Journal of Cellular Biochemistry 19:275–280 (1982)
Tumor Viruses and Differentiation 57–62

Preparation of Rat Monoclonal Antibodies to Epitopes Encoded by the Viral Oncogene (v-*fms*) of McDonough Feline Sarcoma Virus

Soni J. Anderson, Mark E. Furth, Linda Wolff, Sandra K. Ruscetti, and Charles J. Sherr

Laboratory of Tumor Virus Genetics, National Cancer Institute, NIH, Bethesda, Maryland 20205

The McDonough strain of feline sarcoma virus (SM-FeSV) contains a viral oncogene, v-*fms*, transduced from cat cellular genetic sequences designated c-*fms*. Monoclonal antibodies reactive to antigenic determinants encoded by v-*fms* were prepared by immunizing rats with live, syngeneic SM-FeSV-transformed cells, and fusing splenic lymphocytes from a tumor-bearing animal with cultured rat myeloma cells. Culture supernatants from hybrids producing antibodies to epitopes encoded by v-*fms* were identified by immunoprecipitation of radiolabeled polypeptides from SM-FeSV-transformed mink cells. Four positive hybrids were cloned twice in soft agar, established as stable lines, and grown in defined serum-free medium to facilitate purification of homogeneous antibodies. The monoclonal antibodies were used to assay SM-FeSV-specific products by "immunoblotting" of electrophoretically separated proteins, and by fixed-cell immunofluorescence.

Key words: monoclonal antibodies, McDonough feline sarcoma virus, viral oncogene v-*fms*

The McDonough strain of feline sarcoma virus (SM-FeSV) is a replication-defective retrovirus containing the oncogene v-*fms* [1–3]. SM-FeSV was formed, similarly to other acutely transforming type C viruses, by recombination between portions of a replicating leukemia virus and oncogene sequences transduced from the DNA of host cells [2, 3]. The order of genes in SM-FeSV genomic RNA is 5′-*gag-fms-env*-3′, in which the entire leukemia viral *env* gene and most of the *gag* sequence are represented [3]. The v-*fms* gene has a complexity of 3.1 ± 0.3 kilobase pairs, and is nonhomologous to v-*fes*, the transforming gene of two other previously characterized FeSV strains [2, 3].

In cells transformed by virus, or by transfection with molecularly cloned proviral DNA, SM-FeSV-specific glycoproteins of 180 kd (gP180*gag-fms*) and 120 kd (gp120*fms*) can be detected using hyperimmune rat sera [4]. Such sera, produced by rats bearing tumors of syngeneic SM-FeSV-transformed cells, also contain anti-

Received May 10, 1982; accepted June 7, 1982.

0730-2312/82/1903-0275$02.00

bodies that react with viral *gag* antigens, as well as with various cellular determinants. Although absorption of these sera with both feline leukemia virus and rat cellular proteins can render them specifically reactive to v-*fms* encoded antigens [4], the absorbed sera are difficult to produce in bulk, and show appreciable variability from animal to animal in the titer of relevant antibodies. We therefore developed monoclonal antibodies to v-*fms*-encoded epitopes that could serve as reproducible and specific reagents in assays of SM-FeSV gene products.

MATERIALS AND METHODS

Cells

G-2/mink, a nonproducer clone of CCL64 mink lung cells transformed by SM-FeSV, and G-2/NRK, an SM-FeSV nonproducer rat cell clone, have been previously described [4]. The rat myeloma cell line 210RCY3 Ag1.2.3, deficient in hypoxanthine-guanine phosphoribosyl transferase activity [5], was generously provided by C. Milstein, MRC Laboratory of Molecular Biology, Cambridge, England.

Hybrid Fusions and Cell Cloning

The strategy for producing hybrid clones secreting monoclonal antibodies has been described in detail elsewhere [5, 6]. In brief, 10-day-old Osborne-Mendel rats were inoculated with syngeneic SM-FeSV-transformed rat cells, and sera from tumor-bearing animals were assayed 3 weeks later for antibodies to SM-FeSV-encoded proteins [4]. Of two litters of rats inoculated, a single animal showing the highest serum titer of immunoprecipitating antibody against gp120*fms* was selected as a donor for cell fusion. Splenic lymphocytes were fused to 210RCY3 Ag1.2.3 rat myeloma cells in the presence of polyethylene glycol. Lymphocyte-myeloma cell hybrids were selected by growth in the presence of 1.0×10^{-4} M hypoxanthine, 4.0×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine (HAT medium). Antibody-producer hybrid cell cultures were chosen on the basis of gp120*fms*-specific immunoprecipitating activity in the culture media. These cells were subcloned twice in soft agar and established as stable lines.

