Immunogenic Properties of Soluble Cytosol Fractions of Meth A Sarcoma Cells


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Garrett C. DuBois, Ettore Appella, Lloyd W. Law, Albert B. DeLeo, and Lloyd J. Old

ABSTRACT

Tumor-associated transplantation antigen (TATA) was found to be present in fractions derived from the cytosol of the Meth A cell. Meth A ascites cells were disrupted, nuclei and membranes were removed by low- and high-speed centrifugation, and the soluble protein was fractionated by ammonium sulfate precipitation and gel filtration chromatography. The TATA of the soluble cytosol fractions appears to be identical with the TATA solubilized from plasma membranes. The TATA of the cytosol fractions was found to be associated with proteins of an approximate apparent molecular weight of 60,000, specific for the Meth A tumor, and as immunogenic as the membrane-derived TATA. In addition, the most enriched TATA cytosol fraction shows inhibition of an antisera capable of detecting a tumor-specific surface antigen of Meth A. These results suggest that Meth A TATA is not an integral membrane protein and may be related to the tumor-specific surface antigen detected serologically.

INTRODUCTION

Chemically induced neoplasms, such as those induced by polycyclic hydrocarbons, contain individually distinct TATA. These antigens are immunogenic in their syngeneic hosts and provide transplantation immunity only to their respective tumors and not to similarly induced tumors. Transplantation immunity in mice can be imparted in several ways: prior growth and removal of tumor transplants; immunization with irradiated tumor cells; tumor cell membranes; or with solubilized antigen preparations.

Previous studies in this laboratory have used the highly immunogenic MC-induced BALB/c Meth A sarcoma (11) and have shown that TATA can be solubilized from membranes with detergents and partially purified (4, 5, 9). Recently, an individually distinctive TSSA has been defined on Meth A sarcoma with cytotoxic antiserum prepared in BALB/c X C57BL/6 F1 mice (2). This antiserum also recognizes from [35S]methionine-labeled sarcoma cell extracts, an antigen of an apparent molecular weight of 53,000 that appears to be transformation related (1). The relationship of this antigen to TATA or to the TSSA defined by cytotoxic antibody has not been established (1). In the present study, we report that the TATA has been found to be present in fractions derived from the cytosol of the Meth A cell and that it appears to be identical to the TATA found on membranes of Meth A cells. Absorption analysis also suggests a relationship between the serologically defined Meth A antigen and the TATA.

MATERIALS AND METHODS

Mice. Female BALB/c mice, 8 to 12 weeks old, were obtained from the production unit of NIH.

Tumors. Three antigenically distinct MC-induced BALB/c sarcomas were used in these studies: Meth A, CI-4, and CMS4. Sarcoma Meth A was maintained in ascitic form by serial passage in female BALB/c mice and was used as the source of antigen. This in vivo-passaged line does not express murine leukemia virus or its antigens (2). The MC-induced sarcoma designated CI-4 (6) was used as challenge tumor and served as a specificity control. This BALB/c sarcoma possesses its own strong TATA that does not cross-react with Meth A or with any other tumor assayed. Meth A and CMS4 maintained in tissue culture were used for absorption studies of antisera with membranes and chromatographic fractions (2).

Preparation of Subcellular Fractions. The ascites fluid from 95 BALB/c mice (850 ml) was centrifuged for 10 min at 1000 x g. The supernatants were discarded, and the cell pellets were resuspended in 0.155 M NH4Cl, 0.1 mM EDTA and 0.01 M KHCO3 to lyse RBC. The suspensions were stirred by swirling for 10 min and then centrifuged for 10 min at 1000 x g. Supernatants were discarded, and cell pellets were resuspended in additional lysing buffer. This entire procedure was repeated 3 times. Meth A cells were then washed with 0.05 M Tris-HCl (pH 7.2), containing 0.15 M NaCl, 0.1 mM PMSF (Sigma Chemical Co., St. Louis, Mo.) and 0.1% (w/v) aprotinin (Boehringer Mannheim Corp., Indianapolis, Ind.). The washed cells were resuspended to a volume of 600 ml containing 1.18 x 10^8 cells/ml and disrupted in a Stansted cell disruptor. Nuclei and undisrupted cells were removed by low-speed centrifugation (10 min, 1000 x g).

