MURINE MALIGNANT CELLS SYNTHESIZE A 19,000-DALTON PROTEIN THAT IS PHYSICOCHEMICALLY AND ANTIGENICALLY RELATED TO THE IMMUNOSUPPRESSIVE RETROVIRAL PROTEIN, P15E*

By GEORGE J. CIANCIOLO‡, MARK E. LOSTROM, MILTON TAM, and RALPH SNYDERMAN

From the Laboratory of Immune Effector Function, Howard Hughes Medical Institute, Division of Rheumatic and Genetic Diseases, Departments of Medicine, Microbiology, and Immunology, Duke University Medical Center, Durham, North Carolina 27710; and Genetic Systems Corporation, Seattle, Washington 98121

Mononuclear phagocytes can play an important role in restricting the growth of neoplastic cells (1–3), but this function depends upon their ability to localize rapidly at sites of neoplastic transformation. Depression of the intrinsic migratory capabilities of mononuclear phagocytes might very well serve to potentiate the growth or metastasis of tumor cells. Several years ago the observation was made that patients with a variety of neoplastic diseases had depressed monocyte chemotactic responses (4–6) and that these depressed responses were often reversed by surgical removal of the tumor or by immunotherapy (7, 8). These findings led to development of murine models with which it was shown that implantation of a variety of transplantable tumor lines into normal mice resulted in dramatic inhibition of their ability to accumulate macrophages at inflammatory foci (9, 10), to resist bacterial infection (11), and to develop delayed-type hypersensitivity responses (12). A major portion of the inhibitory activity found associated with these tumors was contained in low molecular weight extracts prepared by sonication and ultrafiltration of the tumor cells (9, 10). It was later shown that such activity could not be attributed to contamination with lactate dehydrogenase virus (13, 14) and that not only the tumors but the plasma and urine of mice bearing spontaneous mammary adenocarcinomas (15) or spontaneous lymphomas (16) were able to inhibit macrophage accumulation when injected into normal mice.

In an effort to determine the source of the tumor-associated inhibitors of macrophage accumulation we examined various RNA tumor viruses and found that low molecular weight extracts prepared from certain oncogenic murine leukemia viruses (Friend, Moloney, Rauscher) inhibited macrophage accumulation when they were injected into normal mice (17). The purified retroviral envelope protein P15E had similar inhibitory activity as well as physicochemical characteristics in common with the inhibitor derived from tumor cells. This

* Supported in part by National Cancer Institute grant #PO1-CA-29589-02.

‡ To whom correspondence should be addressed at Box 3892, Duke University Medical Center, Durham, NC 27710.

suggested that P15E could be responsible for the inhibition of macrophage accumulation caused by the retroviruses and perhaps by tumors themselves.

We have now used metabolic labeling and monoclonal antibodies to examine six different murine tumor cell lines to determine whether they synthesize retroviral P15E or related proteins. In addition, using a newly developed competition ELISA assay for P15E, we have examined a spontaneous mammary adenocarcinoma and a primary 20-methylcholanthrene (MCA)[1]-induced fibrosarcoma as well as the ascites fluid of tumor-bearing mice for the presence of P15E-related antigens. We have also determined whether the inhibitors of macrophage accumulation contained in primary murine tumors are antigenically related to P15E.

Materials and Methods

Mice. Male C3H/HeN mice (MMTV-) were obtained from Charles River Kingston, Stone Ridge, NY. Male C3H/FeJ mice were obtained from Jackson Laboratories, Bar Harbor, ME.

Reagents. Double density-gradient purified, Rauscher leukemia virus (RLV; 1.7 mg/ml; 9.3×10^{11} viral particles/ml) and murine mammary tumor virus (MMTV; 1.2 mg/ml; 2.0×10^{11} viral particles/ml) were purchased from Electro-Nucleonics Laboratories, Inc., Bethesda, MD. Tris (hydroxymethyl) aminomethane, deoxycholic acid (DOC), Triton X-100, 20-methylcholanthrene (MCA), polyoxyethylene sorbitan monolaurate (Tween 20), guanidine HCl, bovine serum albumin Fraction V (BSA) and bovine serum albumin radioimmunoassay grade (RIA BSA) were all purchased from Sigma Chemical Co., St. Louis, MO. Sodium dodecyl sulfate (SDS), 2-mercaptoethanol (2ME), ammonium persulfate, acrylamide, N,N′-methylene-bis-acrylamide (BIS), dithiothreitol (DTT) and Bio-Lyte carrier ampholytes were purchased from Bio-Rad Laboratories, Richmond, CA. Glycerol, N,N,N′,N′-Tetramethylethylenediamine (TEMED), glycine, Nonidet P40 (NP40), and Coomassie Brilliant Blue R-250 were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, MD. Bromphenol Blue was obtained from Fisher Scientific, Fair Lawn, NJ, urea from MCB, Norwood, OH, and gelatin from Nutritional Biochemicals Corp., Cleveland, OH.

Cells. Tumor cell lines used included the Hep 129 hepatocarcinoma and BP8 fibrosarcoma that were originally induced by MCA. Three variants (F1, F10, and BL6) of the B16 melanoma were kindly provided by I. J. Fidler of the Frederick Cancer Research Center, and the RL1 lymphoma was provided by C. Whisnant of Duke University. Friend leukemia virus (FLV) producing Eveline cells (fibroblasts) were provided by A. Langlois of Duke University. Nonmalignant cell lines were the STO and 3T3/BALB fibroblast cell lines from the American Type Culture Collection, Rockville, MD, and the 3T3/L1 fibroblast cell line provided by S. Gidwitz of Duke University. Single cell suspensions of normal splenocytes were derived from a male C3Heb/FeJ mouse.

