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Monoclonal antibodies against simian virus 40 nuclear large T tumour antigen: epitope mapping, papova virus cross-reaction and cell surface staining

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Thirty six cloned hybridomas have been isolated which produce monoclonal antibodies directed against simian virus 40 (SV40) large T tumour antigen. They have been shown to recognize at least six different epitopes along the T antigen polypeptide according to their reaction with the various truncated forms of T antigen expressed by adenovirus-SV40 hybrid viruses. Sixteen antibodies cross-react with cells infected by the closely related human BK virus. Only two antibodies, PAb1604 and PAb1614, directed against different epitopes of the SV40 T antigen, cross-react with polyoma large T tumour antigen which has a more limited amino acid sequence homology. This cross-reaction is rarely seen with polyclonal antibodies. Monoclonal antibody PAb1620 gave nuclear immunofluorescence only with murine cells transformed by SV40 and was found to react with a complex of T-antigen and 53 000-dalton host-coded protein. All the monoclonal antibodies react with nuclear T antigen and all but four antibodies stained the surface of SV40-transformed cells. These were four of the five antibodies directed against the central third of the T antigen. Thus the monoclonal antibodies show that cell surface T antigen differs from nuclear T antigen, either in accessibility or structure.

Key words: monoclonal antibodies/SV40/polyoma/BK virus/tumour antigen

Introduction

The early region of the genome of the DNA tumour virus simian virus 40 (SV40) codes for two tumour antigens which are involved in the early phase of lytic infection and for transformation and its maintenance (for review, see Tooze, 1980). The small t tumour antigen (20 000 daltons mol. wt.; 20 kd) is found in the cytoplasm and the large T tumour antigen (90 kd) is found mainly in the nucleus of transformed cells with a minor fraction (10%) on the cell surface (Soule *et al.*, 1982). The large T antigen is present in multiple forms which differ in their state of aggregation, phosphorylation, binding to DNA and complex formation with a host cell coded protein of 53–55 kd (p53) (Fanning *et al.*, 1981; Bradley *et al.*, 1982; Gidoni *et al.*, 1982) which is expressed in many cell lines transformed by various means. The large T antigen is a multifunctional protein (Tooze, 1980) and these properties may be restricted to certain subclasses of T antigen.

The functions of this transforming protein have previously been studied using deletion or temperature-sensitive mutants of the virus and micro-injection of T antigen (Tooze, 1980) or the construction of plasmids with various fragments of the genome coding for the T antigen (Clayton *et al.*, 1982b; Colby and Shenk, 1982). An alternative immunological approach has been the production of antisera against synthetic peptide fragments of the T antigen (Walter *et al.*, 1980) and more recently, the production of monoclonal antibodies against different regions of the tumour antigen (Martinis and Croce, 1978; Gurney *et al.*, 1980; Lane and Hoeffler, 1980; McKay, 1981; Harlow *et al.*, 1981). We have produced a panel of monoclonal antibodies against the SV40 T antigen to study the subclasses and functions of the T antigen. Here we describe the mapping of epitopes recognized by the monoclonal antibodies along the T antigen polypeptide using adenovirus-SV40 hybrid virus T antigen polypeptides. These antibodies have been further subdivided by their cross-reaction with the tumour antigens of related papova viruses, the human BK virus (BKV) and the polyoma virus of mice. To determine which regions of the T antigen are exposed at the cell surface, the reaction of the monoclonal antibodies with the surface of SV40-transformed cell lines was tested by indirect immunofluorescence. Finally, the antibodies were tested against several SV40-transformed cell lines to search for unique forms of T antigen since many cell lines are known to possess a truncated or larger (super-T) form of T antigen due to the deletion or repetition of intrinsic peptides (Chang *et al.*, 1979; Kress *et al.*, 1979; Smith *et al.*, 1979; Spangler *et al.*, 1980; Stitt and Mangel, 1981; Stitt *et al.*, 1981; Chaudry *et al.*, 1982; Lovett *et al.*, 1982; Clayton *et al.*, 1982a).

Results

Production and characterization of monoclonal antibodies

The supernatants from 45 out of 322 cultures derived from the fusion (see Materials and methods) gave nuclear staining of VLM cells by indirect immunofluorescence and was shown to be specific for SV40-transformed cells. These hybridoma cell cultures were cloned by limiting dilution and recloned as necessary, again screening by indirect immunofluorescence. Positive clones were recovered from 36 independent parent hybrids. The monoclonal antibodies produced have been characterized according to Table I. The majority of the antibodies are IgG1. With the exception of PAb1620 (to be discussed later) all gave nuclear staining by indirect immunofluorescence specific for SV40-transformed cells, being positive against VLM, ELONA, SV3T3, IDBR and SV80 and negative against BALB/c 3T3, NIH 3T3, 3T6, W49, VERO and BALB/c mouse embryo cells. They all immunoprecipitated the 90 000 dalton large T antigen from extracts of [^{35}S]methionine-labelled SV80 cells (Table II). Antibodies PAb1620, PAb1626 and PAb1633 precipitated relatively small amounts of T antigen from SV80 cells but efficiently precipitated T antigen from mKSA cells. SV80 cells overproduce free T antigen and no host-coded protein in the region of 55 kd was seen in the SV80 precipitations indicating