Purification of Monoclonal Antibodies

Cell lines producing monoclonal antibodies were transferred to serum-free medium composed of 1:1 Dulbecco's minimum essential medium and Ham's F12 medium supplemented with insulin, transferrin, and bovine serum albumin [6]. Monoclonal immunoglobulins were precipitated from the clarified, spent medium of overgrown cultures by addition of ammonium sulfate to 50% final concentration at 4°C. The precipitates were collected by centrifugation at 10,000g for 30 min, and suspended in and dialyzed against 0.02 M sodium phosphate buffer, pH 6.8. The protein was applied to DEAE cellulose equilibrated in the same buffer, and eluted with a linear NaCl gradient to 0.3 M. Column fractions were monitored for protein by determining their absorbance at 280 nm, and aliquots were assayed for immunoglobulins by electrophoresis and immunodiffusion. Pooled immunoglobulins were dialyzed against 10 mM NH_4HCO_3, lyophilized, and stored at −20°C.

Immunodiffusion

The class and subclass of concentrated monoclonal antibody preparations were determined by the Ouchterlony technique of double immunodiffusion in immuno-

plates (Hyland Division, Travenol Labs) using sera directed against rat IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c}, and IgM (Miles Laboratories).

Labeling of Cytoplasmic Polypeptides and Polyacrylamide Gel Electrophoresis

G-2/mink cells grown to confluence in 75 cm^2 flasks were labeled for 30 min with 200 μCi/ml (^{3}H)-leucine (58 Ci/mmol; Amersham) in 5 ml leucine-free medium. Cells were lysed as described previously, and cytoplasmic polypeptides were immunoprecipitated with either tumor-bearing rat serum and Staphylococcus aureus (Cowan I strain) [3, 4] or supernatants from cultures containing monoclonal antibodies and rabbit anti-rat IgG-coated Staphylococcus aureus [6]. Washed immunoprecipitates were disrupted in SDS-polyacrylamide gel sample buffer containing 10 mM Tris HCl, pH 8, 1% SDS, 1% 2-mercaptoethanol, 1 mM EDTA, 5% glycerol, and 0.02% bromphenol blue, and applied to continuous 6–12% gradient polyacrylamide slab gels containing sodium dodecyl sulfate (SDS-PAGE) [3, 7].

Transfer and Detection of Proteins on Nitrocellulose (Immunoblotting)

G-2/mink cells in culture flasks were rinsed extensively with phosphate-buffered saline (PBS) and lysed directly in SDS-PAGE sample buffer. The lysates were boiled for 4 min, and centrifuged at 150,000g for 30 min to pellet DNA. Approximately 30 μg protein per lane, extracted in this manner or immunoprecipitated as described above, was separated by SDS-PAGE. Proteins were then transferred from gel slabs to 0.2 μ nitrocellulose filters by electrophoresis [8]. Following transfer, the filters were preadsorbed for 3 hr at 37°C in TNE buffer (20 mM Tris HCl, pH 7.5, 0.15 M NaCl, 1 mM EDTA) containing 0.1% Nonidet P40 (Shell Chemicals), 0.3 gm-% bovine serum albumin and 0.1 gm-% bovine gamma globulin (Cohn fractions IV and II, respectively, Sigma Chemicals). Preadsorbed nitrocellulose sheets were incubated in the same buffer for 14 hr at 4°C with 3×10^6 dpm/ml ^{125}I-labeled monoclonal IgGs [9]. The nitrocellulose sheets were extensively rinsed in TNE containing 0.1% Nonidet P40 (5 washes × 20 min at 22°C), permeated with 1.2% glycerol in the same buffer, dried, and autoradiographed.

Immunofluorescence

SM-FeSV-transformed G-2/mink cells or uninfected mink CCL64 cells were grown overnight in gelatin-coated wells of multitest slides (Flow Laboratories), fixed in methanol at −20°C, rinsed with 80% acetone, and air-dried. Fixed cells were reacted for 45 min at 37°C with monoclonal antibodies in PBS containing 0.4 gm-% bovine serum albumin. The wells were rinsed in PBS and incubated for 45 min at 37°C with affinity-purified, rhodamine-conjugated rabbit anti-rat IgG (a generous gift of Dr. J. Wheland, National Cancer Institute). Washed slides were mounted in elvanol under coverslips, visualized by epifluorescence with a Zeiss microscope, and photographed with controlled exposure and developing times [6].