Antibodies. Ascites fluids containing monoclonal antibodies to two different epitopes of the murine retrovirus structural protein P15E were obtained from male $CB6F_1/J$ mice (Jackson Laboratories, Bar Harbor, ME) injected intraperitoneally (i.p.) with 10^7 cultured

[1] *Abbreviations used in this paper:* ABS, absorbance value; APT, aminophenylthioether; BIS, N,N′-methylene-bis-acrylamide; BSA, bovine serum albumin; Con A, concanavalin A; DOC, deoxycholic acid; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FLV, Friend leukemia virus; MCA, 20-methylcholanthrene; 2ME, 2-mercaptoethanol; MMTV, murine mammary tumor virus; MuLV, murine leukemia virus; NDS detergent, 1.0% Nonidet P40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate, 0.4 M sodium chloride, and 0.02 M phosphate, pH 8.0; NGS, normal goat serum; NMS, normal mouse serum; NP40, Nonidet P40; NRS, normal rabbit serum; PHA, phytohemmagglutinin; RIA BSA, radioimmunoassay grade BSA; RLV, Rauscher leukemia virus; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TEMED, N,N,N′,N′-tetramethylethylenediamine; TMB, 3,3′,5,5′-tetramethylbenzidine; Tween 20, polyoxyethylene sorbitan monolaurate; VPE, viral particle equivalents.

hybridoma cells 4 d after a priming injection of 0.5 ml of pristane (2, 6, 10, 14-tetramethylpentadecane; Aldrich Chemical Co., Milwaukee, WI). The two antibodies are designated 19F8 and 4F5 and have IgG_{2b} and IgG_{2a} isotypes respectively (18–20). The ascites fluids for both lines contain ~2.5–4.0 mg specific antibody per ml as the parent myeloma line, BALB/c MOPC21 NS1/1, secretes only light chains. Equal volumes of the two anti-P15E ascites fluids were combined as this technique enhances antibody affinity (21). Both monoclonal anti-P15E isotypes bind *Staphlococcus aureus* (*S. aureus*) protein A. A control monoclonal antibody ascites fluid used was 4F2, an IgG_{2a} antibody that reacts with human and murine cell lines and with mitogen-stimulated lymphoid cells and was kindly supplied by B. Haynes of Duke University.

Rabbit antiserum to P15E was prepared using monoclonal antibody affinity-purified antigen from disrupted RLV. Normal rabbit serum (NRS) was obtained by bleeding the rabbits before the initial immunization. Rabbit antiserum to GP70 was kindly supplied by K. Weinhold of Duke University and rabbit antiserum to goat IgG was obtained from Miles Laboratories, Elkhart, IN. Pooled normal mouse serum (NMS), containing ~3–4 mg/ml of IgG, was obtained from male C3H/HeN mice. Goat antibody to RLV reverse transcriptase was obtained from the Biological Carcinogenesis Branch of the National Cancer Institute. Goat antibodies to RLV GP69/71 and P30 were obtained from the Viral Oncology Branch of the National Cancer Institute. Normal goat serum (NGS) was obtained from Gibco Laboratories, Grand Island, NY.

[^{35}S]Methionine Labeling of Cells. Nonadherent cells were washed once in RPMI 1640 medium (Gibco Laboratories Grand Island, NY) containing 1.0 μM L-methionine and dialyzed 10% fetal bovine serum (FBS; Gibco) (labeling medium). ~2–5 × 10^7 cells were then suspended in 4 ml of labeling medium containing 0.5 mCi of [^{35}S]methionine (>1,000 Ci/mmol; New England Nuclear, Boston, MA), incubated 2 h at 37°C in a humidified 5% CO_2 atmosphere, washed once, resuspended in RPMI 1640 containing 10% FBS and 0.1 mM L-methionine (complete medium), and incubated an additional 2 h at 37°C in humidified 5% CO_2. The cells were then washed twice with cold FBS-free RPMI 1640, the cell pellet resuspended in 2 ml of NDS detergent buffer (1% NP-40, 0.5% DOC, 0.1% SDS, 0.4 M NaCl, 0.02 M PO_4, pH 8.0), and the detergent extract sonicated. The extract was centrifuged for 20 min at 27,000 *g* and 4°C, the supernatant removed and the radioactivity determined. Labeling in this manner usually resulted in ~3–4 × 10^8 cpm of labeled extract, ~95% of which were trichloroacetic acid precipitable. 1 ml of formalin-fixed *S. aureus* (Pansorbin, 7% wt/vol; Calbiochem-Behring Corp., La Jolla, CA) was added for 30 min at 37°C, the Pansorbin pelleted by centrifugation at 40,000 *g* and 4°C for 30 min, the supernatant removed, and the radioactivity determined. This preclearing step routinely removed <5% of the radiolabeled proteins.

Adherent cells (B16, STO, and 3T3 lines) were grown to near confluence, washed twice with labeling medium and then overlaid with 4 ml of labeling medium containing 0.5 mCi of [^{35}S]methionine. The flasks were incubated for 2 h at 37°C in humidified 5% CO_2, the labeling medium removed, the adherent cells washed twice with complete medium, and incubated an additional 2 h at 37°C with 12 ml of complete medium. The adhered cells were washed three times with cold FBS-free RPMI 1640, and then solubilized with 2 ml of NDS detergent buffer.

Mitogen-stimulated Spleen Cells. 1 ml volumes of normal splenocytes at 2 × 10^6 cells/ml in complete medium were incubated for 48 h at 37°C in humidified 5% CO_2 with either Phytohemagglutinin (PHA; 0.5 μg/ml; Wellcome Reagents Limited, Beckenham, England) or concanavalin A (Con A; 1.0 μg/ml; Miles Laboratories, Inc.). The PHA- or Con A-stimulated cells were separately pooled, washed in labeling medium and 2–3 × 10^7 viable cells labeled. Cell viability was >70% by trypan blue dye exclusion.

