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Monoclonal antibodies to Escherichia coli 50S ribosomes

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ABSTRACT.

Hybridoma cell lines that produce monoclonal antibodies directed against 50S ribosomal proteins have been isolated. Spleen cells (from BALB/c mice immunized with 50S ribosomal subunits extracted from Escherichia coli) were fused to mouse myeloma cell line SP2/0-Ag 14. The initial screening for antibody producing hybridomas was carried out by a double antibody sandwich method; hybridomas were subsequently cloned in soft agar. Antibodies were characterized by their specific binding to individual 50S ribosomal proteins separated on phosphocellulose columns and in two-dimensional polyacrylamide gels. The assignments were confirmed with purified single ribosomal proteins. Of four clones analyzed thus far, two are identical with specificity for r-protein L5. The other clones produce two different antibodies directed against r-protein L20. Each monoclonal antibody formed ribosome dimers visualizable in the electron microscope. Dimers could be reacted with a different second antibody to form chains containing 8 or more ribosomes, which may be useful for structural studies.

INTRODUCTION

The specificity of the antigen-antibody reaction has been used to great advantage in the study of ribosome structure and function. Antibodies which react specifically with each of the E. coli ribosomal proteins have been prepared in a number of laboratories (1,2,3). These antibodies have been used for the detection and identification of individual r-proteins (4,5); for the detection of the products of in vitro transcription-translation of ribosomal protein genes (6); to help build models of ribosome structure using immunoelectron microscopy to map components (3); to study the selective inhibition of steps in protein synthesis (7); and to detect changes in the immunochemical accessibility of proteins during in vitro ribosome assembly (8).

The traditional approach does, however, have several problems: (1) a large quantity of a purified individual ribosomal protein is needed to immunize animals and to permit purification of antibody. (2) Contaminants

in the purified antigen used for immunization may produce antibodies with different specificities. (3) Most seriously, multiple antigenic determinants on a single purified ribosomal protein will give rise to a heterogeneous population of antibodies. Thus, in immunoelectronmicroscopy, antibodies to a single protein may bind at several different sites, making it difficult to determine unambiguously whether the protein is "elongated", or the ribosomes are heterogeneous in structure (8).

One approach which bypasses these problems is the use of monoclonal antibodies rather than simple antisera. Monoclonal antibodies can be produced in high yield with reaction specificity at the level of antigenic determinants rather than individual proteins. In this study we describe the isolation and characterization of three monoclonal antibodies to 50S ribosomes.

MATERIALS AND METHODS

Cell cultures.

SP2/0-Ag 14 myeloma cells were used as previously described in (13).

Preparation of ribosomal subunits and ribosomal proteins.

E. coli ribosomal subunits were obtained as described previously (14). Ribosomal proteins were extracted from 50S subunits with acetic acid (14) and the resultant protein solutions were quickly dialyzed against buffer P (50 mM Tris, pH 5.5, 3 mM $MgCl_2$ 125 mM KCl) and stored as such or lyophilized after dialysis into 7% acetic acid. The lyophilized ribosomal proteins were further purified on phosphocellulose columns (14) or by two-dimensional gel electrophoresis (15).

Immunization of mice.

Each female BALB/c mouse was immunized with 1 mg of 50S ribosomal subunits in PBS (10 mM potassium phosphate pH 7.0, 140 mM NaCl) supplemented with complete Freund's adjuvant (1:1 vol). The same animal was boosted using the same procedure after one month and antiribosomal antibodies in the serum were verified by immunoprecipitation. A second boost was given 5 days prior to cell fusion.

Production of anti-ribosomal protein hybridomas.

A modification of the procedure of Galfre *et al* (10) was used to produce hybridomas. Briefly, 10^8 spleen cells from a ribosome-immunized mouse were fused to 1 x 10^7 SP2/0-Ag14 myeloma cells with 50% polyethylene glycol in serum free media. Fused cells were pelleted, resuspended in

complete medium, and distributed into 24-well Costar plates. Cultivation in HAT medium (11) yielded heavy growth of the fused cells within 10 days. After an initial screening (see below), cells from those wells producing anti-ribosomal protein antibodies were cloned in medium containing 0.2% (W/V) Bacto agar (Difco) over mouse 3T3 feeder layers. Isolated colonies were picked after 7-10 days, grown to confluency, and further characterized.

