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Isolation of Monoclonal Antibodies Against Avian Oncornaviral Protein p19

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For the production of monoclonal antibodies against pp60^{src} and the *gag* precursor protein Pr76^{gag}, the spleens of mice bearing tumors that had been induced by avian sarcoma virus Schmidt-Ruppin D-transformed cells were used. One hybridoma culture produced antibodies that were directed against the p19 portion of the *gag* precursor. However, no antibodies directed against pp60^{src} could be detected in any of the hybridoma supernatants. The anti-p19-producing hybridoma culture was cloned twice in soft agar, and a stable clone was used for the production of high-titer ascites fluid in mice. The monoclonal antibodies belonged to the immunoglobulin G subclass 2b. The antibodies precipitated Pr76^{gag} and the processed virion-associated p19, as well as the 75,000-molecular-weight *gag* fusion protein from avian erythroblastosis virus-transformed bone marrow cells. Also, viral ribonucleoprotein complexes were specifically precipitable, indicating that they contain p19 molecules.

Avian sarcoma viruses (ASV) code for genes involved in replication (*gag*, *pol*, and *env*) and in transformation (*src*) (1). Avian cells, such as chicken embryo fibroblasts (CEF), permit replication and transformation and express all viral proteins encoded by the ASV genome (25). In contrast, in mammalian cells transformed by ASV, the transforming gene is expressed, but no virus replication takes place (6). Two virus-specific proteins having molecular weights of 76,000 (76K) and 60,000 (60K) are found in these cells (3, 6). The 76K protein, Pr76^{gag}, is the precursor to the internal core proteins of ASV, however, it is not processed to the structural proteins p19, p12, p27, and p15 (6). The 60K protein, pp60^{src}, is the product of the *src* gene and has been identified as a phosphoprotein with protein kinase activity (5, 13, 22). Parsons et al. (20) showed that, when ASV-transformed mouse tumor cells are injected into syngeneic mice, specific antisera to pp60^{src} can be obtained and suggested the use of the spleen cells from tumor-bearing mice for the establishment of hybridomas producing monoclonal antibodies against this protein by the method of Köhler and Milstein (10). For this purpose, we injected BALB/c 3T3 (A31) cells transformed with ASV Schmidt-Ruppin D (SR-D) into syngeneic newborn mice (purchased from Bomholtgard, Ry, Denmark). Four of five animals developed palpable tumors within 4 weeks. Serum from the tumor-bearing mice precipitated the sarcoma-specific protein pp60^{src} and the unprocessed *gag* precursor Pr76^{gag} from SR-D-transformed chicken cell lysates labeled with [^{35}S]methionine for 3 h. Figure 1A shows the immunoprecipitates after gel electrophoretic analysis and autoradiography. A second bleeding (6 weeks post-inoculation) was analyzed to monitor the increase in antibody titer. One mouse died because of its tumor; mice 2 and 3 were sacrificed for hybridoma production (10, 11). A total of 1×10^8 spleen cells were fused with 2×10^7 cells of the 8-azaguanine-resistant plasmacytoma cell line P3-NS1-1-Ag4-1 (NS-1) by the procedure described by Kennett (9), using polyethylene glycol and dimethyl sulfoxide.

The cell suspension was distributed into five 24-well tissue culture plates and cultured in HAT medium for the selection of hybrid cells (14). After 14 days, the surviving cultures were tested for antibody production in an indirect antibody-binding assay (17, 24, 26). A 96-well plate was first coated with various antigens and then incubated with the supernatant of each culture. Positive cultures were identified by means of an iodinated rabbit anti-mouse immunoglobulin G (IgG). The antigens used were disrupted transforming virus, SR-D, which has been shown to contain pp60^{src} (18; M. K. Owada, P. Donner, A. Scott, and K. Moelling, Virology, in press), a nontransforming virus, avian myeloblastosis virus, as a negative control, the SR-D-transformed BALB/c 3T3 cells that had been used for immunization of the mice, and normal BALB/c 3T3 cells (Fig. 1B). One hybridoma supernatant, from culture no. 52, was found to bind 10- to 20-fold more radioactive anti-IgG

than all others but was not specific for the *src*-containing antigens. The antibody-producing culture no. 52 was analyzed further for its specificity. Various purified viral proteins were used as antigens in an indirect antibody-binding assay (Fig. 2A). The results showed that the antibodies were directed against p19, the N-terminal portion of the *gag* precursor $Pr76^{gag}$, only. Precipitation of $Pr76^{gag}$ from [^{35}S]methionine-labeled cell lysates by the culture supernatant (in particular, after a 100-fold concentration by 50% ammonium sulfate precipitation) supported this result (Fig. 2B).