Radioimmunoprecipitation of Cell Extracts. Cell extracts (~150–300 μl) containing 25 × 10^6 cpm were incubated overnight at 4°C with 10 μl of either anti-P15E, 4F2 antibody, or NMS. 50 μl of Pansorbin was added to each extract for an additional 3 h at 4°C, the Pansorbin pelleted by centrifugation and the pellet washed four times with NDS. The pellet was then resuspended in 150 μl of SDS sample buffer (0.125 M Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 10% 2ME; 0.002% bromphenol blue), allowed to stand 30 min at

room temperature, the Pansorbin pelleted by centrifugation at 12,000 *g* and 4°C for 15 min, the supernatant removed, and the radioactivity determined. Immunoprecipitations with goat antibodies to viral proteins were performed in a similar manner except that 10 μl of rabbit antiserum to goat IgG was added for an additional overnight incubation and 100 μl of Pansorbin was used to bind immune complexes.

SDS-Polyacrylamide Gel Electrophoresis (PAGE). SDS-PAGE gels were run using the method of Laemmli (22) and nonlinear 5–15% gradients. Stained gels were enhanced for autoradiography (EN^{3}HANCE; New England Nuclear) and dried under vacuum using gentle heat. X-Omat AR film (Eastman Kodak, Rochester, NY) was used for fluorography of the dried gels.

Two-dimensional Gel Electrophoresis. [^{35}S]Methionine-labeled extracts of the F10 and BL6 variants of the B16 melanoma and ^{125}I-labeled disrupted RLV were immunoprecipitated with monoclonal anti-P15E or NMS and Protein-A Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). The bound proteins were eluted with 2% SDS and 5% 2ME, adjusted to 4% Triton X-100 and 0.8 M urea, and isoelectric focused for 14 h at 300 V in 12-cm tube gels composed of 4.5% acrylamide, 4% Triton X-100, 8 M urea, and 2% ampholytes (pH 3–10). The focused gels were then electrophoresed on a nonlinear 5–15% gradient SDS slab gel as described above, the slab gels enhanced for fluorography and put up for autoradiography.

SDS-PAGE and Western Blotting of Viral Proteins. Purified RLV was detergent-disrupted and 50 μg of viral protein electrophoresed in each sample lane of a 5–15% nonlinear gradient gel. The proteins were then electrophoretically transferred to activated APT paper (2-aminophenylthioether; Bio-Rad Laboratories) at 4°C for 14 h using 3 V/cm of distance across the electrodes. The blotted proteins were incubated for 1 h at room temperature with 50 ml of either mouse monoclonal or rabbit anti-P15E (1:1,000 in Tris gelatin buffer [0.15 M NaCl, 0.005 M EDTA, 0.05 M Tris-HCl, 0.25% gelatin, 0.05% NP-40, 0.1% BSA, pH 7.4]) or with 50 ml of either NMS or NRS (1:1,000 in Tris-gelatin buffer), washed four times for 15 min with 50 ml of Tris-gelatin buffer, and incubated for 1 h at room temperature in freshly iodinated *S. aureus* protein A (specific activity ~5 μCi/μg) at a concentration of 10^6 cpm/ml in Tris-gelatin buffer. They were then washed, dried, and put up for autoradiography using an intensifying screen.

Detergent-solubilized Cell Extracts. Hep 129, BP8, and the three variants of the B16 melanoma were solubilized at 10^8 cells/ml using NDS detergent buffer. A single cell suspension prepared from a spontaneous mammary adenocarcinoma from a female C3H/HeN mouse was adjusted to 10^8 cells/ml and solubilized. Liver tissue from a sex- and age-matched mouse was homogenized using a tissumizer (Tekmar Co., Cinncinnati, OH) and adjusted to the same packed volume as the mammary adenocarcinoma cells before their solubilization. Fibrosarcomas were induced in the right thighs of 6–8-wk old male C3H/HeN mice by the subcutaneous injection of 5 mg of MCA in 0.5 ml of olive oil. Control mice were injected with the olive oil alone. Tumors developed 8–12 wk after injection, When the tumors were 1.0–1.5 cm in diameter they and control tissues (liver, kidney, spleen) were removed from three tumor-bearing animals. Muscle tissue from the olive oil injected leg and liver, kidney, and spleen were removed from three control mice. The different tissues from either the tumor bearing or control mice were standardized to 200 mg wet weight per ml of NDS detergent buffer, homogenized, sonicated, centrifuged for 20 min at 27,000 *g* and 4°C, and the supernatants removed. All tissue and cell extracts were negative for protease activity using a casein hydrolysis method (Bio-Rad Laboratories).

Competition ELISA Assay for P15E. Material to be tested for P15E (see below) was diluted in NDS detergent buffer. Ascites fluids were first diluted 1:1 with 2 × NDS and then further diluted in 1 × NDS. Diluted samples were mixed 1:1 (vol/vol) with rabbit anti-P15E (1:20,000 in 0.05 M PO_4-saline, pH 7.5, containing 1% RIA BSA and 0.5% Tween 20) or buffer and incubated overnight at 4°C. The control was rabbit anti-P15E (1:20,000) diluted 1:1 with NDS. 50 μl of samples to be tested were then added to each of duplicate wells of a RLV-coated polystyrene plate. The plate was incubated 1 h at 37°C, washed 5× with wash buffer (0.05 M PO_4-saline, pH 7.5, containing 0.5% Tween

20), and 50 μl per well of peroxidase-conjugated goat anti-rabbit IgG (1:3,000 in wash buffer containing 1% RIA BSA) (Tago, Inc., Burlingame, CA) was added for 1 h at 37°C. The plate was washed 5× and 50 μl per well of peroxidase-conjugated swine anti-goat IgG (1:3000; Tago) was added for 1 h at 37°C. The plate was washed 5× and 200 μl per well of TMB substrate (3,3′,5,5′-tetramethylbenzidine; Litton Bionetics, Kensington, MD) in 0.1 M citric acid, pH 4.0, containing 0.3% H_2O_2 was added. The plate was allowed to sit at room temperature to allow color development, and read on a Titertek Multiskan automated plate reader (Flow Laboratories, McLean, VA) with a 405-nm filter. Each plate was read several times during the course of color development. Color development was linear to ~0.5 absorbance units so assays were terminated when the absorbance (ABS) of the positive controls (antibody plus NDS) was ~0.3. Percent inhibition of rabbit anti-p15E binding was calculated as

$$\% \text{ Inhibition} = \frac{\text{ABS}_{405} \text{ Control} - \text{ABS}_{405} \text{ Sample}}{\text{ABS}_{405} \text{ Control}} \times 100.$$