Production of anti-ribosomal antibodies.

The double antibody sandwich method was used to detect ribosome-specific antibody secreted into the growth medium of the hybridoma cells. Polyvinyl chloride microtiter plates (Cooke Lab) were incubated overnight at room temperature with 100 µl per well of 50S total protein solution (2 mg/ml, diluted 20-fold into 20 mM Tris·HCl, pH 7.5 immediately before use). The ribosomal protein solution was removed and the plates were incubated with 200 µl of 2% BSA in PBS for 4 hr, and then with 100 µl of hybridoma medium for another 4 hr. The hybridoma medium was removed and 30,000 cpm of [^{125}I]-labeled goat anti-mouse IgG antibody in 100 µl of PBS containing 2% Fraction V BSA was allowed to react overnight. Each well was counted after washing away unbound labeled antibody

After cloning single anti-ribosomal protein antibody-forming cells, the cloned cells were grown in T-flasks, harvested, and injected into the peritoneal cavity of Pristene-primed mice (10). Cells from one 75 cm^2 T-flask were used to inject 3 mice and after 7-14 days, mouse ascitic fluid was harvested (3-5 ml/mouse). Serum albumin was removed by three successive 40% $(NH_4)_2$ SO_4 precipitations. The resultant solutions were dialyzed and stored in PBS at -20°.

Determination of antigenic specificity.

Forty mg of lyophilized 50S ribosomal protein mixture was separated on a phosphocellulose column (0.7 cm x 40 cm) in the presence of 50 mM NaH_2PO_4, 10 mM methylamine, 6 M urea, 4 mM 2-mercaptoethanol, pH 6.5, according to ref. 14. Fractions of ribosomal protein from the phosphocellulose column were either used to coat microtiter plates (0.5 to 5 µg/well) or spotted on nitrocellulose filter paper (Millipore HA). The microtiter plates or filters were then incubated with [^{125}I]-labeled hybridoma antibody, washed free of unbound antibody, and counted or visualized by autoradiography (see below).

One hundred µg of a mixture of 50S ribosomal proteins was separated by two-dimensional acid-SDS polyacrylamide gel electrophoresis (15). After electrophoresis, gels were soaked in 50 mM NaCl, 2 mM Na_2-EDTA, 4 M urea,

0.1 mM dithiothreitol, 10 mM Tris·HCl, pH 7.0 overnight with two changes of buffer. The proteins were transferred onto two sheets of nitrocellulose filter paper, one on each side of the gel, by a procedure similar to Southern transfer of DNA (9), except that a lead block was placed on top of the gel to facilitate transfer of r-proteins from the gel to the nitrocellulose paper. One of the two sheets was stained with amido black to visualize the protein spots. The other sheet was coated with bovine serum albumin (2% BSA in PBS). Five µl of [^{125}I]-labeled hybridoma antibody (0.12 µg/µl, 300,000 cpm/µg; labeled by the chloramine T method) was added to 10 ml of 2% BSA in PBS containing 1 mM NaN_3, and incubated with the filter paper overnight in a Seal-a-meal (Sears) bag with gentle shaking at room temperature. The filter paper was washed in 2% BSA in PBS twice for 30 min each time and quickly rinsed with distilled water three times to remove residual BSA solution. The filter paper was air dried and autoradiographed with XR-5 film for 2-16 hrs. Spots were identified by alignment with the amino black-stained sheet of filter paper.

Isoelectric focusing (IEF) and antibody class of hybridoma antibodies.

The isoelectric point of hybridoma antibodies was determined by isoelectric focusing (16). The subclass of the monoclonal antibodies was determined by competition radioimmunoassay. Antibodies against each subclass were used to coat microtiter wells. [^{125}I]-labeled IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, and λ and k light chains were mixed individually with various dilutions of monoclonal antibody and this mixture was allowed to react with the antibody-coated wells.