The antibody-producing culture no. 52 was cloned twice in soft agar until 100% of the subclones were antibody producers. One stable subclone, cl52:38:38, was grown in mass culture and used for further characterization. To increase the antibody titer, 10^7 cells from cl52:38:38 were injected intraperitoneally into (BALB/c × C57BL)F_1 hybrid mice (termed CD6) that had been primed with pristane (0.25 ml) 10 and 3 days before to enhance ascites fluid formation (21). The antibody titer of the ascites fluid was about 10^3-fold higher than that of the tissue culture fluid from cl52:38:38. A 10- to 100-fold increase in titer could also be achieved by concentration of culture fluid with 50% ammonium sulfate (Fig. 2C).

Immunoprecipitation of $Pr76^{gag}$ from [^{35}S]methionine-labeled SR-D-transformed CEF with the various culture supernatants reflected the titers of the antibodies (Fig. 2B). It was notable that the clonal supernatants and ascites fluids

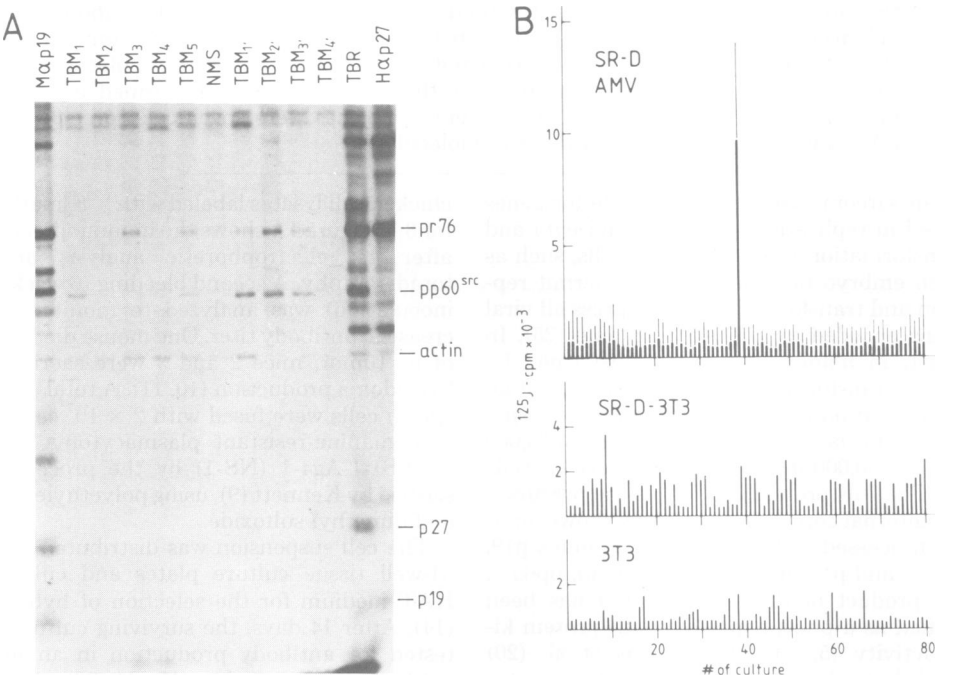

FIG. 1. *(A) Characterization of tumor-bearing mouse (TBM) sera. [^{35}S]methionine-labeled SR-D-transformed CEF were lysed and treated with 5 μl of serum for indirect immunoprecipitation as described elsewhere (15). TBM_{1-5}, TBM sera of five mice (first bleeding); $TBM_{1'-4'}$, second bleeding (one mouse died); $TBM_{2'-3'}$, mice used for fusion at the time of the second bleeding; Mαp19, monospecific anti-p19 mouse serum; TBR, tumor-bearing rabbit serum induced by SR-D (19); NMS, normal mouse serum; Hαp27, sheep anti-p27 serum. (B) Indirect antibody-binding assay for screening of culture fluids. Virus (SR-D and avian myeloblastosis virus [AMV]) and cells (SR-D-transformed 3T3 cells and 3T3 cells) were disrupted in lysis buffer (50 mM Tris-hydrochloride [pH 9.2], 400 mM KCl, 1 mM EDTA, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride) and dialyzed against phosphate-buffered saline. A 50-μl amount containing 2.5 μg of protein was baked as antigens in Limbro plates with 96 wells (14 h at 37°C). The wells were then treated with wash buffer (phosphate-buffered saline containing 5% fetal calf serum, 