The crude membrane fraction was obtained by centrifugation of the low-speed supernatant at 100,000 x g for 50 min. The crude membranes (675 mg of protein) were rehomogenized in 0.01 M Tris-HCl (pH 7.2) containing 0.1 mM PMSF and 0.1% aprotinin (total volume, 22 ml). The membrane suspension was brought to 37% sucrose by addition of a concentrated sucrose solution. The 37% sucrose suspension was placed in nitrocellulose tubes, overlaid with 25% sucrose solution, and centrifuged for 18 hr at 74,000 x g in an SW 27 rotor. Purified membranes were collected from the interface of the 2 sucrose solutions; mixed with 1 volume of 0.01 M Tris-HCl (pH 8.3), 0.1 mM PMSF, and 0.1% aprotinin; and homogenized with 4 strokes of a type B dounce homogenizer. This suspension was centrifuged at 200,000 x g for 60 min in a Beckman 60Ti rotor. Plasma membranes sedimented as a relatively loose pellet overlapping a small tight brown pellet of endoplasmic
reticulum. The membranes, 90 mg of protein, were resuspended in 10 ml of 0.01 M Tris-HCl (pH 7.2) with 0.1 mM PMSF and 0.1% aprotinin and stored at -70°C. The high-speed supernatant, 600 ml, was brought to 55% saturation in ammonium sulfate by slowly adding the calculated amount of salt with stirring at 4°C. The suspension was stirred overnight and the following morning the precipitate was collected by centrifugation at 31,000 x g for 40 min. One-half of the pellets was resuspended in a total volume of 65 ml of 0.01 M Tris-HCl (pH 7.7) containing 0.15 M NaCl, 0.1 mM PMSF, and 0.1% aprotinin and stirred for 3 hr at 4°C. The other half of the pellets was frozen at -70°C. The suspension was then centrifuged for 10 min at 12,000 x g and the supernatant was dialyzed versus 4 liters of the above buffer with one change the following morning. The dialyzed solution was clarified by centrifugation at 12,000 x g for 15 min.

**Sephacryl S-200 Chromatography.** The soluble protein extract was concentrated to a volume of 25 ml containing 3700 mg of protein by ultrafiltration with a Diaflo PM-30 membrane (Amicon Corporation, Lexington, Mass.) and applied to a column (6 x 106 cm) of Sephacryl S-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) previously equilibrated with 0.01 M Tris-HCl (pH 7.2) with 0.15 M NaCl. The column was eluted with the same buffer, and fractions of 11.4 ml were collected. Radioactive marker proteins (200,000 cpm) were added to the sample before application to the column and consisted of 125I-labeled sheep immunoglobulin (M.W. 150,000), 125I-labeled sheep heavy chain (M.W. 53,000) and 125I-labeled sheep light chain (M.W. 23,000). Fractions were pooled in the following approximate apparent molecular weight ranges: S1, 100,000 to 60,000; S2, 60,000 to 40,000, 160 mg; S3, 40,000 to 20,000, 149 mg of protein. A low yield was obtained from the gel filtration chromatography (approximately 50%). This could be the result of aggregation and precipitation of cytosol protein which became apparent after freezing and thawing the samples. The fractions comprising S2 were rechromatographed on a column of Ultrogel AcA54 and yielded a broad peak in the 40,000 to 60,000 region. Two-dimensional gel electrophoresis of this material showed that it consisted of many components with pI's in the range of 5.3 to 7.5.

**In Vivo Tumor Rejection Assay.** TATA activity was assayed using purified plasma membranes, the 3 cytosol fractions from Sephacryl S-200 gel filtration and the Fraction 2 derived from Ultrogel chromatography. In all cases, varying protein concentrations were prepared in phosphate-buffered saline (0.0067 M sodium phosphate, pH 7.2, with 0.15 M NaCl) which had been passed through a 0.45-μm Millipore filter (Millipore Corp., Bedford, Mass.) before use. Female BALB/c mice 8 to 12 weeks of age were immunized s.c. twice at 10-day intervals and challenged 10 days later with the appropriate tumor cells. Mean tumor volumes and the immunogeneic index were calculated as described previously (7, 8). Nonimmunized mice that received phosphate-buffered saline served as controls.