The absorbance values for samples incubated with antibody were determined after subtracting any absorbance generated by samples incubated with buffer only. These latter values never exceeded 10% of the absorbance values of antibody plus sample and generally were <5%. A standard curve was established for inhibition of anti-P15E binding by detergent disrupted RLV using data from four separate experiments. Values for inhibition of binding by cell extracts were compared to the RLV standard curve and expressed as the number of viral particles giving similar inhibition. The values for all experimental samples were calculated from the dilution of sample giving 50% inhibition of rabbit anti-P15E binding. In 50 sets of duplicates examined, the average standard deviation for the mean was <8.4%.

Macrophage Accumulation Inhibition Assay. Macrophage accumulation 2 d after an i.p. injection of 35 μg of PHA was determined for tumor extract treated or control mice as previously described (15). Statistical significance was determined using Student's *t*-test.

Results

Specificity of Murine Monoclonal and Rabbit Polyclonal Anti-P15E. To verify the specificity of the monoclonal anti-P15E, the antibody mixture was reacted with electroblotted proteins of P15E-containing RLV, followed by incubation with ^{125}I-*S. aureus* protein A and autoradiography. Two distinct bands, corresponding to calculated molecular weights of 19,000 and 17,000 daltons, were detected by the anti-P15E (Fig. 1). These bands represent P15E and its degradation product, P12E (23). No reactivity was detected on the blot exposed to NMS. The same pattern of reactivity was seen using either monoclonal antibody alone (not shown) so all further experiments were performed using the combined (γ_{2a} and γ_{2b}) antibodies.

The rabbit antibody to P15E also reacted primarily with the 19,000- and 17,000-dalton protein bands detected by the murine monoclonal anti-P15E (Fig. 1). The reactivity of a rabbit antibody to retroviral GP70 is shown in Fig. 1, lane *E*, in order to verify that the higher molecular weight viral proteins were successfully transferred by the blotting techniques used. No reactivity was detected on the blot exposed to NRS. The enhanced sensitivity obtained by use of a polyclonal antiserum is illustrated by the fact that the autoradiograph of the rabbit anti-P15E required only 30 min of film exposure while that of the monoclonal anti-P15E required 16 h of exposure for comparable intensity.

Radioimmunoprecipitation of Cancerous or Normal Cells with Monoclonal Anti-P15E. Cells that had been metabolically labeled with [^{35}S]methionine were

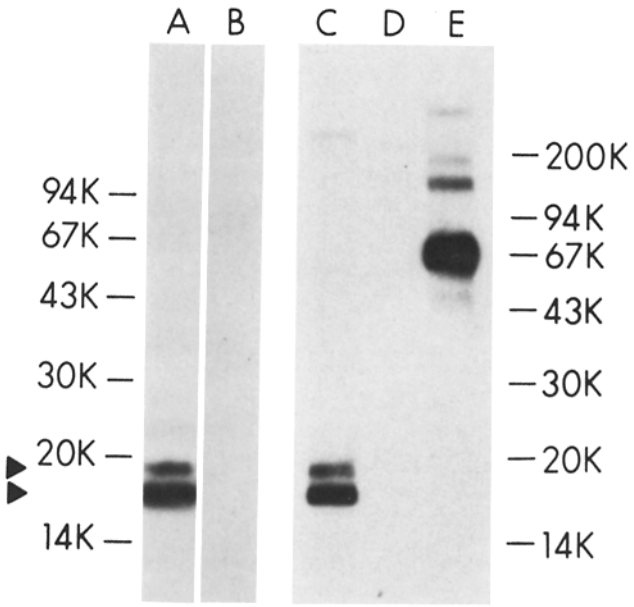

FIGURE 1. Specificity of murine monoclonal and rabbit polyclonal antibodies to P15E. Detergent-disrupted RLV proteins were separated using SDS-PAGE, electrophoretically transferred to APT paper, the paper cut into strips, and the strips incubated with (*A*) murine monoclonal anti-P15E, (*B*) normal mouse serum, (*C*) rabbit polyclonal anti-P15E, (*D*) normal rabbit serum, and (*E*) rabbit anti-GP70. The strips were incubated with ^{125}I-Protein A and then exposed for autoradiography. *K*, kdaltons.

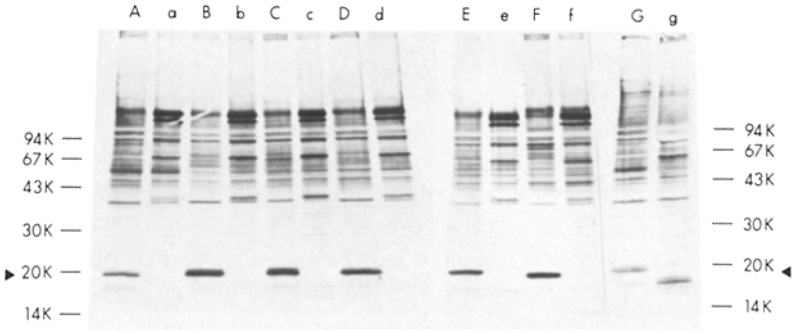

FIGURE 2. Radioimmunoprecipitation of [^{35}S]methionine-labeled cultured murine tumor cells. The following cell lines were labeled, extracted, and immunoprecipitated with monoclonal anti-P15E (*A–G*) or monoclonal 4F2 (*a–g*): Hep 129, $B16_{BL6}$, $B16_{F1}$, $B16_{F10}$, RL1, Eveline, and BP8.