0.1% Triton X-100, 0.02% sodium azide) for 90 min at 37°C. A 50-μl amount of clarified hybridoma supernatant was then added and allowed to bind for 60 min at 37°C. The wells were then washed three times with 200 μl of wash buffer. Subsequently, 50 μl of iodinated rabbit anti-mouse IgG (0.2 μg/ml), corresponding to 3×10^5 cpm, prepared according to Hunter (8) was added to each well for 60 min at 37°C. After washing as above, the radioactivity of the individual wells was solubilized in 100 μl of 2 M NaOH (30 min, 37°C) and counted in a gamma counter.*

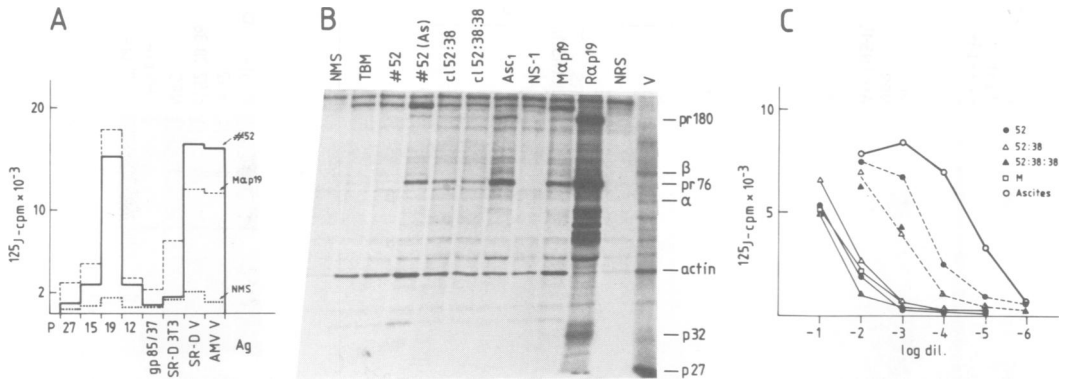

FIG. 2. *(A) Indirect antibody-binding assay with purified antigens. A 0.25-μg amount of purified p27, p19, p15, p12, and gp85/37 (16) was used as antigens (Ag) according to the procedure described in the legend to Fig. 1B. The supernatant of the hybridoma culture (no. 52) was compared with a monospecific mouse anti-p19 serum (Mαp19) and normal mouse serum (NMS). (B) Immunoprecipitation of viral polyproteins from transformed CEF as in Fig. 1A, using 50 μl of culture fluid from culture no. 52, from a clone, cl52:38, derived from it, and from a subclone, cl52:38:38. The culture fluid of no. 52 was also tested after 100-fold concentration by ammonium sulfate (AS). Asc_1, Ascites fluid (5 μl); NS-1, culture fluid (5 μl) serving as a negative control for the hybridoma supernatants; Rαp19, monospecific rabbit anti-p19 antibodies; NRS, normal rabbit serum; V, [^{35}S]methionine-labeled SR-D as a marker; NMS, normal mouse serum; TBM, tumor-bearing mouse serum. (C) Comparison of antibody titers in an indirect antibody-binding assay. Tenfold serial dilutions of the hybridoma supernatant no. 52 (●——●) and the subclones cl52:38 (△——△) and cl52:38:38 (▲——▲) and serum from tumor-bearing mice (M, □——□) were prepared. Their antibody-binding ability was compared with that of supernatants after 10-fold (△- - -△, ▲- - -▲) and 100-fold (●- - -●) concentrations by ammonium sulfate precipitation (50%, vol/vol). Ascites fluid (○——○) was obtained from F_1 hybrid mice after pristane treatment and injection of clone 52:28:38 cells.*

