The use of monoclonal antibodies to study the proteins specified by the transforming region of human adenoviruses

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Monoclonal antibodies against two of the proteins specified by one of the transforming genes (early region 1B) of human adenovirus type 2 have been produced and characterized. Two clones (RA1 and PA6), generated by fusion of mouse myeloma NSO cells with splenocytes from rats immunized with whole-cell lysates of an adenovirus-transformed rat cell line (F19), secreted antibodies against a 58 kDa protein. Another clone (DC1) produced antibodies against the same protein, and resulted from fusion of immune rat splenocytes with the rat myeloma Y3.Ag.1.2.3. Immunoprecipitation studies showed that all three antibodies recognized [³⁵S]-methionine-labelled 58 kDa protein, and phosphorylated derivatives of the 58 kDa protein labelled with [³²P]orthophosphate present in infected human cells. One clone (EC3) produced antibody against a 19 kDa protein also encoded by early region 1B, but not sharing sequence homology with 58 kDa. The identity of the 19 kDa protein recognized by the EC3 antibody was established by immunoprecipitation from lysates of labelled-infected cells and from products of cell-free translation directed by mRNA isolated from adenovirus 2-infected cells. Indirect immunofluorescent-antibody staining of infected human cells using the RA1 and EC3 antibodies revealed a nuclear location of the 58 kDa protein and a mainly cytoplasmic location of the 19 kDa protein.

Human adenoviruses are DNA tumour viruses that have the ability to transform normal rodent cells to a malignant phenotype (Pettersson & Akusjärvi, 1983). Studies on transformation mediated by DNA fragments generated by restriction endonucleases revealed that the genetic information necessary and sufficient to establish the transformed state is located in the left 11% of the linear 36 500-base-pair viral genome (Graham *et al.*, 1975). This region of the genome coincides with one of the four regions expressed early after infection of human cells, hence it is termed 'early region 1' (E1). Two transcriptional units have been identified within E1, the first being from 1.5% to 4.5% (E1A) and the second (E1B) from 4.5% to 11.5% (Chow *et al.*, 1979).

Co-operation of E1A polypeptides with E1B products is essential for the expression of para-

meters associated with the fully transformed phenotype (Jones & Shenk, 1979). The functions of E1B proteins in cellular transformation and infection remain to be clarified. E1B proteins of 58 and 19 kDa have been identified in infected and transformed cells, and represent the major adenovirus transformation proteins (Lassam *et al.*, 1980; Gilead *et al.*, 1976). A minor product of 21 kDa has been recently mapped to E1B. The 58 and 19 kDa proteins do not share common peptides, although the 21 and 58 kDa proteins appear to possess peptide homology (Matsuo *et al.*, 1982). These studies have relied on the use of sera from hamsters bearing tumours induced by transformed cells or have used antisera raised in rats hyperimmunized with lysates of transformed cells. In order to study the functions of individual E1B proteins, monospecific antibodies are required. Tumour and hyperimmune sera suffer from the disadvantage of being polyspecific, containing antibodies against the entire repertoire of transformation proteins. We report here the production of standardized rat monoclonal antibodies for the 58 kDa and 19 kDa proteins and describe their use in studies on their

Abbreviations used: FITC, fluorescein isothiocyanate; E1A, E1B, early region 1A or 1B of the adenovirus genome; SDS, sodium dodecyl sulphate.

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biosynthesis and subcellular location in infected cells.

Experimental

Materials

[³⁵S]Methionine (sp. radioactivity 1000 Ci/mmol) and [³²P]orthophosphate (carrier-free) were obtained from Amersham International.

AS rats were provided by the University of Leicester Animal Services. Cell-culture media, heat-inactivated foetal- and newborn-calf serum and sterile culture plastics were obtained from Gibco, Irvine, Ayrshire, Scotland, U.K. The myeloma cell lines NSO and Y3.Ag.1.2.3 (Galfré & Milstein, 1981) were provided by Dr. C. Milstein, MRC Laboratory of Molecular Biology, Cambridge, U.K. and the adenovirus-transformed cell line F19 by Dr. P. H. Gallimore, Department of Cancer Studies, University of Birmingham, Birmingham, U.K. Rabbit antisera against rat immunoglobulins and FITC-conjugated rabbit anti-rat IgG were from Miles, Stoke Poges, Slough, Berks., U.K. Bacterial protein A adsorbent was prepared as previously described (Kessler, 1975).