Table I. Characterization of the monoclonal antibodies

Monoclonal antibody[a]	Heavy chain class and light chain	Immunofluorescence						
		Nuclear			Surface	Nuclear[h]		
		SV40[b]	BKV[c]	Py[d]	VLM[e]	Ad2+ ND4	Ad2+ ND2	Ad2+ ND1
PAb1601	γ1, κ	+	+	−	++[g]	−	−	−
PAb1602	γ1, κ	+	+	−	+	+	−	−
PAb1603	γ1, κ	+	−	−	++	+	−	−
PAb1604	γ1, κ	+	+	+	++	+	−	−
PAb1605	γ1, κ	+	−	−	++	+	+	+
PAb1606	γ2a,κ	+	+	−	+	+	−	−
PAb1607	γ1, κ	+	−	−	++	−	−	−
PAb1608	γ1, κ	+	−	−	++	+	+	+
PAb1609	γ1, κ	+	−	−	++	+	−	−
PAb1610	γ1, κ	+	−	−	++	+	−	−
PAb1611	γ1, κ	+	−	−	+	−	−	−
PAb1612	γ1, κ	+	−	−	+	+	+	+
PAb1613	γ1, κ	+	−	−	++	+	−	−
PAb1614	γ1, κ	+	+	+	++	−	−	−
PAb1615	γ1, κ	+	−	−	+	−	−	−
PAb1616	γ1, κ	+	+	−	++	−	−	−
PAb1617	γ1, κ	+	+	−	+	+	+	+
PAb1618	γ1, κ	+	+	−	+	+	+	+
PAb1619	γ1, κ	+	−	−	++	+	−	−
PAb1620	γ2a,κ	(+)[f]	−	−	+	−	−	−
PAb1621	γ1, κ	+	+	−	++	+	+	+
PAb1622	γ1, κ	+	+	−	++	−	−	−
PAb1623	γ1, κ	+	+	−	−	+	+/−	−
PAb1624	γ1, κ	+	+	−	++	−	−	−
PAb1625	γ1, κ	+	−	−	++	+	−	−
PAb1626	γ1, κ	+	+	−	++	−	−	−
PAb1627	γ1, κ	+	−	−	++	+	+	+
PAb1628	γ1, κ	+	−	−	−	+	−	−
PAb1629	γ1, κ	+	+	−	++	+	+	+
PAb1630	γ2b,κ	+	−	−	+/−	+	−	−
PAb1631	γ1, κ	+	−	−	+	+	−	−
PAb1632	γ1, κ	+	−	−	++	+	+	+
PAb1633	γ1, κ	+	+	−	++	−	−	−
PAb1634	γ3, κ	+	−	−	+/−	+	+/−	−
PAb1635	γ1, λ	+	−	−	++	−	−	−
PAb1636	γ1, κ	+	+	−	+	+	+	+

[a]Nomenclature according to Crawford and Harlow (1982).
[b]SV40-transformed cell lines (see text).
[c]BKV-infected W49 cells.
[d]Polyoma-infected 3T6 cells.
[e]Indirect immunofluorescence of cell surface of EDTA harvested VLM cells.
[f]See text.
[g]Intensity of cell surface staining: ++, bright; +, weak; +/−, borderline; −, undetectable.
[h]Hybrid-virus infected Vero cells. Staining of Ad2+ND1 and Ad2+ND2 infected cells was perinuclear.

Table II. Epitope mapping by immunoprecipitation

Monoclonal antibodies	SV80 T	Ad2+ND4					Ad2+ND2		Ad2+ND1
	90K	85K	74K	72K	64K	60K	56K	42K	30K
PAb1601	+	−	−	−	−	−	−	−	−
PAb1602	+	+	+	+	−	−	−	−	−
PAb1603	+	+	+	−	−	−	−	−	−
PAb1604	+	+	+	+	−	−	−	−	−
PAb1605	+	+	+	+	+	+	+	+	+
PAb1606	+	+	+	+	−	−	−	−	−
PAb1607	+	−	−	−	−	−	−	−	−
PAb1608	+	+	+	+	+	+	+	+	+
PAb1609	+	+	+	−	−	−	−	−	−
PAb1610	+	+	+	+	−	−	−	−	−
PAb1611	+	−	−	−	−	−	−	−	−
PAb1612	+	+	+	+	+	+	+	+	+
PAb1613	+	+	+	+	+	+	−	−	−
PAb1614	+	−	−	−	−	−	−	−	−
PAb1615	+	−	−	−	−	−	−	−	−
PAb1616	+	−	−	−	−	−	−	−	−
PAb1617	+	+	+	+	+	+	+	+	+
PAb1618	+	+	+	+	+	+	+	+	+
PAb1619	+	+	+	−	−	−	−	−	−
PAb1620	+	−	−	−	−	−	−	−	−
PAb1621	+	+	+	+	+	+	+	+	+
PAb1622	+	−	−	−	−	−	−	−	−
PAb1623	+	+	+	+	+	+	+	−	−
PAb1624	+	−	−	−	−	−	−	−	−
PAb1625	+	+	+	−	−	−	−	−	−
PAb1626	+	−	−	−	−	−	−	−	−
PAb1627	+	+	+	+	+	+	+	+	+
PAb1628	+	+	+	+	+	+	+	−	−
PAb1629	+	+	+	+	+	+	+	+	+
PAb1630	+	+	+	+	+	+	+	−	−
PAb1631	+	+	+	+	+	+	+	−	−
PAb1632	+	+	+	+	+	+	+	+	+
PAb1633	+	−	−	−	−	−	−	−	−
PAb1634	+	+	+	+	+	+	+	−	−
PAb1635	+	−	−	−	−	−	−	−	−
PAb1636	+	+	+	+	+	+	+	+	+

no direct reaction against the host-coded protein in a complex with T antigen.

