Monoclonal Antibody Specific for Avian Sarcoma Virus Structural Protein p27

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SUMMARY

A hybridoma cell line which secretes antibody to the Rous sarcoma virus (RSV) structural protein p27 has been established. The hybrid resulted from the fusion of NS-1 myeloma cells with spleen cells from a Balb/c mouse which was immunized with RSV-transformed mouse cells. Antibodies produced by the hybrid clone immunoprecipitated p27 and \textit{gag} precursor proteins (Pr180^{gag,pol}, Pr76^{gag} and Pr66^{gag}) from [35S]methionine-labelled chicken embryo fibroblasts transformed by the Schmidt–Ruppin strain of RSV. When Schmidt–Ruppin virus was radioactively labelled with [35S]methionine, p27 was the only virus structural protein immunoprecipitated. Antibody production by the hybrid clone (designated 7-29-D6) has remained stable for longer than 12 months at a level of 50 IgG/ml medium. A highly sensitive method to determine the subclass specificity of monoclonal antibodies is described. In this procedure, the clone is incubated with [35S]methionine, and radiolabelled antibody is precipitated with affinity-purified, subclass-specific rabbit anti-mouse serum and \textit{Staphylococcus aureus}. The advantages of this procedure are discussed.

Avian retroviruses are well-characterized oncogenic agents which cause a variety of tumours in animals. Their relatively simple genome consists of three genes involved in virus replication, \textit{gag}, \textit{pol} and \textit{env}. In addition, the avian sarcoma viruses (ASV) possess a single gene, \textit{src}, which is responsible for oncogenic transformation (Hanafusa, 1977; Vogt, 1977). Monoclonal antibodies directed against the gene products of these viruses could be used to analyse the life cycle of these viruses and investigate their mechanism of tumour production.

Two monoclonal antibodies against avian retrovirus structural protein have recently been described. Sovovh et al. (1980) immunized Balb/c mice with the Carr–Zilber strain of ASV and obtained a monoclonal antibody which precipitated the \textit{gag} precursor protein Pr76 from Prague-C, ASV-transformed chick embryo fibroblasts (CEF). It is not known which of the processed products of Pr76^{gag} (p27, p19, p12, p15) are recognized by the antibody. More recently, Greiser-Wilke et al. (1981) produced a monoclonal antibody against the virion phosphoprotein p19. To date, monoclonal antibodies recognizing the transforming protein of ASV, pp60^{src}, the envelope glycoproteins or the reverse transcriptase have not been reported. To produce monoclonal antibodies against various Rous sarcoma virus (RSV) components, I have immunized Balb/c mice with Schmidt–Ruppin RSV (SR-RSV)-transformed mouse cells and isolated a hybridoma which secretes antibody against the RSV core protein p27.

Immunizations were performed by injecting female 10- to 12-week-old Balb/c mice (Jackson Laboratory, Bar Harbor, Me., U.S.A.) intraperitoneally with 10^7 SR-RSV-transformed Balb/c mouse cells in 0.5 ml serum-free media. Booster injections were performed in the same manner 4, 8 and 9 weeks later. Seventy-two h after the final injection the mouse spleen was removed for fusion with NS-1 myeloma cells. The myeloma cell line was kindly provided by Dr D. McFarlin (NINCDS, NIH, Bethesda, Md., U.S.A.). The fusion procedure was that of Gefter et al. (1977) as modified by Ozato et al. (1980). After fusion, the pelleted cells were resuspended at a density of 10^8 spleen cells/40 ml HAT selection
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medium (Littlefield, 1964). The medium was high glucose (4500 mg/l) Dulbecco's modified Eagle's (DME; Gibco) with 10% heat-inactivated foetal calf serum (Gibco), 10% NCTC 109 (MA Bioproducts, Bethesda, Md., U.S.A.), 1% glutamine (Gibco), 5% conditioned medium (DME with 10% calf serum) from cultured rat fibroblasts, 1 mm-sodium pyruvate, 1% non-essential amino acids 100× (Gibco), 100 μM-hypoxanthine, 10 μM-aminopterin and 30 μM-thymidine. All chemicals were obtained from Sigma. The cell suspension was seeded in 24-well Costar plates using 0.5 ml/well. Four days later, 0.5 ml HAT medium was added to each well and, thereafter, the volume of media per well was maintained at 1 ml. Two weeks after the fusion, hypoxanthine, aminopterin and thymidine were removed, and the medium was subsequently designated S+. Wells showing vigorous macroscopic growth were screened for ability to immunoprecipitate [35S]methionine-labelled proteins from CEF transformed by SR-RSV, subgroup D (SR-CEF), using the procedure of Brugge & Erikson (1977). Hybrid wells that were positive in initial screening were first expanded into four wells of a 24-well Costar plate, then to a 60 mm Petri dish, and subsequently cloned in soft agar (MacPherson, 1973).