Buffers

Phosphate-buffered saline contained 150 mM-NaCl and 22 mM-sodium phosphate buffer, pH 7.1. Buffer A contained 20 mM-Tris/HCl, pH 7.5, 100 mM-NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS and 1 mM-phenylmethanesulphonyl fluoride.

Cell and virus growth

Human Hep2 cells and adenovirus type 5-transformed hamster H14b cells (Williams, 1973) were grown in monolayer culture in DMEM (Dulbecco-modified Eagle medium) with 10% (v/v) newborn-calf serum. F19 cells, a line of AS rat-embryo brain cells transformed by adenovirus type (Harwood & Gallimore, 1975), were grown in DMEM/JMEM (Joklik-modified Eagle medium) (1:1, v/v), containing 5% foetal-calf serum. The mouse myeloma NSO and the rat myeloma Y3.Ag.1.2.3 were grown in spinner culture in DMEM with 2.5% foetal-calf serum before use in cell fusion.

Human adenovirus types 2 and 5 were grown in Hela S₃ cells (maintained in spinner culture in JMEM with 5% newborn-calf serum). Virus was isolated and titrated as previously described (Russell *et al.*, 1967).

Cell labelling and immunoprecipitation

Confluent monolayers of Hep2 cells grown in 90 mm-diameter dishes were infected with adenovirus at a multiplicity of 50 plaque-forming units/cell and incubated for 8 h at 37°C. Metabolic

labelling of proteins was then performed for 3 h at 37°C in either methionine-free DMEM containing 50 µCi of [³⁵S]methionine/ml or phosphate-free DMEM containing 200 µCi of [³²P]orthophosphate/ml.

Labelled cells were washed in phosphate-buffered saline, resuspended in buffer A at a density of 10⁷ cells/ml and maintained on ice for 1 h. The cell lysates were then centrifuged at 15 000 g for 5 min at 4°C and the supernatant used for immunoprecipitation. A portion (10 µl) of serum or 200 µl of antibody-containing culture medium was incubated with 1.0 ml of cell supernatant at 4°C for 15 h. In certain cases 20 µg of rabbit anti-rat IgG was added and incubation was continued for a further 1 h at 4°C. Immune complexes were collected by adsorption to bacterial protein A adsorbent (Kessler, 1975). Immunoprecipitates were washed in buffer A and analysed by SDS/polyacrylamide-gel electrophoresis as previously described (Russell & Blair, 1977).

Production of sera and monoclonal antibodies

Tumour-bearing hamster sera were prepared by inoculating neonatal golden hamsters with 10⁶ H14b cells in 0.1 ml of phosphate-buffered saline. After approx. 3 weeks, palpable tumours were evident and serum was collected by cardiac puncture.

Female AS rats (4–6-weeks old) were immunized by subcutaneous injection with 10⁸ F19 cells emulsified with complete Freund's adjuvant. This was followed 2-weeks later by intraperitoneal injection with 10⁸ F19 cells in complete Freund's adjuvant. After a further 3 weeks, 10⁸ F19 cells sonicated in phosphate-buffered saline were introduced by intravenous injection. At 1 week after this injection, animals were bled from the tail vein and the test serum was analysed by immunoprecipitation using extracts of [³⁵S]methionine-labelled adenovirus-infected and uninfected Hep2 cells. Appropriate immune animals were then rested for at least 4 weeks. At 4 days before cell fusion, immune rats were boosted by intraperitoneal injection of 10⁸ F19 cells sonicated in phosphate-buffered saline and again with the same number of cells on the next day by intravenous route. After 3 days, splenocytes were prepared and fused with cells of either the mouse NSO or the rat Y3.Ag.1.2.3 line. Mouse-rat hybrid cells were prepared by the method of Davis *et al.* (1982) whereas rat-rat hybrids were prepared as described by Galfré & Milstein (1981). Culture supernatant medium was removed from wells containing vigorously growing hybrids and used to construct two-dimensional pools of supernatants by using the approach adopted by Cepko *et al.* (1981). Portions of these pools were used in

immunoprecipitation with extracts of [35 S]-methionine-labelled adenovirus-infected Hep2 cells.

Hybrids identified as positive in the immunoprecipitation assay were cloned in soft agar (Galfré & Milstein, 1981).