Mapping of antigenic determinants

To map the location of epitopes along the T antigen recognized by the panel of monoclonal antibodies, non-defective adenovirus-SV40 hybrid viruses were used (Tooze, 1980; Deppert *et al.*, 1981; Harlow *et al.*, 1981; Mercer *et al.*, 1983). The various lengths of the SV40 early region genome integrated into the adenovirus genome produce T antigen polypeptides of increasing length extending from the carboxy-terminal end towards the amino-terminal (Figure 1). This data is derived from Prives *et al.* (1982). The amino termini of the hybrid virus T antigen polypeptides are not precisely known. The Ad2+ND4, 60 K, 64 K, 72 K and 74 K polypeptides have not been mapped. The T antigen polypeptides produced by each hybrid virus is indicated in Table II. The 56 K and 42 K polypeptides are also produced by Ad2+ND4 but in low abundance. The reaction of the monoclonal antibodies with the T antigen polypeptides produced in hybrid-virus infected Vero cells was examined by immunofluorescence (Table I) and immunoprecipitation of extracts of [^{35}S]-methionine-labelled hybrid-virus infected HeLa cells (Table II). Controls with adenovirus wild-type infected cells were negative. The only discrepancy was that the group of antibodies precipitating the 56 K but not the 42 K T antigen polypeptide of Ad2+ND2 failed to give clearly positive immunofluorescence of Ad2+ND2 infected cells. As an example of the results of the mapping studies Figure 2 shows the immunoprecipitation of the T antigen polypeptides of Ad2+ND4 by various monoclonal antibodies. The antibodies could be divided into six groups according to whether the smallest T antigen polypeptide they could precipitate was the Ad2+ND1 30 K, Ad2+ND2 56 K, Ad2+ND4 60 K, 72 K or 74 K polypeptide or the complete 90 K T antigen.

Cross-reaction with tumour antigens of related papova viruses

To further subdivide the monoclonal antibodies mapping to the various regions of the T antigen they were examined by immunofluorescence for cross-reactions with the tumour antigens of polyoma virus and BKV, using infected 3T6 and W49 cells respectively (Table I). Sixteen antibodies stained the nuclei of BKV-infected cells. Only two (PAb1604 and PAb1614) gave nuclear staining of polyoma-infected cells and were directed against different regions of the SV40 T antigen according to the mapping studies. Control, uninfected cells were negative. Both antibodies immunoprecipitated a 92 kd polyoma large T antigen (Figure 3). In addition, PAb1614 precipitated a 53 kd protein from the polyoma-infected 3T6 cells which was not precipitated by anti-polyoma tumour sera. PAb1614 was not observed to precipitate a 53 kd protein from extracts of labelled uninfected 3T6 cells (not shown). It is not clear whether PAb1614 is directed against polyoma large T antigen or the 53 kd protein, as the two proteins could form a complex. Precipitation of polyoma middle T and small t antigen was not tested.

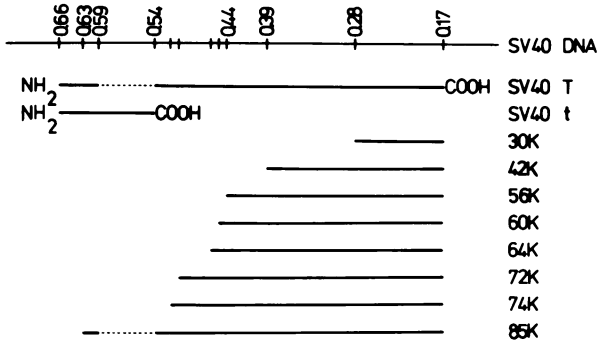

Fig. 1. The T antigen polypeptides produced by the adenovirus-SV40 hybrid viruses (and their mol wt. x 10^{-3}) together with the region, in map units, of the SV40 genome coding for them.

Antibodies against unique forms of T antigen

The VLM cell line used for immunization is known to contain normal and super-T antigen (Soule and Butel, 1979). In an attempt to isolate antibodies which react with a unique form of large T antigen all monoclonal antibodies were tested by nuclear immunofluorescence against several SV40-transformed cell lines, namely VLM, ELONA, SV80, IDBR and SV3T3. All were positive with the exception of PAb1620 which gave nuclear staining only of VLM, SV3T3 and in addition mKSA. It failed to stain BALB/c 3T3, VERO and hybrid-virus infected cells. This antibody could be directed against a form of T antigen unique to these three cell lines, or detect a determinant on T antigen or the p53 host protein unique to murine cells, or react with a determinant on T antigen restricted to certain strains of SV40 used for cell transformation. This was tested by immunoprecipitation. Antibody PAb1620 immunoprecipitated the 90 000-dalton large T antigen from the SV80 cell line with low efficiency. Using a labelled extract of mKSA cells, PAb1620 appeared to preferentially precipitate the T antigen complexed to a 53-kd protein (Figure 4) whereas anti-SV40 tumour sera precipitated