The autoradiogram (Fig. 1) compares the proteins precipitated from radiolabelled SR-CEF cell extracts using hybridoma supernatant or antisera from tumour-bearing rabbits (TBR), rats (TBr) or mice (TBM). The preparations of TBr and TBR have been described previously (Jay et al., 1978; Brugge & Erikson, 1977). The TBM sera were obtained from the mice immunized for fusions. No proteins were specifically precipitated by S+ media alone (lane 1). TBr sera (lane 2) and media harvests from hybridoma 7-29 at three stages of expansion (lanes 5 to 7) precipitated the viral gag precursor proteins PrI80, Pr76 and Pr66. Initial screening experiments were conducted using 10% SDS-polyacrylamide gels 9 cm in length. It could not be determined which of the processed virion core proteins was recognized by the positive hybridoma because all processed proteins (having mol. wt. <30000) ran at the dye front (Fig. 1). Although pp60src was recognized by all sera from tumour-bearing animals (lanes 2 to 4), it was not precipitated by hybridoma 7-29 supernatant. The precipitation was specific: media from two other hybrid wells in the same fusion (lanes 8 and 9) failed to precipitate any labelled proteins from SR-CEF. Moreover, no [35S]methionine-labelled proteins were precipitated from uninfected CEF by the hybridoma media (data not shown).

After cloning hybridoma 7-29 by limiting dilution in soft agar, four positive clones were isolated, grown in mass culture and rescreened for their ability to precipitate gag proteins from SR-CEF using 12.5% SDS-polyacrylamide gels. The four subclones of 7-29 had the same specificity and precipitated p27 (data not shown). Only one of these clones, designated 7-29-D6, was selected for further characterization.

To confirm the antibody specificity and to examine the precursor-product relationship of gag proteins in RSV-infected cells using monoclonal antibody, a pulse labelling experiment was performed. SR-CEF were labelled with [35S]methionine for 15 min, and then ‘chased’ by incubating in medium with 50-fold excess of unlabelled methionine for 2 h. At various times, cells and virus were harvested separately, detergent-lysed and precipitated with the monoclonal antisera. The results are shown in Fig. 2 (A, B). In each part (a) represents a 4 h continuous labelling of cells and virus, followed by immunoprecipitation with various sera or monoclonal antibody; (b) shows pulse-labelled cells and virus precipitated with monoclonal antibody; (c) shows pulse-labelled cells and virus precipitated with TBR sera.

In Fig. 2 (A, part b) at the earliest time point (zero time), which represents pulse labelling only, the most prominent protein precipitated by the monoclonal antibody was Pr76gag. Also apparent was Pr180gag,pol and a higher mol. wt. component of approx. 220000 (220K). The 220K protein, which also appeared as a contaminant in the virus preparations (Fig. 2B), was found to bind non-specifically to normal rabbit sera (NRS)—Staphylococcus aureus complexes and was not considered further. Pr180gag,pol did not show significant decay
Fig. 1. Immunoprecipitation of [35S]methionine-labelled SR-CEF. SR-CEF (8 × 10⁶ cells/100 mm Petri dish) were labelled with 1 mCi [35S]methionine (Amersham International, sp. act. > 1000 Ci/mmol) in 4 ml methionine-free Eagle's medium for 3 h at 37 °C. Cells were lysed using 1 ml modified RIPA buffer (Collett et al., 1979) per dish and clarified at 100,000 g for 30 min. For immunoprecipitation, 0.2 ml of radiolabelled cell extract was incubated at 0 °C for 30 min with 0.25 ml hybridoma supernatant or 10 μl antisera from tumour-bearing animals in a total vol. of 1 ml modified RIPA buffer. Immune complexes were absorbed to 100 μl formalin-fixed S. aureus (Kessler, 1975) which was prepared as described by Richert et al. (1979). After extensive washing, the proteins were eluted from the bacteria by boiling in SDS-sample buffer (Brugge & Erikson, 1977) and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gels (Laemmli, 1970). ¹⁴C-labelled standards (New England Nuclear) were used as markers. After fluorography (Laskey & Mills, 1975), the dried gel was exposed to Kodak X-Omat film at -70 °C for 12 h. Immunoprecipitation of SR-CEF was carried out with the following: lane 1, S+ media; lane 2, TBr sera; lane 3, TBR sera; lane 4, TBM sera. Lanes 5 to 7 are precipitates of SR-CEF using media from the first expansion stage, second expansion stage, or the original well of hybrid 7-29 respectively. Lanes 8 and 9 are SR-CEF precipitates using media from hybrid clones 7-44 and 7-45.

