificity of these hybridomas.

Using the same Western blot method, many supernatants from uncloned cultures with positive ELISA-reaction were tested without finding any antibodies directed against protein M2. This could either be due to the lack of such antibodies or else reflect the fact that the preparation of ribonucleotide

reductase used as antigen contains an excess of protein M1 over protein M2² and therefore M2 positive clones could be lost in the first ELISA selection.

To get anti-M2 antibodies there is a need of protein M2 antigen, free of protein M1, preferably already when immunizing the mice but even more critical when screening the cultures with the ELISA-test. The supply of such M2 preparations has been very limited earlier but can now be obtained using anti-M1 antibodies coupled to Sepharose to remove M1 from ribonucleotide reductase preparations.

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