Preparation of samples for electron microscopy.

Partially purified dimers were prepared according to Tischendorf et al. (12). These dimers were mixed with a second antibody to promote the formation of chains. Ribosomes and antibody were visualized either by positive staining with uranyl acetate on parlodion support grids, or by negative staining by a double carbon sandwich method (12).

RESULTS

Detection of anti-ribosomal antibody.

In a typical fusion experiment, cell hybrids were obtained in 46 of 48 wells, of which four (9%) showed strong positive radioimmunoassays against total 50S r-proteins. Three additional wells showed weaker binding of radioactive antibody. These proved to bind non-specifically to several

r-proteins, and are not discussed further here. The radioimmunoassay could detect 30 ng of each r-protein per well.

Detection of antibody reaction with specific r-proteins.

Many r-proteins form aggregates, especially in mixtures, so that initial attempts to determine antibody specificity by immunoprecipitating r-proteins were frustrated by nonspecific precipitation. Satisfactory results were obtained by screening antibodies against ribosomal proteins anchored to solid phase supports. Fig. 1 (top panel) shows a standard partial separation of r-proteins on phosphocellulose. The optical density profile is shown, and the elution positions of individual proteins are indicated. (These assignments are in accord with published patterns, and were confirmed by electrophoretic mobilities). After spotting each column fraction on a nitrocellulose filter, the filter was incubated with a solution of radioactive hybridoma antibody, washed and autoradiographed. This approach suggested that L20 probably carried the antigenic determinant recognized by antibody L20A4A1 (Fig. 1). However, because of the high sensitivity of the

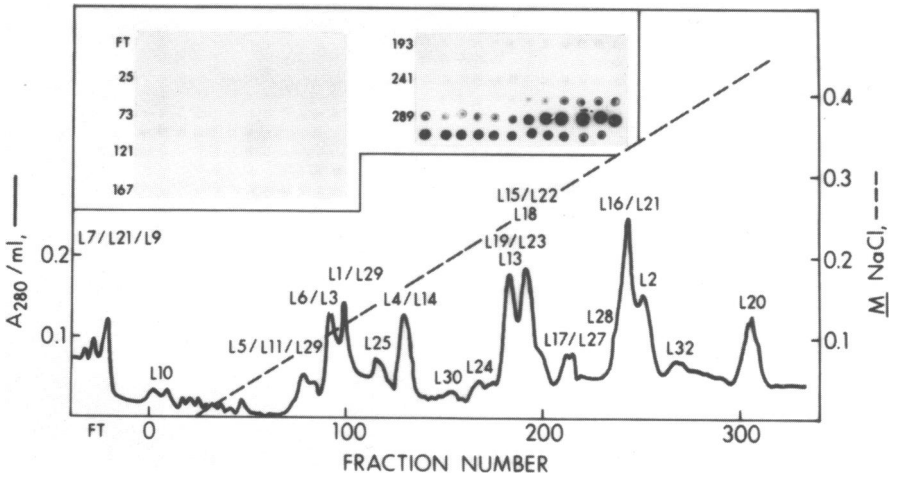

Fig. 1. Identification of the antigenic specificity of monoclonal antibodies. A mixture of 50S r-proteins was separated on a phosphocellulose column. The optical density of the flow-through (FT) and eluates is shown. The protein content of individual peaks was confirmed by SDS-polyacrylamide gel electrophoresis (Methods). 25 µl of every other fraction (as specified in the insert) was spotted on nitrocellulose filter paper. [125I]-labeled antibody L20A4A1 was allowed to react with the filter paper. After washing away unbound material, the filter paper was dried and autoradiographed. Positive reaction was shown by fractions coinciding with the elution position of r-protein L20.

assay, the observed peaks tended to be diffuse, and column fractions usually contained more than one r-protein. Comparable results were obtained with other antibodies (data not shown). We therefore exploited the sensitivity of this nitrocellulose assay using very small amounts of r-protein separated on 2D gels (see Methods).