throughout the 2 h chase, in agreement with the results of Oppermann et al. (1977) indicating a slow rate of turnover. After a 15 min chase, the monoclonal antibody detected p27 in both cell extracts and extracellular virus. Thereafter, there was a decrease in Pr76⁸⁸ and a corresponding increase in labelled p27 which confirms the precursor–product relationship described by Vogt et al. (1975). Several intermediate cleavage products of Pr76⁸⁸ were also detected by the monoclonal antisera. Pr66⁸⁸ and three smaller intermediates (46K, 48K and 50K) were more apparent in the 4 h continuously labelled cell extracts (Fig. 2A, part a, lane 4) than in the pulse-labelled cells. There was no precipitation of a Pr60⁸⁸ intermediate by the monoclonal antibody from either pulse-labelled or continuously labelled cells. It is not known whether sera from tumour-bearing animals detect this intermediate because pp60src would co-migrate in this region. The ‘dead-end’ cleavage product Pr32⁸⁸ was precipitated by TBR sera (Fig. 2A, part a, lane 3 and part c, lanes 30, 60 and 120 min) but was not detected by
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(A)  

1 2 3 4  0 15 30 60 120  0 15 30 60 120

Pr180<sup>aaa</sup>,<sup>p11</sup>  
Pr90<sup>aa+a</sup>  
Pr76<sup>aa+a</sup>  
Pr66<sup>aa+a</sup>  
pp60<sup>aa+a</sup>  

Pr32<sup>aa+a</sup>  
p27  

(B)  

1 2 3 4  0 15 30 60 120  0 15 30 60 120

p27  
p19  
p12/15
Fig. 3. Characterization of hybridoma Ig. $^{35}$S]methionine-labelled hybridoma antibody was immunoprecipitated using subclass-specific RAM sera. Hybridoma antibody was labelled by incubating clone 7-29-D6 with 1 mCi $^{35}$S]methionine in 4 ml S+ media. After 18 h at 37 °C, the culture supernatant was harvested and clarified of cell debris by centrifugation at 3000 rev/min for 10 min. Antisera against whole mouse IgG was affinity-purified using mouse IgG (both from Cappel Laboratories) coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia). Affinity-purified RAM specific for mouse IgG2a was purchased from Litton Bionetics, and RAM against mouse IgG2b, IgG3, mouse $\kappa$ light chain and mouse $\lambda$ light chain were obtained from Miles Laboratories (Elkhart, Ind., U.S.A.). For immunoprecipitation, 10 $\mu$l RAM sera were absorbed to 25 $\mu$l S. aureus for 30 min at 0 °C in 1 ml modified RIPA. The bacteria were pelleted at 3000 rev/min for 10 min and unbound sera removed. To block any remaining protein A sites on the bacteria, the RAM/S. aureus pellets were resuspended in 1 ml buffer and incubated with an excess (10 $\mu$l) of NRS for 30 min at 0 °C prior to adding 50 $\mu$l $^{35}$S]methionine-labelled hybridoma antibody. The immunoprecipitates were washed extensively, and labelled proteins were separated by 10% SDS-PAGE. After fluorography, dried gels were exposed to film for 2 h at -70 °C. The precipitation of $^{35}$S]methionine-labelled hybridoma antibody with S. aureus alone (lane 1) or with rabbit antiserum--S. aureus complexes are shown as follows: NRS (lane 2), RAM IgG (lane 3), RAM IgG2a (lane 4), RAM IgG2b (lane 5), RAM IgG3 (lane 6), anti-mouse $\kappa$ (lane 7) and anti-mouse $\lambda$ (lane 8).

the monoclonal antibody presumably because it contains an altered p27 (Eisenman & Vogt, 1978).

Fig. 2 (B) shows that the only virus core protein precipitated by the monoclonal antibody was p27, which confirms its specificity. All of the virus structural proteins were precipitated