less efficiently precipitated p19-related intermediate *gag* precursors, three of which ranged between 50 and 70K. These polyproteins were readily detected with the monospecific rabbit anti-p19 serum used as a control. Since the putative *gag-pol* fusion protein Pr180 was precipitated by the ascites fluid, it appears that the recognition of fusion proteins may depend on the accessibility of their p19 portion to the antibodies that are directed against one specific antigenic determinant. In SR-D-transformed CEF labeled for 3 h, the amount of labeled processed viral structural protein (in particular, of p19) was relatively small.

To analyze whether the monoclonal antibodies recognized the processed virion-associated p19 molecule, [^{35}S]methionine-labeled virus was lysed and used for immunoprecipitation. As shown in Fig. 3A (left), p19 was precipitated from virus particles and was efficiently competed for by the addition of purified p19. In addition, the α and β subunits of reverse transcriptase were also precipitated with the monoclonal antibodies. We thought that this phenomenon might be due to the coprecipitation with the ribonucleoproteins which are in a complex with the polymerase, some p27, p12, and a small amount of p19 (4, 23). To prove this, virus was disrupted, and the soluble (supernatant) and insoluble (pellet) materials were separated by ultracentrifugation, applying the method described by Chen et al. (4). The monoclonal anti-p19 serum could efficiently coprecipitate reverse transcriptase from the pellet, but not from the supernatant. Also, the specific precipitation of p19 with the monoclonal antibodies could not be competed for by purified p27 or polymerase (Fig. 3A). In addition, the monoclonal anti-p19 serum did not react with purified polymerase in the antibody-binding assay (data not shown). These findings strongly support the data of Sen and Todaro (23), who reported that very few molecules of p19 are associated with RNA.

The monoclonal anti-p19 antibodies were tested further for their ability to precipitate the putative transformation-specific fusion proteins of acute leukemia viruses, the N-terminus of which is p19. In avian erythroblastosis virus-transformed bone marrow cells, the virus codes for a protein of 75K, which is probably transformation specific and fused to p19 (2, 7). This fusion protein is detected in [^{35}S]methionine-labeled transformed bone marrow cells by the monoclonal antibodies. Since these cells also produced the helper virus protein $Pr76^{gag}$, this protein was removed by preprecipitation. As it is very similar in size, it would otherwise prevent the detection of the 75K protein (Fig. 3B).