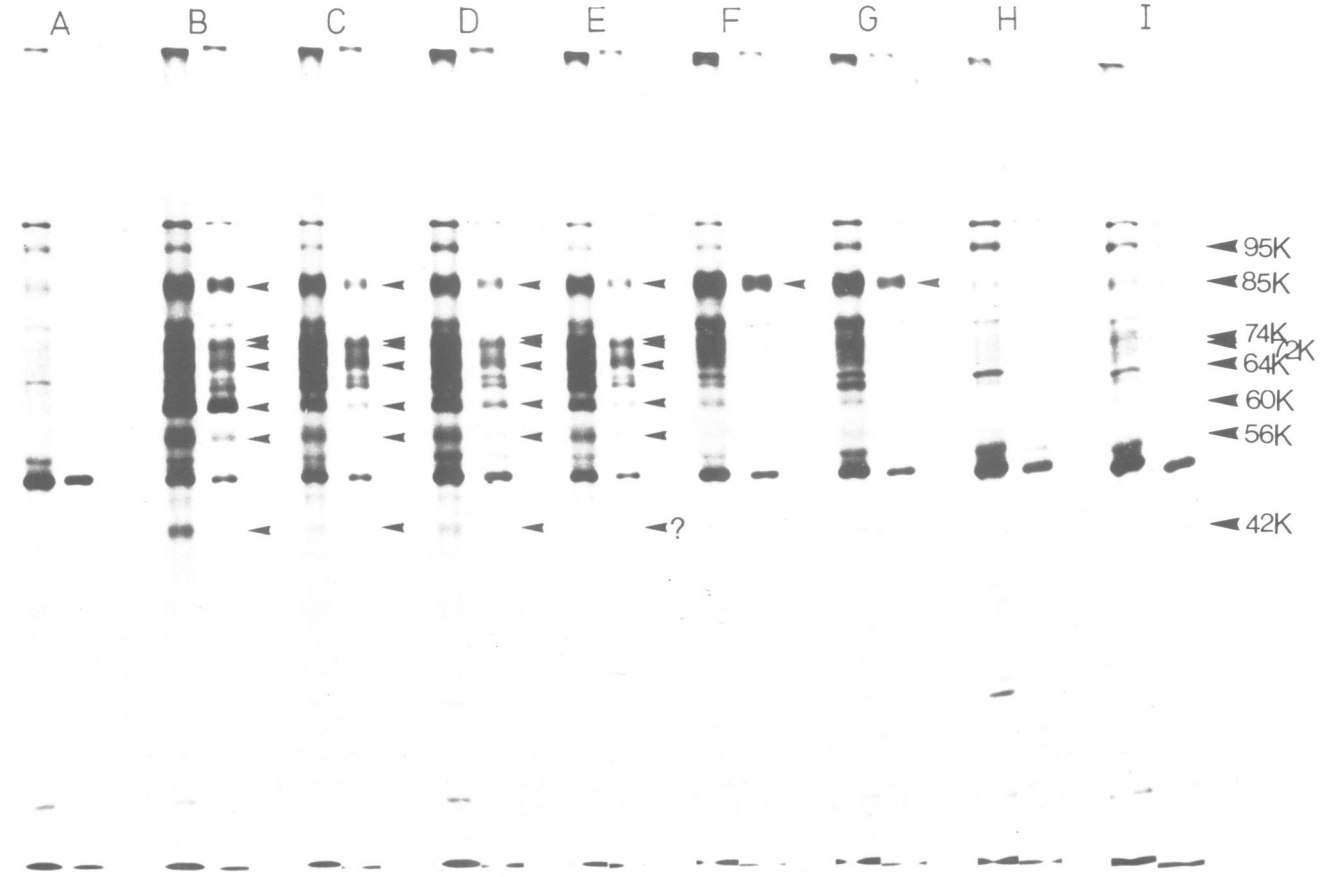

Fig. 2. Extract of [^{35}S]methionine-labelled, Ad2^{+}ND4 infected HeLa cells immunoprecipitated with **(A)** normal mouse serum, **(B)** BALB/c anti-VLM tumour serum, **(C)** PAb1608, **(D)** PAb1612, **(E)** PAb1634, **(F)** PAb1604, **(G)** PAb1619, **(H)** PAb1614, **(I)** PAb1607. A 10% SDS-polyacrylamide gel. Mol. wts. indicated on right. Prominent hybrid-virus T antigen polypeptides are indicated by arrows. Exposures of 48 h and 16 h in adjacent lanes.

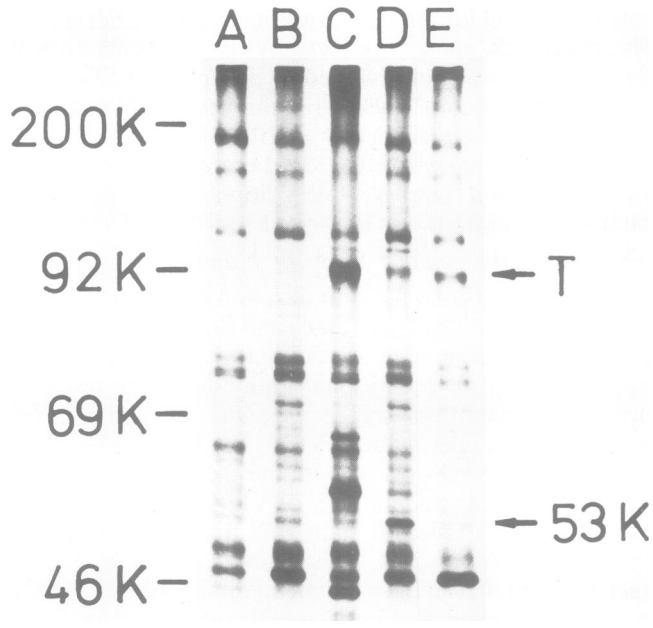

Fig. 3. Extract of [^{35}S]methionine-labelled, polyoma-infected 3T6 cells immunoprecipitated with **(A)** normal rat serum, **(B)** monoclonal antibody 25-36-F ($\gamma 1, \kappa$ with no SV40 T antigen reactivity), **(C)** rat anti-polyoma tumour serum, **(D)** PAb1614, **(E)** PAb1604. A 10% SDS-polyacrylamide gel. Mol. wt. of marker proteins (x 10^{-3}) on the left.