Fig. 2. Precipitation of labelled SR-CEF cells (A) and virus (B). (a) Four h continuous labelling of SR-CEF and virus. Third passage SR-CEF were labelled for 4 h with $^{35}$S]methionine as described in Fig. 1. To obtain labelled virus, the culture media were harvested, clarified at 10000 g for 20 min, and then centrifuged at 100000 g for 90 min. The virus pellet was lysed in 1 ml modified RIPA and clarified at 100000 g for 60 min. Cell extracts were prepared as described in Fig. 1. Cell and virus extracts (0-1 ml) were precipitated with 10 $\mu$l TBr sera (lane 1), NRS (lane 2), TBR (lane 3), or 100 $\mu$l monoclonal antibody from clone 7-29-D6 (lane 4). (b, c) Pulse-labelled SR-CEF and virus. SR-CEF were pulse-labelled with $^{35}$S]methionine for 15 min (0 time). The dishes were rinsed twice with Tris-buffered saline pH 7-2, and then switched to minimal essential medium containing excess unlabelled methionine (750 mg/l) for 15, 30, 60 or 120 min. At the times indicated, cell and virus extracts were prepared as described above and precipitated with 100 $\mu$l monoclonal antisera (b) or TBR sera (c). Mol. wt. markers are shown in lanes between (a) and (b) and between (b) and (c).
by TBr sera and weakly precipitated by TBR sera (Fig. 2B, part a, lanes 1 and 3). Tumour-bearing sera also showed a protein migrating faster than p27 with an estimated mol. wt. of 23000. This may be the 23K protein described by Pepinsky & Vogt (1979) which is related to p19, and results from an abnormal cleavage. It is not detected by the monoclonal antibody.

The hybridoma antibody from clone 7-29 was initially determined to be IgG from the mol. wt. of the immunoglobulin H chain on SDS–polyacrylamide gels. Further characterization was performed by labelling the clone with [35S]methionine and immunoprecipitating the labelled hybridoma antibody using affinity-purified, subclass-specific rabbit anti-mouse (RAM) sera and S. aureus. The results (Fig. 3) show that the labelled hybridoma IgG precipitated with S. aureus alone (lane 1). This precipitation was blocked, however, if excess NRS were first preabsorbed to the protein A sites on the bacteria (lane 2). When subclass-specific RAM sera were bound to S. aureus, then reacted with excess NRS, and finally with [35S]methionine-labelled hybridoma antibody, the labelled IgG was precipitated only by sera recognizing whole mouse IgG (lane 3), mouse IgG2a (lane 4) and mouse κ light chains (lane 7). No labelled IgG was precipitated by RAM sera specific for subclass IgG2b, IgG3 or λ light chains (lanes 5, 6 and 8 respectively). Thus, the isotype of the hybridoma antibody is IgG2aκ.

The sensitivity of this procedure offers several advantages over the Ouchterlony diffusion method currently used to determine monoclonal antibody specificity. First, even ng quantities of antibody can be detected by this method, eliminating the requirement for large-scale concentration and purification of monoclonal antibody. Secondly, if two or more clones having different specificities are present in a positive well, this can be detected prior to cloning in soft agar. After cloning, one can immediately determine whether all of the hybrids have been isolated or whether recloning is necessary prior to expansion and growth as ascites.

Since the time of isolation of hybridoma 7-29-D6, the clone has been passaged more than 100 times on a twice-weekly schedule and expanded to roller bottles without loss of antibody titre. From the intensity of the Coomassie Brilliant Blue band on SDS–polyacrylamide gels, the amount of monoclonal antibody secreted by this clone has been estimated at 50 μg/ml medium. Subsequent fusions using the same mouse immunization procedure have produced two other hybrid clones specific for p27, although it is not known whether they recognize the same or different determinants on this virus antigen. Thus, the virus gag proteins appear to be highly immunogenic in the mouse. It is interesting that although pp60src is the predominant protein precipitated by polyclonal antisera from immunized mice (Fig. 1, lane 4), the majority of hybrids resulting from the fusion secrete antibodies to virus structural proteins. We are now attempting fusions using mouse lymph nodes in addition to spleen cells to generate antibodies specific for pp60src. Alternatively, the detection of such monoclonal antibodies may require a more sensitive screening method.

Recent evidence has demonstrated that certain avian leukaemia viruses, e.g. MC29 (Bister et al., 1977) and avian erythroblastosis virus (Hayman et al., 1979), as well as replication-defective ASVs, e.g. PRC II (Breitman et al., 1980; Neil et al., 1980), Y73 (Kawai et al., 1980) and Fujinami sarcoma virus (Hanauska et al., 1980), are capable of transforming CEF in vitro in the absence of an src gene. Rather, these viruses synthesize a gag-containing polyprotein (mol. wt. >100 000) which is linked to transformation-specific ‘onc’ sequences derived from the host cell. Like pp60src (Collett & Erikson, 1978; Hunter & Sefton, 1980), the transformation-specific proteins are phosphoproteins which possess an intrinsic or closely associated tyrosine kinase activity (Kawai et al., 1980; Neil et al., 1981; Feldman et al., 1980). Although gag sequences are probably not required for the transforming function of these proteins (Ghysdael et al., 1981; Pawson et al., 1981), monoclonal antibodies against gag determinants may be useful in the characterization and purification of these proteins.
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REFERENCES


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