**Antisera.** The antisera used in this study, CB6F; aMeth A and CB6F; αCMS4 have been described previously (2, 3). These sera contained cytotoxic antibodies which defined non-cross-reacting TSSA on each of these sarcomas.

**Absorption Analysis Assays.** The membrane, Sephacryl S-200, and Ultrogel AcA54 chromatographic fractions were assayed for the serologically defined Meth A antigen by absorption of Meth A. Appropriate dilutions of antisera were mixed with equal volumes of membrane and chromatographic fractions and incubated for 30 min at 4°C. The mixtures were centrifuged in a Beckman Microfuge for 5 min, and the supernatant was assayed for residual cytotoxic activity against Meth A (ascites cells) in the microcytotoxicity test (2). As a negative control for these experiments, absorption of αCMS4 by membrane and chromatographic fractions of Meth A sarcoma were also done.

**RESULTS**

**Fractionation of Cytosol Protein.** Meth A ascites cells (7 x 10^10 cells) after disruption, centrifugation, and (NH_4)_2SO_4 precipitation yielded 7600 mg of soluble protein. The yield of purified plasma membranes was 90 mg of protein. One-half of the crude soluble protein was chromatographed on a Sephacryl S-200 column and produced an A_280 profile shown in Chart 1. Fractions were pooled in the following approximate apparent molecular weight range: S1, 100,000 to 60,000, 182 mg; S2, 60,000 to 40,000, 160 mg; S3, 40,000 to 20,000, 149 mg of protein. A low yield was obtained from the gel filtration chromatography (approximately 50%). This could be the result of aggregation and precipitation of cytosol protein which became apparent after freezing and thawing the samples.

The fractions comprising S2 were rechromatographed on a column of Ultrogel AcA54 and yielded a broad peak in the 40,000 to 60,000 region. Two-dimensional gel electrophoresis of this material showed that it consisted of many components with pI's in the range of 5.3 to 7.5.

**Tumor Rejection Activity of Sephacryl S-200 Fractions S1, S2, and S3.** Fractions S1, S2, and S3 as well as purified plasma...
Absorption Analyses of Meth A by Membrane and Soluble Cytosol Fractions of Meth A Sarcoma. Two non-cross-reacting systems of TSSA on Meth A and an antigenically unrelated MC-induced BALB/c sarcoma, CMS4, have been defined with cytotoxic antisera prepared in CB6F, mice (2, 3). Absorption tests identified the Meth A antigen on 1 of 20 BALB/c sarcomas (Meth A) and the CMS4 antigen on 2 of 20 BALB/c sarcomas (CMS4 and 11). CMS4 served as a specificity control for absorption analysis of αMeth A by membrane and chromatographic fractions of soluble cytosol of Meth A sarcoma. Purified plasma membranes of Meth A sarcoma completely absorbed the cytotoxicity of αMeth A (Chart 2). The partial inhibition of αCMS4 by undiluted membranes can be attributed to anticomplementarity of this preparation which was readily diluted out. All of the fractions from Sephacryl S-200 and Ultrogel AcA54 chromatography were assayed for the serologically defined Meth A antigen by absorption analysis of αMeth A. Only the Sephacryl S-200 Fraction S2 showed partial absorption of cytotoxicity (Chart 3), and this was demonstrated in 3 separate absorption experiments and with 2 separate preparations of Sephacryl S-200 Fraction S2.