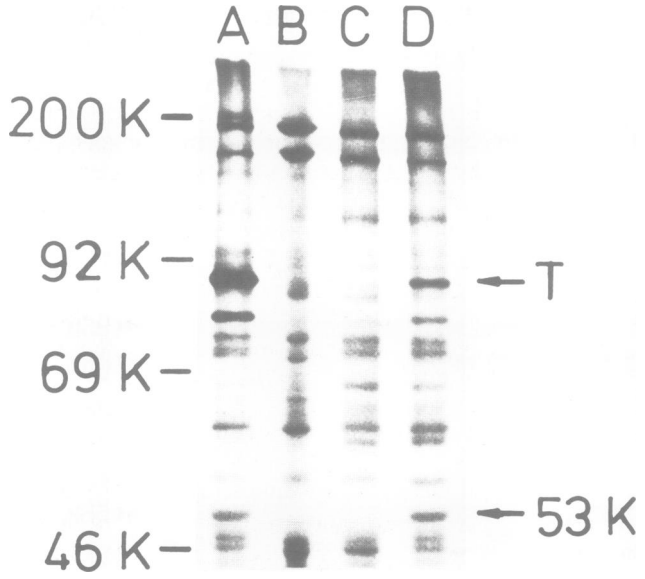

Fig. 4. Extract of [^{35}S]methionine-labelled mKSA cells immunoprecipitated with **(A)** BALB/c anti-VLM tumour serum, **(B)** normal mouse serum, **(C)** 25-36-F, **(D)** PAb1620, A 10% SDS-polyacrylamide gel.

more T antigen relative to the amount of 53-kd protein precipitated. The PAb122 monoclonal antibody (supplied by C.Burger, Konstanz) against the T antigen-associated 53-kd host-coded protein (Gurney *et al.*, 1980) was used to precipitate the p53-T antigen complex from mKSA cells and to directly precipitate p53 from Meth A cells but antibody PAb1620 did not precipitate p53 from Meth A (data not shown). PAb1620 has independently been found to precipitate p53 from Meth A (C.Burger and E.Fanning, personal communication). The immunofluorescence results may reflect the relative abundance of this antigen in different SV40-transformed cell lines.

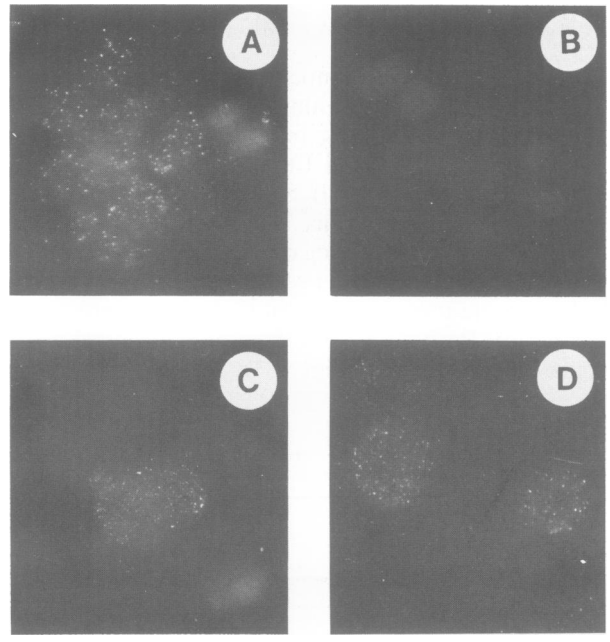

Fig. 5. Cell surface staining of VLM cells with anti-T antigen antibodies. EDTA harvested VLM cells were stained in suspension by indirect immunofluorescence using a 1:100 dilution of **(A)** anti-VLM tumour serum, **(B)** normal mouse serum, or by undiluted culture supernatant from **(C)** clone PAb1627 or **(D)** clone PAb1632 as described in Materials and methods. None of the antibodies stained control BALB/c 3T3 cells (not shown).

Cell surface staining of SV40-transformed cells

Sera from BALB/c mice bearing a solid subcutaneous tumour of VLM cells (anti-tumour sera) were found to give speckled staining of the cell surface of VLM cells by indirect immunofluorescence with a titre of 1 in 8000 (Figure 5). This staining was specific for SV40-transformed cells as VLM, SV80, mKSA and SV3T3 were positive and BALB/c 3T3, NIH 3T3, Meth A, B16/BL6, P815, F9 and PyA31 were negative. An exception was the failure to stain ELONA cells for unknown reasons. In addition to staining in suspension after harvesting with EDTA, VLM cells could be stained when mechanically harvested. In previous reports, fixed monolayer cells could be stained only with antisera against purified, SDS-denatured T antigen but not with anti-tumour sera, whereas unfixed cells were stained with neither. Cells harvested into suspension by various means could be stained with both antisera (Deppert *et al.*, 1981; Henning *et al.*, 1981; Lange-Mutschler *et al.*, 1981).