RESULTS AND DISCUSSION

Of 200 wells of hybrid myeloma-lymphocyte cultures plated, 185 cultures were confluent after 1 week of growth in HAT medium. Sixteen cultures produced antibodies that precipitated radiolabeled proteins from lysates of G-2/mink cells. Of these, nine produced antibodies reactive to the SM-FeSV-encoded polypeptides gP180*gag*-

fms and gp120*fms*. Five other cultures produced antibodies reactive with cellular polypeptides present both in SM-FeSV-transformed and in uninfected mink cells. One culture produced antibodies reactive to a non-SM-FeSV-encoded polypeptide of G-2/mink cells, and one produced antibodies against a protein detected only in CCL64 cells.

Of the nine cultures producing antibodies apparently specific for v-*fms*-encoded proteins, four were chosen for cloning by virtue of superior cellular growth characteristics and supernatant antibody titers. These cells were cloned twice in soft agar, and four cell lines (designated SM1.32.6, SM3.19.4, SM5.15.4, and SM4.23.5) were characterized in greater detail. Antibodies produced by SM1.32.6, SM3.19.4, and SM5.15.4 were found to be IgG (γ_1) by Ouchterlony immunodiffusion tests, and by their apparent molecular weights, before and after reduction of disulfide bonds, on SDS-PAGE. In contrast, SM4.23.5 produced IgM. None of the monoclonal immunoglobulins precipitated labeled proteins from rat, mink, or cat cells infected with

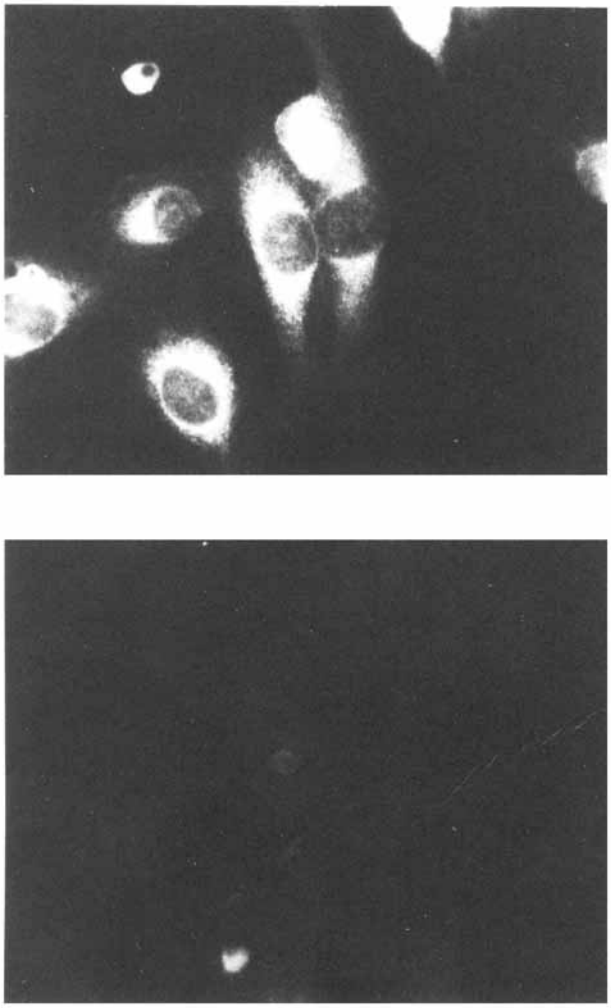

Fig. 1. Fixed G-2/mink cells (top) and CCL64 cells (bottom) were reacted with 30 μg/ml SM5.15.4 monoclonal IgG followed by reaction with rhodamine-conjugated rabbit antirat IgG. The two panels represent equal exposure and developing times.

different subgroups of FeLV. Moreover, none of the antibodies reacted with polyproteins encoded by the Snyder-Theilen and Gardner-Arnstein strains of FeSV, both of which contain a different viral oncogene (v-*fes*) [2–4]. Thus, unlike serum from tumor-bearing rats, the monoclonal immunoglobulins reacted specifically to epitopes encoded by v-*fms*, and lacked reactivity to rat cellular antigens or to antigenic determinants specified by the SM-FeSV *gag* or *env* genes.