detergent extracted and immunoprecipitated with anti-P15E and formalin-fixed *S. aureus*. The immunoprecipitated proteins were analyzed by SDS-PAGE followed by gel fluorography. Control antibodies used were monoclonal 4F2 or NMS. As shown in Fig. 2, lane *F*, Eveline cells, which are FLV-producing fibroblasts, synthesize a 19,000-dalton protein corresponding to P15E. Because these cells are actively producing viral particles this result was expected. Fig. 2 also shows that the Hep 129, BP8, RL1, and three B16 melanoma variants also produced the 19,000-dalton band corresponding to P15E. The same number of

radioactive counts (80K) was applied to each of the lanes in Fig. 2. The amounts of radioactivity precipitated by anti-P15E and monoclonal 4F2 for the Eveline and tumor cell lines were essentially the same for each cell type. The radioactivity precipitated by NMS was approximately one-third to one-sixth of that precipitated by anti-P15E or 4F2. In other experiments not shown, gels containing the same amounts of radiolabeled counts from anti-P15E and NMS precipitates demonstrated that the 19,000-dalton band was specific for anti-P15E. Because the lower amounts of radioactivity associated with NMS precipitates necessitated much longer fluorography times these samples were not routinely run on the gels. The precipitates obtained with 4F2 are shown to illustrate the proteins that are precipitated in reactions involving a monoclonal antibody that recognizes determinants other than P15E. All of the cell lines shown in Fig. 2 except RL1 were examined by electron microscopy for the presence of type C viral particles. Viral particles were readily detectable in the FLV infected Eveline cells whereas none were found in the other five cell lines examined (data not shown). In addition, autoradiograms of gels of the three B16 cell lines that had been immunoprecipitated with goat antibodies to RLV reverse transcriptase, GP69/71 or P30 did not indicate the presence of any of these proteins in the malignant cells (data not shown).

Fig. 3 illustrates autoradiograms of immunoprecipitated extracts from normal or mitogen-stimulated splenocytes or from three different nontransformed murine fibroblast cell lines. None of the nonmalignant cells contained sufficient amounts of radiolabeled P15E to be detected by immunoprecipitation using monoclonal anti-P15E. The same number of radioactive counts (70K) was applied to each lane for all three splenocyte samples but the number of counts precipitated from the fibroblast extracts made it necessary to standardize these samples to 30K per lane and use a correspondingly longer autoradiography developing time. For reference purposes, lane *A* of Fig. 3 contains the radiolabeled immunoprecipitate from a $B16_{BL6}$ extract incubated with monoclonal anti-P15E.

Inhibition of Immunoprecipitation of Tumor Cell-Associated P15E by RLV Extract. To verify that the 19,000-dalton protein precipitated from tumor cell

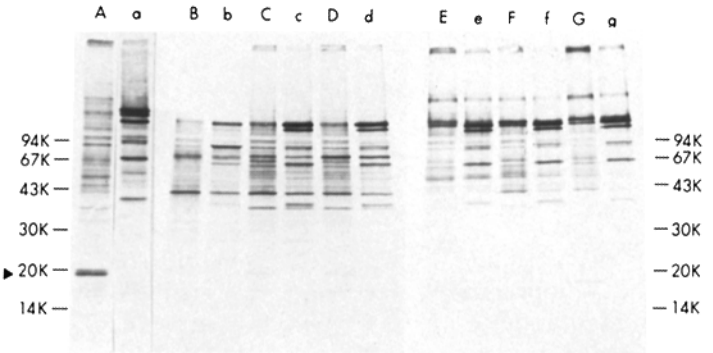

FIGURE 3. Radioimmunoprecipitation of [^{35}S]methionine-labeled cultured murine splenocytes and fibroblasts. The following cells were labeled, extracted, and immunoprecipitated with monoclonal anti-P15E (*A–G*) or monoclonal 4F2 (*a–g*): $B16_{BL6}$, unstimulated cultured splenocytes, PHA stimulated splenocytes, Con A-stimulated splenocytes, STO, 3T3/BALB, and 3T3/L1 fibroblasts.

extracts was antigenically related to P15E, we immunoprecipitated a [^{35}S]methionine-labeled extract of $B16_{BL6}$ melanoma cells with anti-P15E that had been preincubated for 30 min at 37°C with either buffer, 800 μg of detergent-disrupted RLV, or 800 μg of detergent-disrupted MMTV. Preincubation of anti-P15E with buffer or MMTV, a virus that does not contain P15E (24), had no effect on its ability to precipitate the 19,000-dalton protein from the B16 extract (Fig. 4). However, preincubation of the anti-P15E with P15E containing RLV prevented the precipitation of most of the 19,000-dalton protein synthesized by the tumor cells.

Two-dimensional Gel Electrophoresis of Tumor and Viral Anti-P15E Precipitates. Anti-P15E immune precipitates from $B16_{F10}$ and $B16_{BL6}$ [^{35}S]methionine-labeled extracts and ^{125}I-labeled disrupted RLV were separated by isoelectric focusing in a pH 3–10 gradient and then subjected to electrophoresis into a nonlinear 5–15% gradient SDS slab gel. As shown in Fig. 5, both the tumor-derived and viral precipitates contained proteins of ~19,000 daltons that focused between pH 7.4 and pH 8.4. Precise comparison of isoelectric points cannot be made however, since the RLV and tumor extracts, by necessity, were not radiolabeled with the same isotope. Nonetheless, the strong similarity between the tumor and virus profiles suggest that the 19,000-dalton proteins present in both are not only antigenically related but physicochemically similar as well.