The tumour sera also immunoprecipitated T antigen and stained nuclear T antigen by immunofluorescence, but this is not conclusive proof that cell surface T antigen is recognized, as cross-reactions have also been found with antigens on the surface of foetal cells using anti-tumour sera (Shevinsky *et al.*, 1981). This was examined by testing culture supernatants from the panel of monoclonal antibodies. Table I shows that all but four of the antibodies gave cell surface immunofluorescence on VLM and were negative on control BALB/c 3T3 cells. The intensity of staining varied between experiments and the brightest staining obtained is recorded. The four monoclonal antibodies giving borderline (PAb1630, PAb1634) or no staining (PAb1623, PAb1628) were retested using culture supernatants concentrated 25-fold, or using antibody in the form of ascites fluid with a titre >1 in 10^3 for

Table III. Summary of epitope mapping, papova virus cross-reaction and surface T antigen staining of the panel of monoclonal antibodies

BK	Py	90 K	74 K	72 K	60 K	56 K	30 K
+	+	PAb1614		PAb1604			
+	−	PAb1601		PAb1602		PAb1623(sT$^-$)	PAb1617
		PAb1616		PAb1606			PAb1618
		PAb1622					PAb1621
		PAb1624					PAb1629
		PAb1626					PAb1636
		PAb1633					
−	−	PAb1607	PAb1603	PAb1610	PAb1613	PAb1628(sT$^-$)	PAb1605
		PAb1611	PAb1609			PAb1630(sT$^-$)	PAb1608
		PAb1615	PAb1619			PAb1631	PAb1612
		PAb1620	PAb1625			PAb1634(sT$^-$)	PAb1627
		PAb1635					PAb1632

nuclear T antigen staining, with similar negative results. These four antibodies also gave bright nuclear staining of fixed monolayers of SV40-transformed cells with a titre comparable to the rest of the panel of culture supernatants. These antibodies were four out of the five antibodies which precipitate the 56 K Ad2$^+$ND2 T antigen polypeptide and not the 42 K polypeptide. Thus, only a minority of the determinants recognized by the panel of monoclonal antibodies against nuclear T antigen are not detectable on the cell surface of SV40-transformed VLM cells. For all 36 monoclonal antibodies Table III presents a summary of the epitope mapping according to the smallest T antigen polypeptide immunoprecipitated, cross-reaction with BK and polyoma virus tumour antigens and inability to stain the cell surface of SV40-transformed cells (sT$^-$).

Discussion

The mapping of the epitopes recognized by the monoclonal antibodies was restricted to the smallest polypeptide that could be precipitated. These were six different polypeptides, hence there must be at least six different epitopes each contained within that polypeptide. Negative precipitation results were not used. It should be noted that only the amino-terminal boundary of the polypeptide sequence within which the determinant lies can be defined using the hybrid viruses. The group of antibodies which precipitate only the complete 90 K T antigen and not the largest 85 K polypeptide of Ad2$^+$ND4 may not necessarily map to the amino terminus of the T antigen as they may recognize conformational determinants not expressed on the hybrid-virus proteins. Direct evidence would be the precipitation of small t antigen being of identical amino acid sequence to the amino terminus of large T. The Ad2$^+$ND4 T antigen polypeptides of 60 K, 72 K and 74 K have an amino-terminal within the region between 0.44 and 0.54 map units. This effectively subdivides the T antigen epitope previously defined as the sequence present in the Ad2$^+$D2 107 K protein but absent from the Ad2$^+$ND2 56 K protein (Harlow *et al.*, 1981).

The antibodies reacting with each of these regions could be further subdivided according to whether they cross-reacted with the tumour antigens of the related papova viruses, BKV and polyoma. The amino acid sequences of the large T antigen of BKV and polyoma (proposed from the DNA sequence) show ~75% and 40% homology respectively with SV40 T antigen (Tooze, 1980; Friedmann *et al.*, 1979). Frequent serological cross-reactions would be expected with BKV (as seen in antisera) and indeed 16 out of the 36 monoclonal antibodies cross-react. In contrast, anti-tumour sera against SV40 T antigen normally fail to cross-react with polyoma. Similarly only a minority of two monoclonal antibodies from the panel, PAb1604 and PAb1614 (which are directed against different regions) cross-reacted with polyoma large T antigen. A similar cross-reaction has been reported where a rabbit antiserum against the D2 protein coded by the adenovirus-SV40 hybrid-virus Ad2$^+$D2 cross-reacted with polyoma large T and not middle or small t antigen (McCormick *et al.*, 1982). The interpretation of the structural basis of these cross-reactions is difficult since an antibody is restricted neither to linear amino acid homologies nor to a determinant in the same location as on the SV40 T antigen immunogen.