The high resolving power of two-dimensional gel electrophoresis (15) combined with a gel transfer method (9) (see Materials and Methods) was

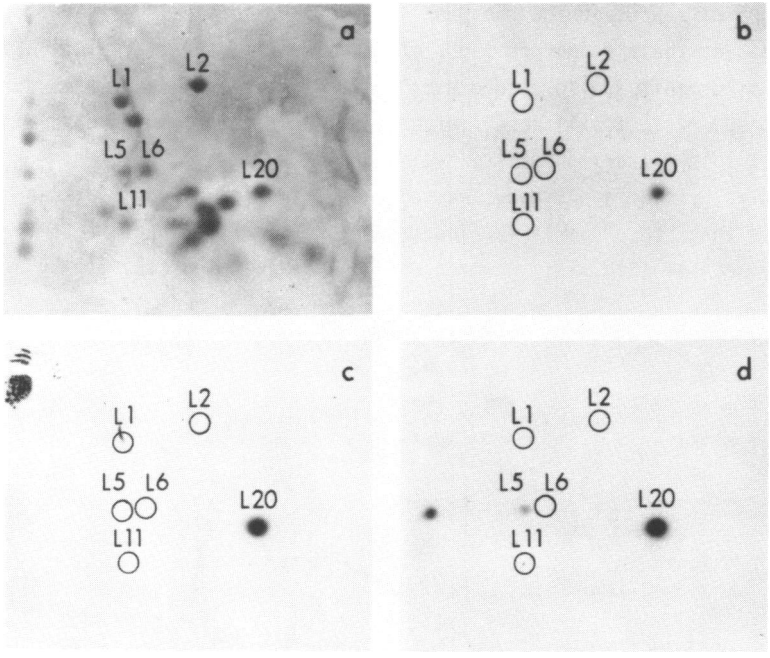

Fig. 2. Detection of the antigenic specificity of monoclonal antibodies by a gel transfer method. 100 µg of a mixture of 50S ribosomal proteins was separated by acid-SDS two-dimensional polyacrylamide gel electrophoresis. After soaking the gel to remove SDS, ribosomal proteins were transferred to two sheets of nitrocellulose filter paper, one on each side of the gel (9). One sheet of filter paper was stained with amido black (panel a); relevant ribosomal proteins are indicated . The other filter paper was soaked in 2% BSA in PBS. [^{125}I]-labeled monoclonal antibody in 2% BSA in PBS was added and incubated overnight with gentle shaking. After unbound labeled antibody was removed, the filter paper was dried and autoradiographed.
Second and third labeled antibodies were reacted in the same way. b. first antibody, L20D4B1 c. second antibody, L20A4A1 d. third antibody, L5B6D6. Some L5 remained at the origin of the second dimension of electrophoresis resulting in a spot at that position in d.

adapted to simplify the determination of antigenic specificity. The filter paper prepared from one gel can be used repeatedly with different antibodies to determine the specificities of additional antibodies without losing previously bound antibody and requiring no more than 0.5 μg of each r-protein. Fig. 2 shows an example. Using this test, two hybridoma antibodies were identified as specific for ribosomal protein L20 (clones L20A4A1 and L20D4B1); and two others, for r-protein L5 (clones L5B6D6 and L5D3C5).

Since all r-proteins have been purified to homogeneity, unequivocal confirmation of the antigenic specificity of each antibody was possible using pure protein samples kindly provided by Drs. W. Strycharz and M. Nomura of the U. of Wisconsin. Proteins were bound to nitrocellulose filter paper and incubated with [^{125}I]-labeled hybridoma antibody. An example is shown in Fig. 3. (Note that r-protein L6, which migrates very near L5 in gel electrophoresis, did not react with these antibodies).