Indirect immunofluorescent-antibody staining

Hep2 cells were grown on 2 cm $\times$ 2 cm glass coverslips, infected with adenovirus and incubated for 8 h at 37°C. The coverslips were washed by dipping them in phosphate-buffered saline and they were fixed in 100% methanol at -20°C for 10 min. After further washing in phosphate-buffered saline, 25 μ l of antibody-containing culture medium was spotted and spread over the coverslip, which was then incubated for 60 min in a humidified box at 37°C. Coverslips were washed in phosphate-buffered saline and 25 μ l of a 1:50 dilution of FITC-conjugated rabbit anti-rat IgG was applied. After a further 60 min incubation, coverslips were washed in phosphate-buffered saline and mounted in gelvatol medium (Rodriguez & Deinhardt, 1960).

Determination of the class and subclass of secreted heavy chain

Heavy-chain class and subclass of secreted monoclonal antibody was determined by Ouchterlony double diffusion with monospecific antisera against rat immunoglobulins (kindly provided by Professor H. Bazin, Experimental Immunology Unit, University of Louvain, Louvain, Belgium). Culture medium was concentrated 10-fold for this assay with 50%-satd. (NH₄)₂SO₄ precipitation. The class and subclass of secreted immunoglobulin was also confirmed by immunoprecipitation as described by Richert (1982).

Translation in vitro of mRNA from adenovirus-infected cells

mRNA was isolated from adenovirus-infected Hep2 cells and translated in a micrococcal-nuclease-treated rabbit reticulocyte lysate as previously described (Zajdel Blair & Blair, 1981, 1982).

Results and discussion

Production of monoclonal antibodies to adenovirus transformation proteins

In order to elicit an immune response against adenovirus transformation proteins, syngeneic AS rats were immunized with whole-cell lysates of F19 cells, a line of adenovirus 2-transformed AS rat-embryo brain cells that contain only the transforming genes of the virus (Gallimore *et al.*, 1974). The antibody concentrations in individual rats

were assessed by immunoprecipitation with test sera and [35 S]-methionine-labelled cell extracts of adenovirus-infected and uninfected Hep2 cells (Fig. 1). Test rat sera precipitated polypeptides of 58 and 19 kDa from extracts of adenovirus-infected, but not from uninfected, Hep2 cells. Serum from hamsters bearing tumours induced by the adenovirus-transformed hamster cell line H14b precipitated a polypeptide of M_r identical with that of the 58 kDa species precipitated by immune rat sera. It may be noted that immune responses to the 58 and 19 kDa proteins varied between individuals; for example, test serum from rat 17 failed to immunoprecipitate the 19 kDa protein from infected cells (Fig. 1, track 6), whereas rat 16 clearly produced antibodies against the 19 kDa polypeptide. Some evidence was obtained, with sera from certain rats, for production of antibodies against a 21 kDa polypeptide (Fig. 1, track 6) described as a product of E1B (Matsuo *et al.*, 1982). It may then be concluded that immune responses can be induced in rats against the 58 and 19 kDa

Fig. 1. Proteins immunoprecipitated from extracts of [35 S]-methionine-labelled adenovirus-infected and uninfected Hep2 cells by immune sera

Immunoprecipitates were prepared and analysed by SDS/polyacrylamide-gel electrophoresis as described in the Experimental section. Track 1, methyl- 14 C-labelled marker proteins (Amersham) of M_r ($\times 10^{-3}$) 100, 92.5, 69, 46, 30 and 14.3. Tracks 2–6, immunoprecipitates using the following sources of antigen and antibody: track 2, infected cells and tumour-bearing-hamster serum; track 3, uninfected cells and test serum from rat 16; track 4, uninfected cells and test serum from rat 17; track 5, infected cells and test serum from rat 16; track 6, infected cells and test serum from rat 17.

polypeptides of E1B, and that individual animals may be chosen to given increased chances of isolation of monoclonal antibodies against either of these proteins.

The monoclonal antibodies described here resulted from three independent fusions, two using the mouse myeloma NSO with splenocytes from rats 16 and 17, and one using the rat myeloma Y3.Ag.1.2.3 and splenocytes from a rat that displayed an immune response against the 58 kDa protein. The rat-mouse fusions yielded the cell lines designated RA1, PA6 and EC3, whereas the cell line DC1 was derived from a rat-rat fusion. The frequency of isolation of hybrid cell lines secreting antibody recognizing adenovirus transformation proteins was low. These four cell lines were isolated from a total of approx. 300 growing hybrids.

Immunodiffusion analysis of culture media allowed the determination of the class and subclass of the secreted antibodies. In all cases the heavy chain belonged to the IgG class (Table 1). With the exception of the antibody secreted by the EC3 cell line, all antibodies bound to protein A.