The monoclonal antibodies were used to localize v-*fms*-encoded antigens in SM-FeSV-transformed cells. A typical pattern of fixed-cell immunofluorescence is shown in Figure 1. Bright, granular cytoplasmic fluorescence with an apparent perinuclear concentration was observed in SM-FeSV-transformed mink cells (Fig. 1, top), but not in untransformed mink cells (Fig. 1, bottom). No appreciable nuclear staining was observed.

Radioiodinated IgG from lines SM1.32.6 and SM3.19.4, but not from line SM5.15.4, could be used to detect SM-FeSV-encoded proteins immobilized on a solid

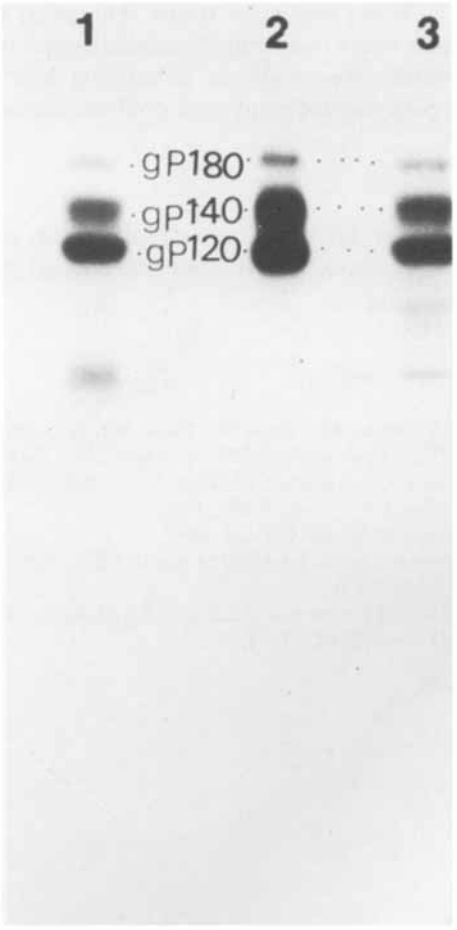

Fig. 2. Proteins either were first immunoprecipitated from cytoplasmic extracts of G-2/mink cells with hyperimmune rat serum (lane 2) or were applied to gels as total cellular extracts of G-2/mink cells (lanes 1 and 3). The proteins, separated by SDS-PAGE, were transferred from gel slabs to nitrocellulose sheets and reacted with ^{125}I-labeled monoclonal antibody from clone SM1.32.6 (lane 1) or clone SM3.19.4 (lanes 2 and 3).

substrate. In this assay, unlabeled proteins, extracted from cells and separated electrophoretically in SDS-PAGE, were transferred to nitrocellulose sheets and reacted with purified ^{125}I-labeled monoclonal IgGs. Figure 2 (lanes 1 and 3) shows results obtained with SM1.32.6 and SM3.19.4 IgGs, respectively. For comparison, lane 2 shows the position of proteins immunoprecipitated from unlabeled lysates of G-2/mink cells prior to denaturation, SDS-PAGE, and transfer to nitrocellulose. Three glycoproteins (designated gP180*gag-fms*, gp140*fms*, and gp120*fms*) were detected, all of which were entirely absent from lysates of CCL64 cells (not indicated in figure). A glycoprotein (gp140*fms*) not seen in earlier metabolic labeling studies [3, 4] is a processed form of gp120*fms* which can be detected after long kinetic labeling periods (our unpublished observations).

The availability of monoclonal antibodies therefore facilitates studies of steady-state levels of v-*fms* encoded antigens in transformed cells. In particular, the immunoblotting procedure, being independent of kinetic labeling, provides the first semiquantitative assay of SM-FeSV-transforming glycoproteins, and enables independent measurements of their different molecular forms. This assay can potentially complement cell fractionation or other biochemical manipulations in localizing SM-FeSV-encoded glycoproteins within the cytoplasm, in defining their association with particular organelles, and in purifying the viral gene products themselves.

ACKNOWLEDGMENTS

We thank Tom Shaffer for technical assistance with cell culture, Dr. Jurgen Wheland for help with fluorescence microscopy and photography, and Dr. Gilbert Jay for advice on immunoblotting.

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