**DISCUSSION**

TATA was found to be present in fractions derived from the cytosol of the Meth A cell; this activity was found in each of 3 different preparations. The cytosol TATA appears to be as active as the TATA that has been solubilized from plasma membranes of Meth A sarcoma (4, 7, 9). Based on the tumor rejection assay, the cytosol TATA was also specific for Meth A. Tumor rejection was observed only against Meth A, resembling the activity of intact cells, membranes, and detergent-solubilized membranes (7, 9). Previous studies on TATA solubilized from Meth A membranes have also shown that TATA is associated with proteins with an approximate molecular weight of 60,000 (7); similar results were obtained in the present study. It appears that most of the TATA is associated not with the plasma membranes but with the cytosol of the cell. These results strongly suggest that the Meth A TATA is not an integral membrane protein, but a cytosol protein that is most probably peripheral to the membrane and thus exhibits a strongly immunogenic response. Chromatography of the cytosol extract of Meth A on an agarose-hexylamine column followed by gel filtration on Ultrogel AcA34 in 0.5% Nonidet P-40 clearly has shown that the TATA antigen elutes in a molecular weight range of 43,000 to 67,000 which is the same range of the membrane-bound TATA.4 More recent findings in our laboratory have shown that TATA of the CI-4 sarcoma can also be isolated from the cytosol and from fractions obtained from gel filtration of cytosol. The TATA of CI-4 cytosol fractions is specific; immunization protects against challenge with CI-4 but not against Meth A challenge.5, 6

The presence of TATA in the cytosol fraction is not unexpected. We have reported previously that TATA could be prepared in good yield and in soluble form from an SV40-induced sarcoma, mKSA, without resorting to the usual extraction procedures such as limited proteolysis, 3 mM KCl extraction, or detergent extraction of intact cells (16). Cell disruption,
and by purified plasma membranes of Meth A sarcoma. Each antiserum was absorbed by its respective cell: 9, Meth A; A, CMS4. The dilutions of purified antigen preparation that was extremely potent in the tumor rejection assay and retained all of the immunogenic character properties of the purified molecules.

The possibility that tumor antigens are modified macromolecular components present on normal cells has received increased attention by many investigators. One proposal is that TATA may be oncogen-modified normal alloantigens or derepressed alloantigens. The work of Parmiani et al. (12, 13) with the MC-induced tumor in BALB/c mice designated C-1, provides a convincing example of the expression of alien H-2 antigens that appear to "have tumor rejection activity." However, recent work in this laboratory has shown, through chromatographic analyses, that for detergent-solubilized C-1 membranes, a clear separation of the normal and alien H-2 antigens from TATA can be obtained (8, 15). The chromatographic behavior of the TATA from C-1 on both gel filtration and lectin affinity chromatography closely resembles the characteristics of the unique TATA isolated from Meth A sarcoma membranes (6). These similarities suggest that the TATA of these 2 MC-induced sarcomas may reside on the same or similar types of molecules. The TATA derived from the cytosol may also be related to the TSSA defined serologically since the most enriched TATA fraction prepared from gel filtration Sephacryl S-200 also partially inhibited the cytotoxicity of αMeth A. The TSSA antigen is restricted to the ascites and solid forms of Meth A sarcoma, and to tissue culture lines derived from Meth A. The TATA is likewise restricted to the ascites, solid forms, and tissue culture lines derived from Meth A. In addition, the TATA of Meth A is unique and is non-cross-reactive with any other MC-induced BALB/c tumor. Definite conclusions, however, as to the identity of TATA and TSSA antigens must await purification and biochemical characterization.

ACKNOWLEDGMENTS

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REFERENCES


Table 2

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<th>Group</th>
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<th>No. challenged</th>
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* See Table 1, Footnotes a, b, and c.

† Numbers in parentheses, tumor volume (cu mm).

* The difference between the frequency of takes in the 20- to 40-μg-immunized groups and controls is significant at p < 0.01.

† The 50% tumor lethal dose of CI-4 is 10^3 cells.

Chart 2. Absorption analysis of αMeth A (A) and αCMS4 (B) by tumor cells and by purified plasma membranes of Meth A sarcoma. Each antiserum was absorbed by its respective cell: O, Meth A; △, CMS4. The dilutions of purified plasma membranes were: O, undiluted; △, 1/10 dilution; A, 1/100 dilution; and V, 1/1000 dilution. Protein concentration of purified plasma membranes was 22.4 mg/ml.

Chart 3. Absorption analysis of αMeth A (A) and αCMS4 (B) by soluble cytosol fractions of Meth A sarcoma. The cytosol fractions are: A, Ultrogel AcA54, Fraction 2; O, Ultrogel AcA54, Fraction 1; △, Ultragel AcA54, Fraction 2. O, unabsorbed serum. Protein concentrations were 18.4 mg/ml for Sephacryl S-200 fraction and 5.7 and 2.1 mg/ml, respectively, for the AcA54 fractions.

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