Characteristics of a monoclonal antibody to the adenovirus 19 kDa protein

Antibody-containing culture medium from the EC3 cell line was used to study the biosynthesis of the 19 kDa protein of E1B *in vivo* and *in vitro* (Fig. 2). The 19 kDa protein represents a minor product of protein synthesis in adenovirus-infected Hep2 cells (Fig. 2, track 5). The EC3 antibody immunoprecipitated the 19 kDa protein from metabolically labelled infected cells, but not from uninfected cells (Fig. 2, tracks 9 and 6 respectively). Omission of rabbit anti-rat IgG in the immunoprecipitation procedure led to loss of the 19 kDa protein from the immunoprecipitate (Fig. 2, track 8), showing that the EC3 antibody did not bind to protein A. A pooled immune serum from rats reacted with both the 58 and 19 kDa proteins (Fig. 2, track 7). The

19 kDa protein appears to be an abundant product of translation of mRNA isolated from adenovirus-infected cells (Fig. 2, track 2). The 19 kDa protein synthesized *in vitro* could be immunoprecipitated by the pooled rat immune serum, and with much greater efficiency by the EC3 antibody (Fig. 2, tracks 3 and 4 respectively). It should be noted that the 58 kDa polypeptide is very poorly translated *in vitro*, as is shown by the failure to immunoprecipitate this entity from cell-free translation products, although it was readily precipitated from proteins synthesized *in vivo*. This is presumably the result of initiation of protein synthesis at the second AUG from the 5'-terminus of the mRNA encoding the 58 kDa protein (Bos *et al.*, 1981). Another point worthy of note is that there is no detectable difference in M_r between the 19 kDa protein synthesized during pulse-labelling *in vivo* and that synthesized *in vitro*, implying that post-translational processing events are not required for the generation of mature 19 kDa protein.

Fig. 2. Immunoprecipitation of the 19 kDa protein synthesized *in vitro* and *in vivo* by monoclonal antibody EC3. See the Experimental section for details. Track 1, ^{14}C -labelled marker proteins (see the legend to Fig. 1); track 2, total reticulocyte '*in vitro*' translation products synthesized by using [^{35}S]methionine, directed by polyadenylated RNA from adenovirus-infected Hep2 cells; track 3, immunoprecipitate of *in vitro* translation products with pooled immune rat serum; track 4, immunoprecipitate of *in vitro* translation products with EC3 culture medium; track 5, total proteins of adenovirus-infected Hep2 cells pulse-labelled with [^{35}S]methionine; track 6, uninfected Hep2 cells labelled with [^{35}S]methionine, immunoprecipitated with EC3 culture medium; tracks 7-9, labelled proteins of adenovirus-infected Hep2 cells immunoprecipitated with: track 7, pooled immune rat serum; track 8, EC3 culture medium, but omitting rabbit anti-rat IgG; track 9, EC3 culture medium including the rabbit anti-rat IgG addition.

Table 1. Immunochemical properties of the monoclonal antibodies

Antibody	Heavy-chain subclass*	Protein A binding†	Adenovirus E1B polypeptide recognized
RA1	γ_1	+	58 kDa
DC1	γ_{2b}	+	58 kDa
PA6	γ_{2a}	+	58 kDa
EC3	γ_{2b}	-	19 kDa

* Determined by Ouchterlony immunodiffusion.

† Determined by binding to bacterial protein A adsorbent.

Characteristics of monoclonal antibodies to the adenovirus E1B-58kDa protein

Culture medium from the RA1, PA6 and DC1 cell lines were used in immunoprecipitation experiments with uninfected and adenovirus-infected Hep2 cells (Fig. 3). Since the 58kDa protein is known to be phosphorylated (Levinson & Levine, 1976), cells were pulse-labelled with either [³⁵S]-methionine or [³²P]orthophosphate. The results showed that the RA1, PA6 and DC1 antibodies precipitated the 58kDa polypeptide labelled with [³⁵S]-methionine from infected, but not from uninfected, cells (Fig. 3a). No requirement from the addition of rabbit anti-rat IgG was noted, implying that all three antibodies bound to protein A. Antibodies RA1 and PA6 precipitated 58kDa phosphoproteins from infected, but not from uninfected, cells, the 58kDa phosphoprotein co-migrating in polyacrylamide gels with 58kDa species precipitated with tumour-bearing-hamster serum (Fig. 3b, track 3). The DC1 antibody also precipitated the phosphorylated 58kDa protein (results not shown). The antibody secreted by the PA6 cell line was notably less effective than either

RA1 or DC1 in precipitating the 58kDa protein. This may be due to a lower level of antibody production or a lower affinity of the PA6 antibody for protein A.