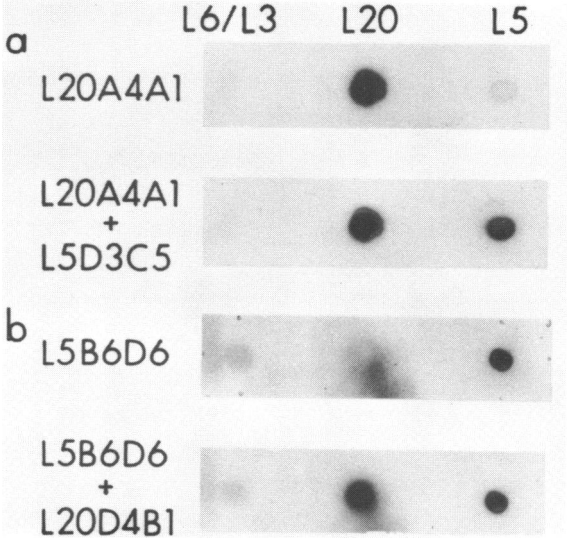

Fig. 3. Confirmation of antigenic specificity using purified r-proteins. One μg of purified r-proteins (L5,L20 and a mixture of L6 and L3) were spotted on nitrocellulose filter paper as indicated. The filter paper was immersed in 2% BSA in PBS, and reacted with [^{125}I]-labeled monoclonal antibodies in two steps, as described in the legend to Fig. 1. a. first antibody, L20A4A1; second antibody, L5D3C5; b. first antibody L5B6D6; second antibody, L20D4B1.

Monoclonality of the hybridoma antibodies.

The antibodies were characterized by analysis in an isoelectric focusing gel (Fig. 4). Each exhibited the small number of "spaced" bands characteristic of monoclonal antibodies (16). The two anti-L5 antibodies, from a single animal, were indistinguishable by IEF, with a pI of 5.5 (L5B6D6, L5D3C5). The two antibodies against L20, however, were distinct, with pI's of 7.0 (L20A4A1) and 6.2 (L20D4B1).

The antibodies were further characterized by determing their antibody subclass, using competition radioimmunoassay. Each of the four antibodies

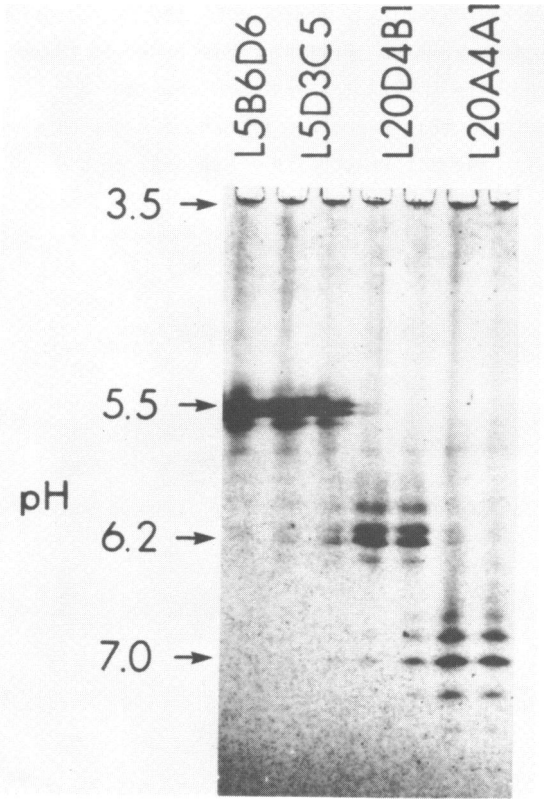

Fig. 4 Isoelectric focusing of hybridoma antibodies. Ammonium sulfate cuts from antibody preparations were focused in a gel containing urea and 5% acrylamide-diallytartardiamide with a pH range of 3.5 to 10.0. The anolyte buffer was 0.5% phosphoric acid and the catholyte buffer was 0.5% diethanolamine. Focusing was performed at 2.5 mA constant current until the voltage peaked (usually 700-900 volts) at room temperature. The gel was fixed with trichloroacetic acid and stained with Coomassie blue.

was, as expected, uniform and all belonged to the IgG_1, k subclass.

Visualization of ribosome-monoclonal antibody complex.

In a number of preliminary experiments, ribosome-antibody dimers were formed with one antibody. When a second antibody to a different protein was added, chains of 4 to 9 or more ribosomes have been observed: Fig. 5 shows examples.