Large T antigen has been demonstrated at the cell surface both by lactoperoxidase-catalysed radioiodination (Soule *et al.*, 1982; Schmidt-Ullrich *et al.*, 1982) and by indirect immunofluorescence (Deppert *et al.*, 1980; Henning *et al.*, 1981; Deppert and Walter, 1982). To approach the questions of whether cell surface and nuclear T antigen are structurally identical and how much of the T antigen is exposed at the cell surface, the panel of monoclonal antibodies were examined for their ability to stain the cell surface of SV40-transformed cells. Serological differences were found between nuclear and surface T antigen since two of these anti-nuclear T antigen antibodies gave no detectable cell surface staining (PAb1623 and PAb1628) and two gave equivocal staining (PAb1630 and PAb1634), all four antibodies recognizing the 56 K but not the 42 K Ad2$^+$ND2 T polypeptide. Serological differences have previously been found where certain antisera immunoprecipitated surface and not nuclear T antigen (Soule *et al.*, 1980). Again, certain determinants on nuclear T antigen have been undetectable at the cell surface using peptide inhibition of staining of adenovirus-SV40 hybrid-virus infected cells with antisera (Deppert and Walter, 1982). These results have several possible interpretations. Surface T antigen may have a different conformation or even a different amino acid sequence. A candidate would be the so far undetected protein product of a third species of SV40 early mRNA (Mark and Berg, 1980) but nuclear and surface T antigen have recently been claimed to have peptide homology (Schmidt-Ullrich *et al.*, 1982). Certain determinants could be hidden because of steric hindrance due to association with the plasma membrane, glycosylation or complexing with adjacent membrane components. In one report surface T antigen has been shown to be a glycosylated form of nuclear T antigen (Schmidt-Ullrich *et al.*, 1982) which in addition forms a complex with a 55-kd, host-coded protein at the cell surface (Schmidt-Ullrich *et al.*, 1982; Santos and Butel, 1982). A fraction of T antigen in the plasma membrane has also been shown to be covalently bound to palmitic acid (Klockmann and Deppert, 1983). Subclasses of T antigen have been defined by their failure to react with particular monoclonal antibodies (Gurney *et al.*, 1980; Harlow *et al.*, 1981) hence the above results could also be explained by the absence of certain subclasses of T antigen from the cell surface.

Various technical problems should be noted. The process of fixation of the monolayer could lead to an increase in the expression of certain epitopes on nuclear T antigen or that certain determinants on cell surface T antigen are present below the level of detection of the assay. Cell surface T an-

tigen has been found to have a very short half-life of 2.5 h (Soule *et al.*, 1982) and extracts of T antigen readily adsorb to the cell surface (Lange-Mutschler and Henning, 1982). Since it is difficult to evaluate the leakage of nuclear T antigen it is not yet established whether T antigen present at the cell surface has been deliberately inserted as an intrinsic or extrinsic membrane protein or is merely passively adsorbed. The latter could possibly represent the situation of the release of T antigen in necrotic tumours. The reaction with the panel of monoclonal antibodies suggests that the majority of the serologically defined determinants on the T antigen are detectable at the cell surface with the exception of certain epitopes near the centre of the large T antigen. Each group of antibodies directed against any one of the six epitopes defined by the reaction pattern with various T antigen polypeptides has been further subdivided according to cross-reaction with related papova virus tumour antigens and staining of the cell surface of SV40-transformed cells. Such an approach indicated an even larger number of at least 13 epitopes although they have not yet been structurally defined.

Materials and methods

Cell lines, viruses and virus infection

The following SV40-transformed cell lines were obtained as indicated: VLM, mouse (BALB/c) embryo cells from W.Deppert; ELONA, hamster fibroblasts (Brandner *et al.*, 1977); SV80, human fibroblasts from E.Fanning; SV3T3, mouse (BALB/c 3T3) fibroblasts from G.Walter; IDBR, rabbit fibroblasts from G.Darai and mKSA-ASC, mouse (BALB/c) kidney cells from R.Henning. Other cell lines were obtained as follows: 3T6 and BALB/c 3T3, mouse fibroblasts from G.Walter; NIH 3T3, mouse fibroblasts from G.Schütz; PyA31, polyoma transformed BALB/c 3T3 fibroblasts from H.Werchau; Vero, African green monkey cells from Flow Laboratories; HeLa, human cervical carcinoma cells from K.Scheidtmann; W49, diploid human embryo fibroblasts from H.Berthold. A.Brownbill, Ciba-Geigy, supplied: Meth A, methylcholanthrene-induced BALB/c sarcoma; B16-BL6, C57BL/6 murine melanoma; P815, DBA/2 murine mastocytoma and F9, murine (129/SV) embryonal carcinoma cells. Cells were cultured in Dulbecco's modified Eagle's medium plus 10% heat-inactivated foetal calf serum, 50 μg/ml gentamycin, 100 units/ml penicillin and streptomycin, 4 mM L-glutamine, (DFCS). Cell lines were regularly checked for mycoplasma contamination using the Hoechst 33258 dye (Russell *et al.*, 1975).

Viruses were obtained as follows: BKV (strain VR-837) from the American Type Culture Collection; adenovirus 2 wild-type from H.zur Hausen; polyoma and the adenovirus-SV40 hybrid viruses $Ad2^+ND1$, $Ad2^+ND2$ and $Ad2^+ND4$ from G.Walter. Commencing with a 1-h period of serum-free virus adsorption 3T6 cells were infected with polyoma virus for 24 h, Vero or HeLa cells with adenovirus or adenovirus-SV40 hybrid virus for 40 h and W49 cells with BK virus for 5 days using monolayer cultures.