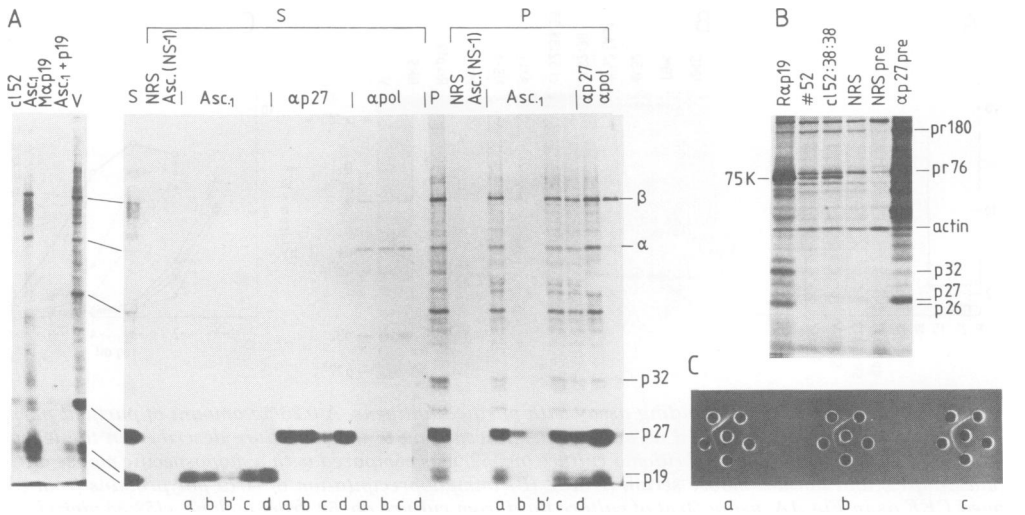

FIG. 3. *(A) Immunoprecipitation of viral antigens from [^{35}S]methionine-labeled SR-D. The virus was lysed with RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 0.1 M NaCl, 0.05 M Tris-hydrochloride [pH 7.2]) containing 0.8 M NaCl and applied directly to the gel (V) or incubated with the indicated antibody samples (abbreviations as in Fig. 2). For the competition assay, purified p19 (5 μg) was added to the ascites fluid (Asc.$_1$ + p19). Alternatively, the labeled virus was disrupted by the method of Chen et al. (4) and centrifuged in an SW50.1 rotor at 40,000 rpm for 60 min at 4°C to separate the soluble material (S) from the pellet (P) (4). Samples of both were applied directly to the gel (S and P) or used for immunoprecipitation. Absorption of the sera (5 μl each) was performed with no antigen (a), with 1 μg of p19 (b), with 10 μg of p19 (b'), p27 (c), and purified reverse transcriptase (d) as antigens (5 μg each). Mouse ascites fluid produced with NS-1 cells [Asc. (NS-1)] served as the control; α and β indicate the two subunits of the reverse transcriptase. (B) Immunoprecipitation from [^{35}S]methionine-labeled avian erythroblastosis virus-transformed bone marrow cells (3 h). The lysates were first preprecipitated with normal rabbit serum (NRS) and αp27 serum (pre) and subsequently treated with hybridoma supernatants. (C) Agar gel diffusion test for identification of the immunoglobulin class. The central well contained: (a) 15 μl of 100-fold-concentrated supernatant of culture no. 52; (b) 20 μl of 10-fold-concentrated cl52:38:38; and (c) 20 μl of 10-fold-concentrated cl52:38 supernatants. The other wells contained (starting from the top, counterclockwise) 10 μl of: rabbit anti-total mouse IgG serum, rabbit anti-mouse IgG2b, rabbit anti-mouse IgG2a, and rabbit anti-mouse IgG1 (the last well was empty). The photograph was taken after 4 h at 37°C.*

In an agar gel diffusion analysis with immunoglobulin class-specific reagents purchased from Miles Laboratories, Inc., Elkhart, Ind., it was shown that the antibodies produced by the original culture belonged to two IgG classes, to IgG2b and, to a less extent, to IgG1. The supernatants of the clones were specific and reacted positively with anti-IgG2b only (Fig. 3C).

The isolation of monoclonal antibodies against p19 will prove to be useful for analyzing the location and function of p19 in virus particles and of p19-containing fusion proteins in virus-infected cells. This may be helpful in understanding the role of fusion proteins of acute leukemia viruses in transformation and differentiation. Also, since only monoclonal antibodies have high concentrations of pure and specific antibodies, they may be used for microinjection into cells and allow an analysis of the mechanisms of transformation and differentiation. Furthermore, monoclonal antibodies may be useful for studying properties of the reverse transcriptase in ribonucleoprotein particles (4) and the interaction of p19 with RNA which has been suggested as being relevant in the splicing of viral mRNA's (12).

Although the first intention was to make monoclonal antibodies against the transformation-specific protein pp60^{src}, the indirect antibody-binding assay showed a higher sensitivity for the viral structural proteins than for pp60^{src}. Therefore, the probability of finding hybridomas producing monoclonal antibodies against the transformation-specific pp60^{src} protein was very low. The use of partially purified pp60^{src} protein as an antigen would improve the sensitivity of the assay.

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