DISCUSSION

The results presented here indicate the advantages of monoclonal antibodies over conventional antisera, both for the preparation of pure antibody and for structural studies. The possibility of using mixed antigens for immunization combined with the gel transfer technique described here may be even more useful in less well characterized systems where antigen purification is much more difficult or impossible (for example, with membrane antigens or with proteins composed of multiple subunits) and for the study of developmental systems (for example when the amount of different proteins, separated in 2D gels, is measured at different times in the life

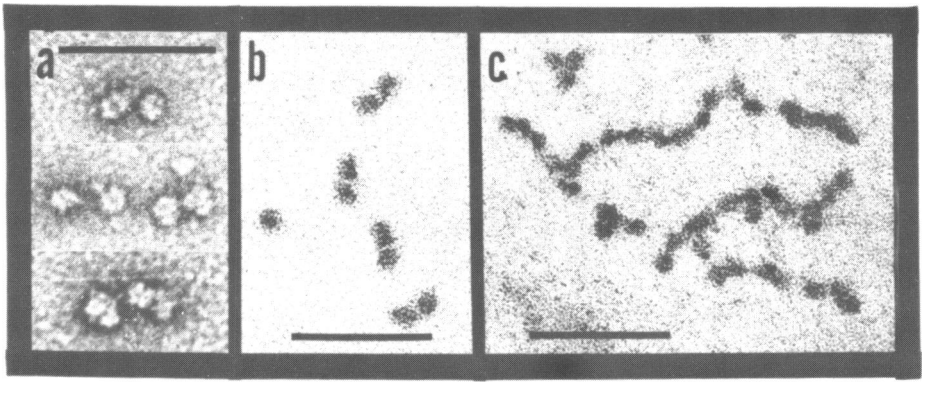

Fig. 5. Immunoelectron microscopy of monoclonal antibody-ribosome complexes. 50S ribosomal subunits (10 optical density units) were reacted with monoclonal antibody (100 µg) L20D4B1 in 20 mM Tris·HCl, pH 7.5, 0.2 mM $MgCl_2$, 60 mM NH_4Cl and 6 mM 2-mercaptoethanol. Dimers were separated from monomers by centrifugation in a 10-30% sucrose gradient in the same buffer. A small amount of the dimers was then reacted with monoclonal L5D3C5 antibody. Electron microscopic samples were prepared by positve or negative staining. Negative stain: a. Dimers with L20D4B1 (190,000 X). Positive srain: b. Dimers with L20D4B1 (170,000X). c. Oligomers with L20D4B1 and L5D3C5 (170,000X). Bars indicate 100 nm.

of an organism).

The most promising use for the monoclonal antibodies we have prepared is in the extension of the analysis of ribosome structure. Lake (2,3) has used conventional antisera against different individual r-proteins to form antibody-ribosome dimers, permitting the localization of specific r-proteins on the ribosome by immunoelectron microscopy. Because the monoclonal antibodies are specific for a single antigenic determinant rather than for an entire protein, they should make it possible to distinguish extended r-proteins from a heterogeneous ribosome population (see Introduction). Also, if the conditions can be optimized, long fibers of ribosome-antibody dimers (Fig. 5) could be prepared. Since alternate pairs of ribosomes would have the same orientation in these fibers, superposition of the microscopic images by computer might improve electron microscopic resolution of ribosome structure.

One result reported here was unexpected. Although the four hybridomas described are all of subclass IgG_1, the most common for protein antigens (17), they were directed against only two r-proteins. The two anti-L5 antibodies have the same pI (Fig. 4), and they are probably identical products of one B cell cone; but the two anti-L20 antibodies have distinct isoelectric points and so are distinct antibodies. Since all r-proteins are highly immunogenic when tested separately, and mice were immunized with subunits containing 34 different r-proteins, one would have expected a greater range of antibody specificity. Many explanations are possible, and only a few clones have been analyzed thus far, but it is conceivable that certain determinants on L5 and L20 may be especially immunogenic when in the ribosome, perhaps as a result of surface exposure.

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