Immunocytochemical staining of adenovirus-infected Hep2 cells with monoclonal antibodies to adenovirus 19 and 58kDa proteins

The availability of monoclonal antibodies to adenovirus transformation proteins of 58 and 19kDa permitted an unequivocal assignment of the subcellular location of these proteins. Culture medium from the RA1 cell line was used in indirect immunofluorescent-antibody staining of fixed adenovirus-infected or uninfected Hep2 cells. An exclusively nuclear staining pattern was observed in infected cells (Fig. 4c and 4d), with discrete areas of the 58kDa protein evident within the nucleus. Essentially no cytoplasmic staining of infected cells was observed. However, culture medium from the EC3 cell line gave a mainly cytoplasmic and diffuse staining pattern. No staining of uninfected Hep2 cells was obtained with either RA1 or EC3 culture medium (results not shown).

Fig. 3. Immunoprecipitation of the 58kDa protein with monoclonal antibodies from extracts of adenovirus-infected Hep2 cells labelled with [³⁵S]-methionine or [³²P]-orthophosphate

See the Experimental section for details. (a) Track 1, ¹⁴C-labelled protein markers (see the legend to Fig. 1); tracks 2–5, uninfected Hep2 cells pulse-labelled with [³⁵S]-methionine, immunoprecipitated with: track 2, standard culture medium; track 3, DC1 culture medium; track 4, PA6 culture medium; and track 5, RA1 culture medium. Tracks 6–9, adenovirus-infected Hep2 cells pulse-labelled with [³⁵S]-methionine, immunoprecipitated with: track 6, standard culture medium; track 7, DC1 culture medium; track 8, PA6 culture medium; track 9, RA1 culture medium. (b) Track 1, ¹⁴C-labelled protein markers; tracks 2–9, immunoprecipitates from extracts of [³²P]-orthophosphate-labelled Hep2 cells using the following sources of antigen and antibody: track 2, uninfected cells and tumour-bearing-hamster serum; track 3, infected cells and tumour-bearing-hamster serum; track 4, uninfected cells and RA1 culture medium; track 5, infected cells and RA1 culture medium; track 6, uninfected cells and PA6 culture medium; track 7, infected cells and PA6 culture medium; track 8, uninfected cells and standard culture medium; track 9, infected cells and standard culture medium.

Fig. 4. Phase-contrast and fluorescence microscopy of adenovirus-infected Hep2 cells. In each pair, the micrograph on the right (b,d) is a phase-contrast view of the same area shown on the left with an FITC-conjugated second antibody (a,c). Micrographs (a) and (b) were obtained by using an initial incubation with EC3 culture medium; (c) and (d) were obtained by using RAI culture medium. Magnification $\times 800$.

Thus the 19 kDa transformation protein occupies a predominantly cytoplasmic location within the cell, whereas the 58 kDa protein is nuclear in location.

Conclusions

Here we describe the use of rat monoclonal antibodies to two proteins specified by the adenovirus transforming region in studies on their synthesis and subcellular location in virus-infected cells. The protein of 58 kDa, to which three monoclonal antibodies (RA1, PA6 and DC1) were produced, is a phosphoprotein located in the nucleus of infected cells. Perhaps this location reflects an as-yet-unidentified function for the 58 kDa protein in gene expression.

Other protein products of viral oncogenes, e.g. polyoma large T antigen, have a nuclear location within infected and transformed cells (Griffin & Dilworth, 1983) as do the products of the viral and cellular *myc* oncogenes (Alitalo *et al.*, 1983).

The 19 kDa protein is not phosphorylated (Gaynor *et al.*, 1982) and, by use of the monoclonal antibody EC3, was shown to be cytoplasmic in location. Proteins specified by the transforming genes of certain retroviruses have been shown to be associated with the plasma membrane of transformed cells (Willingham *et al.*, 1980). Further studies with the anti-(19 kDa protein) monoclonal antibody may define the location of the 19 kDa protein within infected and transformed cells with greater precision. Recent genetic studies on mis-sense mutants in the 19 kDa-protein gene have pointed to an essential role for this protein in cellular transformation (Chinnadurai, 1983).

Purification of the 58 and 19 kDa proteins by standard chromatographic techniques has proved difficult. Monoclonal antibodies to these proteins may therefore be important reagents in their purification, leading to a better understanding of their function in infected and transformed cells. Possible interactions between these viral proteins and cellular proteins or subcellular structures will also be amenable to analysis with monoclonal antibodies.

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