Quantification of P15E Associated with Cancerous Cells or Ascites. Cell extracts were prepared from Eveline, Hep 129, BP8, three variants of the B16 melanoma, and a spontaneous mammary adenocarcinoma and were tested for the presence of P15E antigen using a competition ELISA assay. For comparative purposes, the inhibition of anti-P15E binding by cell extracts or ascites is given as viral particle equivalents (VPE); i.e., the number of detergent-disrupted RLV particles giving similar inhibition. As previously stated however, except for the Eveline cells, the tumor lines used were free of any detectable viral particles. The results shown in Table I indicate that the cultured murine tumor cells contained material

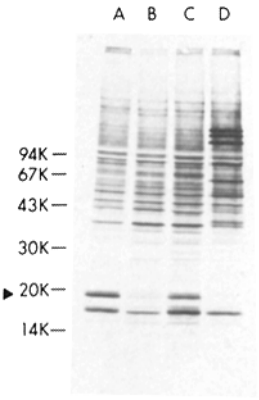

FIGURE 4. Inhibition of precipitation of [^{35}S]methionine-labeled P15E from $B16_{BL6}$ cells by disrupted RLV. Extract prepared from [^{35}S]methionine-labeled $B16_{BL6}$ cells was incubated with monoclonal anti-P15E that had been preincubated with either (*A*) buffer, (*B*) disrupted RLV, or (*C*) disrupted MMTV. Lane *D* is the precipitate obtained from the $B16_{BL6}$ extract incubated with monoclonal 4F2 and is shown to illustrate the non-P15E related binding.

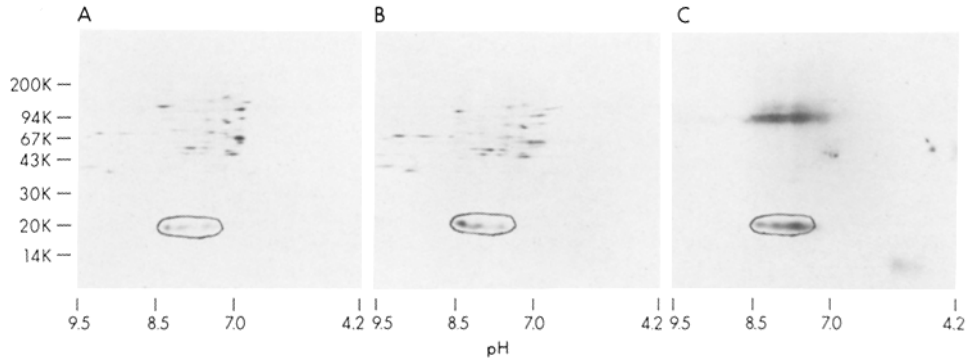

FIGURE 5. Two-dimensional gel electrophoresis of anti-P15E immunoprecipitates of [^{35}S]-methionine-labeled $B16_{F10}$ (*A*), $B16_{BL6}$ (*B*), or of ^{125}I-labeled detergent disrupted RLV (*C*). The isoelectric focusing and electrophoresis in the second dimension were carried out as described in the text.

TABLE I

Quantification of P15E Associated With Murine Neoplasms

Source of material*	Viral particle equivalents‡
Eveline cells	2.4×10^{10}
Hep 129 cells	4.4×10^{9}
$B16_{F1}$ cells	3.3×10^{9}
$B16_{F10}$ cells	2.5×10^{9}
BP8 cells	2.0×10^{9}
Spontaneous mammary tumor cells	1.8×10^{9}
Hep 129 ascites	6.8×10^{8}
$B16_{BL6}$ cells	5.9×10^{8}
Liver	None detected§
Control ascites	None detected
Normal mouse serum	None detected
Protein A purified immunoglobulins‖	None detected

* Cells standardized to 10^8 cells/ml and solubilized in NDS detergent.
‡ Number of detergent-disrupted RLV particles giving equivalent inhibition of rabbit anti-P15E binding per milliliter of extract.
§ Lower limit of detection $\sim 3 \times 10^7$.
‖ Purified from 4F2 ascites fluid.

reactive with anti-P15E. The virus-producing cell line, Eveline, contained at least five times as much P15E antigen as the other cell lines tested. The spontaneous mammary adenocarcinoma cells contained at least 20 times as much P15E as liver tissue that had $<3.0 \times 10^7$ VPE, the lower limit of detection for our assay. Cell-free ascites fluid from C3H/HeN mice injected 7 d earlier with 1×10^6 Hep 129 cells contained an amount of P15E antigen per milliliter of ascites fluid that would be equal to that found in $\sim 15 \times 10^6$ Hep 129 cells. Since each mouse contained ~5–6 ml of ascites fluid the amount of P15E recovered would be equivalent to ~ 75–90×10^6 Hep 129 cells. We estimate from previous studies that the number of Hep 129 cells in the peritoneal cavity at this time would be $\sim 150 \times 10^6$. Noncancerous ascites fluid, which was obtained from the peritoneal cavities of C3H/HeN mice injected 7 d earlier with 1 ml of complete Freund's

TABLE II

Quantification of P15E Associated With Methylcholanthrene-induced Fibrosarcomas

Tissue*	Viral particle equivalents‡
I. *Tumor-bearing mice*	
Fibrosarcoma	1.4×10^{10}
Liver	None detected§
Kidney	None detected
Spleen	None detected
II. *Control (olive oil-injected) mice*	
Muscle	None detected
Liver	None detected
Kidney	None detected
Spleen	None detected

* Tissue standardized to 200 mg wet weight/ml and solubilized.

‡ Number of detergent disrupted RLV particles giving equivalent inhibition of rabbit anti-P15E binding per ml of extract.

§ Lower limit of detection $\sim 3 \times 10^7$.