Production of monoclonal antibodies

Female BALB/c mice (Zentralinstitut für Versuchstiere, Hannover) each received a s.c. injection of 10^6 syngeneic VLM cells obtained from solid tumours and were boosted 4 weeks later with a s.c. injection of 10^7 *in vitro* cultured cells. Three days later their spleen cells were fused as described previously (Herbst and Braun, 1981) with the hybridoma cell line Sp2/0-Ag14 (immunoglobulin non-producing, Schulman *et al.*, 1978) kindly provided by Dr. G.Köhler. The fusion of cells from eight spleens was distributed in 322, 2-ml cultures in Costar tissue culture plates (type 3524), with the addition after the fusion of 2 x 10^5 BALB/c spleen cells/ml as filler cells in HAT medium (DFCS plus 13.6 mg/l hypoxanthine, 0.191 mg/l aminopterin, 3.87 mg/l thymidine). The hybridoma culture supernatants were screened 3 weeks later for nuclear staining of fixed VLM cells by indirect immunofluorescence. Cells from positive cultures were cloned by limiting dilution in 0.2 ml cultures in Costar 96-well tissue culture plates (type 3596) in HT medium, again using filler cells. Clones were screened 2 weeks later by nuclear immunofluorescence. Selected clones were adapted to DFCS medium and expanded in large cultures. Culture supernatants were concentrated by ultrafiltration using Minicon A25 or B125 concentrators (Amicon). BALB/c mice injected i.p. with 0.5 ml pristane mineral oil (Potter *et al.*, 1972) 4–14 days previously were given 10^6 hybridoma cells i.p. to produce high-titred ascites fluid. Anti-tumour antisera were prepared by immunizing BALB/c mice with VLM cells essentially as described for cell fusion. The heavy chain class and light chain of the monoclonal antibodies were determined by double diffusion using 1.5% agarose gels containing 2.5% polyethylene glycol (mol. wt. 6000) and rabbit antisera from Litton Bionetics (Kensington, MD), after concentrating the culture supernatant ten-fold.

Nuclear immunofluorescence assay

Subconfluent cells grown on acid-washed microscope slides were rinsed in PBS, fixed in methanol:acetone, 1:1 for 10 min at −20°C, air-dried and stored at −70°C. Within a grid drawn with nail polish, 5 μl of monoclonal antibody or antiserum were incubated for 1 h at 37°C then washed with PBS. This was followed by 15 μl of a 1/30 dilution of FITC-coupled rabbit anti-mouse immunoglobulin (Nordic) in BSS medium (balanced salt solution without glucose + 10% heat inactivated FCS + 0.2% NaN_3) incubating and washing as above. A coverslip was mounted with 60% glycerol in PBS with 0.064% NaN_3 and sealed with nail polish.

Cell surface immunofluorescence

The antibodies in culture supernatant or antisera were deaggregated by ultracentrifugation at 100 000 *g* for 10 min in a Beckman airfuge immediately prior to use. In the case of adherent cells, subconfluent cultures in plastic tissue culture flasks (150 cm^2) were washed with PBS and incubated for 10 min at room temperature with 5 ml of 0.1% EDTA in PBS (pH 7.2) with gentle lateral shaking to harvest the cells. The cells were immediately diluted in 25 ml of cold DFCS and washed three times in cold BSS medium. To ensure high viability the cells and reagents were kept at 4°C throughout. For the assay 2 x 10^5 cells in 100 μl BSS medium were distributed in V-bottom, 96-well rigid polystyrene microtiter plates, centrifuged at 120 *g* for 2 min and the supernatants removed by a short downward flick of the inverted plate. The cells were resuspended in 50 μl of the first antibody (monoclonal or antiserum), incubated for 30 min on ice, washed three times with 200 μl of BSS medium resuspending with a multichannel pipette. This was followed by 50 μl of a 1:10 dilution of FITC-coupled rabbit anti-mouse immunoglobulin antiserum with incubation and washing as before. After the last wash sufficient medium remains to resuspend the cells and use 5 μl for a wet mount. For fluorescence microscopy and photography a Zeiss microscope with epifluorescence illumination was used in combination with a x63 planapo oil immersion phase objective. Using Ilford HP5 (400 ASA) film immunofluorescence pictures were taken with a 30-s exposure.

Labelling, extraction and immunoprecipitation

Subconfluent cultures in 9 cm Petri dishes were washed with PBS and labelled for the last 4 h of culture with 300 μCi [^{35}S]methionine (800 Ci/mmol, Amersham), in 1 ml methionine-free Eagle's minimal essential medium plus gentamycin and 10% dialysed FCS. After washing with cold PBS the cultures were lysed with 1 ml lysis buffer (10 mM Tris, 0.14 M NaCl, 3 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 0.5% NP40, 1% Trasylol, Bayer, pH 8.0) on ice for 30 min. The extract was cleared by centrifuging 10 min in an Eppendorf centrifuge and 10^7 TCA-precipitable c.p.m. were used for each immunoprecipitation sample. 10 μl antiserum or 400 μl culture supernatant were added, incubating 90 min on ice, followed by 10 μl rabbit anti-mouse immunoglobulin (Nordic) for 1 h on ice. 50 μl of a pre-washed 10% w/v suspension of formaldehyde-fixed *Staphylococcus aureus* (Pansorbin, Calbiochem) in NET buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 7.4) were added and mixed for 30 min at 4°C. The bacteria were washed twice with NET wash buffer (NET + 0.5 M NaCl, 1% sodium deoxycholate, 0.5% NP40 and 30% w/v sucrose), twice with a 10-fold dilution of NET buffer and 50 μl of sample buffer (125 mM Tris, 4.5% SDS, 20% 2-mercaptoethanol, 10% glycerol, pH 6.8) were added and heated to 100°C for 3 min. Samples were run on 1 mm thick 10% SDS-polyacrylamide gels at 20 mA. Gels were fixed and stained with Coomassie Blue, destained and prepared for fluorography according to Bonner and Laskey (1974) using Kodak XOmat AR film.

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