TABLE III

*Effect of Monoclonal Anti-P15E on Inhibitory Activity of Tumor Extracts**

Expt	Mice injected with:	Accumulated Mϕ ($\times 10^6$ + SEM)	Percent inhibition	*P*
1	Buffer	6.6 ± 0.8		
	Tumor extract	1.2 ± 0.2	82	<0.001‡
	Spleen extract	7.1 ± 0.9	0	>0.6‡
	Tumor extract absorbed with anti-P15E	4.5 ± 0.4	32	<0.001§
2	Buffer	1.9 ± 0.1		
	Tumor extract	0.3 ± 0.1	84	<0.001‡
	Tumor extract absorbed with anti-P15E	1.8 ± 0.3	5	<0.001§
	Tumor extract absorbed with IgG_{2a}	0.5 ± 0.1	74	>0.3§

* Extracts were incubated with buffer or buffer containing antibody for 1 h (expt. 1) or 3 h (expt. 2) at 4°C, absorbed with Protein A Sepharose, and the extracts then injected into the thighs of five normal C3H/HeN mice which were 30 (expt. 1) or 5 (expt. 2) wk old. Peritoneal macrophage accumulation in response to 35 μg of PHA was calculated as previously described (15).

‡ Compared with buffer control.

§ Compared with tumor extract.

adjuvant and which contained ~33% more protein than the Hep 129 ascites, had no detectable levels of P15E. Normal mouse serum, adjusted to the same protein concentration as the Hep 129 ascites, and protein A-purified immunoglobulins from a 4F2 ascites were also both negative for P15E.

MCA-induced primary fibrosarcomas and various control tissues were examined for the presence of P15E using the competition ELISA assay. The carcinogen-induced tumor tissue contained the equivalent of 1.4×10^{10} RLV particles per milliliter of tissue while the control tissues from both tumor-bearing and

control mice were below the limit of detection (Table II).

Effect of Monoclonal Anti-P15E on Macrophage Inhibitory Activity of MCA-induced Fibrosarcomas. Extract prepared from 40 μg wet weight of MCA induced fibrosarcoma was incubated for 1 or 3 h at 4°C with either buffer, monoclonal anti-P15E (8 ng), or myeloma immunoglobulins of the same isotype and concentration. Immunoglobulin was removed by Protein A Sepharose and the extracts injected into the thighs of normal mice, followed 24 h later by an i.p. injection of PHA. As shown in Table III, tumor extract incubated with buffer alone inhibited macrophage accumulation by 82–84%. Extract prepared from the spleens of the tumor bearing animals did not inhibit macrophage accumulation. Incubation of the tumor extract with monclonal anti-P15E removed 61% of the inhibitory activity in experiment 1 and 94% of the inhibitory activity in experiment 2. The lower number of accumulated macrophages in experiment 2 can be attributed to the younger age (5 w) of mice used in this experiment. Incubation of the tumor extract with myeloma IgG_{2a} had no significant effect on the tumor associated inhibitory activity.

Discussion

We have previously shown that murine tumor cells and certain oncogenic murine viruses contain low molecular weight factors capable of inhibiting macrophage accumulation at inflammatory foci (9, 10, 13, 15, 17). The inhibitory activity of the oncogenic viruses was found to reside in the retroviral envelope protein P15E (14). Of interest were the similar physicochemical characteristics, of P15E and the antiinflammatory agent from tumor cells, in regards to molecular size and charge (25). We therefore determined whether murine tumor cells contained P15E. Using metabolic labeling followed by immunoprecipitation with monoclonal antibodies, we have now shown that all six different murine tumor cell lines examined synthesize a protein that is antigenically related to retroviral P15E. Immunoprecipitation of the P15E-related protein was blocked by preincubation of monoclonal anti-P15E with detergent-disrupted RLV, a virus known to contain P15E (19). In contrast, preincubation of the anti-P15E with non-P15E containing MMTV had no effect. The immunoprecipitated P15E-related protein was found only in malignant cells; unstimulated and mitogen-stimulated splenocytes as well as three different murine fibroblast cell lines were all negative. Furthermore, in the two malignant cell lines examined, the tumor associated 19,000-dalton proteins showed a pattern in isoelectric focusing similar to that of RLV P15E, suggesting that the tumor and viral proteins were not only antigenically but physicochemically similar. Thus many commonly used tumor cell lines synthesize a protein that is at least very similar, if not identical with, the immunosuppressive protein P15E. Type C viral particles were not detected by electron microscopy in our cultured tumor lines although they were readily seen in virally infected Eveline cells. We cannot rule out the possibility, however that the tumor cell lines contain a lower number of C-type viruses than are detectable by electron microscopy. However, immunoprecipitation of [^{35}S]methionine-labeled extracts of the three B16 melanoma lines with goat antibodies to RLV reverse transcriptase, GP69/71, or P30 did not result in specific precipitation of these proteins from the tumor extracts. Under identical conditions these same

antibodies precipitated the appropriate proteins from ^{125}I-labeled RLV. Thus we consider it highly unlikely that the synthesis of the P15E related protein is associated with viral particle formation. In any case, investigators must be cognizant of the probability that murine tumor lines routinely synthesize a protein antigenically related to an immunosuppressive viral protein even in the absence of detectable virus particles.

The possibility that oncogenic transformation in vivo leads to the expression of a retroviral related protein is confirmed by the data showing expression of a P15E-related antigen in both a spontaneous mammary adenocarcinoma and a carcinogen induced-primary fibrosarcoma. The use of metabolic labeling and immunoprecipitation to identify this antigen in these tissues was precluded by the poor viability of cells obtained from dissected tumors and their relatively low incorporation of the methionine label. The development of a sensitive competition ELISA assay for P15E utilizing a polyclonal rabbit antiserum did, however allow the quantification of anti-P15E reactive material not only in the tumors, but in a variety of other tissues. Although the limits of the assay prevented the conclusion that noncancerous tissues contained no P15E related antigen, the data indicates that neoplastic tissues contain substantially higher levels of this protein.

Previously, investigators have reported finding type C virus particles in some cell lines (26, 27) as well as in transplanted tumors (28, 29). Other investigators have shown that retroviruses or retroviral proteins can be induced in a variety of cultured mouse cell lines by chemical mutagens such as 5-azacytidine (30) or by mitogens combined with 5-bromo-2′-deoxyuridine (31) and that messenger RNAs corresponding to retroviral genes can be elicited from carcinogen- or radiation-transformed fibroblasts (32). Fischinger et al. (33) have recently reported the expression of a unique surface murine leukemia virus recombinant-like glycoprotein on a virus-free, X ray-induced lymphoma. The mechanisms leading to the synthesis of the 19,000-dalton, P15E-related protein by the tumor cells examined in the present study were not determined, but intact viruses were seen only in the FLV-infected Eveline cells. The inability to detect other viral proteins suggests that this protein, although antigenically and physicochemically similar to RLV P15E, may be cellular in origin. Although we did not examine the culture supernatants of the various cell lines to determine whether they were secreting as well as synthesizing P15E related proteins, the data showing such materials in the ascites fluid of mice injected intraperitoneally with Hep 129 cells suggests that this occurs in vivo.

The demonstration in common laboratory tumor cell lines of an immunosuppressive, virally related protein is certainly of interest, but of greater importance is the finding of high levels of these proteins in primary tumors arising in vivo. We have previously postulated that tumors might subvert immune surveillance through the release of soluble antiinflammatory factors and that such factors might play their most important role during the early stages of tumor growth (9, 10, 13). Our previous studies indicated that as little as 2 ng of disrupted RLV could inhibit macrophage function in normal mice (17). The present studies have found the equivalent of more P15E antigen than that derived from 100 ng of whole RLV per mg of tumor tissue in carcinogen-induced primary tumors.

Furthermore, 40 μg wet weight of such tissue inhibited macrophage accumulation in normal mice by >80%. The inhibitory activity was almost completely removed by absorption with monoclonal antibody to P15E, suggesting that the P15E related tumor proteins are also immunosuppressive as we have previously shown for retroviral P15E (17). Thus, it is conceivable that even at the earliest stages of neoplastic growth enough viral P15E or its cellular counterpart is released to affect immune function either systemically or locally. While our previous studies have shown that P15E inhibits macrophage function, others have demonstrated that it also inhibits lymphoproliferative responses in cats and man (34) and disrupted RLV has been shown to suppress the Con A response of murine lymphocytes (35). Thus the production of this or a closely related protein by malignant cells might allow them to curtail many immunological defenses against tumor cells.

Recently, we made the observation that human cancerous effusions contained low molecular weight factors that inhibited the responses of normal monocytes to chemotactic stimuli (36). Interestingly, any of three different monoclonal antibodies to P15E absorbed the inhibitory activity from these cancerous fluids (36). This suggested that human cancerous effusions contained a protein antigenically related to a known immunosuppressive murine retroviral protein. P15E has recently been reported by Thiel et al. (37) to have been well-conserved in viral phylogeny. Furthermore, Martin et al. (38) have reported that human DNA contains sequences that hybridize to an MuLV reactive fragment of cloned African green monkey DNA and Repaske et al. (39) recently reported the isolation of 26 MuLV-related clones from a human DNA library. There has been a great deal of recent interest in oncogenes and their ability to neoplastically transform normal cells. We now propose the hypothesis that clinically relevant carcinogenesis might require the induction of additional gene products to those which are responsible for neoplastic transformation alone; i.e., synthesis of substances that subvert defensive processes of the host and allow the transformed cells to escape surveillance mechanisms.

Summary

Murine tumors contain low molecular weight factors that inhibit macrophage accumulation at inflammatory foci. Certain oncogenic murine leukemia viruses contain similar inhibitory activity and the active component of the retroviruses was shown to be the envelope protein P15E. A number of murine malignant and nonmalignant cell lines, as well as primary tumors, have now been examined to determine whether production of retroviral P15E or a related protein is characteristic of neoplastic cells. Tumor lines examined included the Hep 129 hepatocarcinoma, BP8 fibrosarcoma, RL1 lymphoma, and three variants of the B16 melanoma. Tumor lines were virus negative by electron microscopy. Nonmalignant cells examined included ST0, 3T3/BALB, and 3T3/L1 fibroblasts and unstimulated, as well as mitogen-stimulated murine splenocytes. Cells were pulse-labeled with [^{35}S]methionine, proteins immunoprecipitated with two monoclonal antibodies to P15E and analyzed by SDS-PAGE and gel fluorography. All tumor lines synthesized a ~19,000-dalton protein that co-migrated with retroviral P15E on SDS-PAGE. None of the nonmalignant cells synthesized this

protein. Two-dimensional gel electrophoresis of the proteins precipitated from two B16 melanoma lines by monoclonal anti-P15E showed them to be physicochemically similar to P15E from Rauscher leukemia virus. A competition ELISA assay for P15E was developed and confirmed the results obtained by metabolic labeling and demonstrated P15E-related antigens in the tumor cell lines and also in the ascites fluid of mice injected with Hep 129 cells. More importantly, P15E antigens were expressed in both a spontaneous mammary adenocarcinoma and in a primary methylcholanthrene-induced fibrosarcoma. Nonmalignant tissues from animals bearing these tumors contained no detectable P15E antigen. Extracts from the primary fibrosarcomas, when injected into the thighs of mice, inhibited the intraperitoneal accumulation of inflammatory macrophages. The inhibitory activity was specifically removed by absorption with monoclonal antibody to P15E.

These results suggest that synthesis of the immunosuppressive retroviral protein P15E, or a very similar protein, routinely occurs during the growth of murine neoplastic cells. This P15E-related protein is present in spontaneous murine primary tumors as well as in all murine tumor cell lines tested. The expression of such proteins by transformed cells in vivo could confer a selective advantage for their sustained growth since they would be more likely to escape immune surveillance.

We wish to thank Dr. Juan Vergara, Department of Anatomy, Duke University, for performing the electron microscopic examination of the various tumor cell lines. We also acknowledge the excellent technical assistance of Deborah Cone, Jessica Emery, and Donna Phipps and the expert secretarial assistance of Sharon Goodwin and Jodi Telander.

Received for publication 11 April 1983 and in revised form 13 June 1983.

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