Epithelial Origin of Polyoma Salivary Tumors in Mice: Evidence Based on Chromosome-Marked Cells

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of recipients on the basis of the predominant allotype marker of most clusters and the sizes of these clusters.

Clusters of immunocytes in the repopulated spleen arise as a result of cell proliferation; this is clear since the number of cells containing donor allotype in a recipient's spleen alone often exceeds the total number of transferred leukocytes (*4*). It is not known whether the precursors of these clusters are already committed to giving rise to daughters that produce only one parental allotype or the other. If the precursors are already committed before culture in vitro, the apparent stimulation of proliferation of b9 cells by exposure to antibody to b9 may be due to enhanced survival of their precursors before transfer, to an absolute increase in the number of such precursors during culture, or to an earlier initiation of proliferation and differentiation of b9 cells compared with b5 cells. It is known that antibodies to one parental allotype stimulate only a fraction of rabbit peripheral blood lymphocytes from heterozygotes to transform into blast cells (*9*). If the precursors of clusters formed in recipients' spleens are among those cells transformed, it may be that the action of antibodies to b9 is to initiate the proliferation and differentiation processes of appropriately committed cells in vitro before transfer.

Whether the transfer into an in vivo milieu allows the visualization of the eventual outcome of blast transformation of some cells in vitro is still unknown. Since discrete clusters of immunocytes can contain mixtures of cells making antibodies of different specificities, it is likely that they do not represent clones (*4*). The relative homogeneity of most of the donor cell clusters in both the control and experimental groups of recipients with respect to cells making one or the other parental marker suggests that some type of selective aggregation may occur in vivo after transfer or even before, during the in vitro culture period. The opposite presumption, that precursors may be uncommitted with respect to the allotype that their daughters will express, would change the interpretation of much of the above. Precursors may be influenced by antibody to b9 to give rise to daughters that produce b9 rather than b5 molecules. This mechanism should result in fewer b5 cells and molecules in recipients receiving treated cells compared with controls, but we have not found such a trend. The observations reported here may provide an insight into the mechanism of "allotype suppression" (*10*) which is the alteration in vivo of allelic expression resulting from the exposure of neo- or prenatal rabbits to antibodies to the corresponding allotype. Moreover the system may offer a practical approach to the selective expansion of immunocytes making a particular class, type, allotype, or idiotype of immunoglobulin from a mixed population of precursor cells.

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Epithelial Origin of Polyoma Salivary Tumors in Mice: Evidence Based on Chromosome-Marked Cells

Abstract. *Trypsin dissection of epithelium from mesenchyme of salivary gland rudiments allows reassembly of glands having either epithelium or mesenchyme selectively marked by T_6 chromosomes. Virus-induced tumors in such glands invariably bear the karyotype of the epithelial component. The method solves a specific example from a group of classical problems concerning epithelial as opposed to mesenchymal origin of neoplasms.*

Identification of tissue and cell type of origin of neoplasms has traditionally depended on recognition of one or more phenotypic characters of the neoplastic cells. These characters may be morphological, biochemical, or functional. For example, an islet cell tumor of pancreas may be characterized in part by the resemblance of its cells to β cells, by the demonstration of insulin production by the tumor, or by manifestations of hyperinsulinism in the host.

When specific characters are lacking, or when those available conflict with one another in nosologic significance, other methods of identification must be devised. We report here a method in which a karyotypic marker is used for the identification of the tissue type of origin of a problematic neoplastic group, that is, the polyoma virus–induced neoplasms of mouse submandibular gland.

The method (Fig. 1) combines use of the T_6 chromosome–marker system (*1*) with the trypsin technique (*2*) for separating epithelium from mesenchyme of gland rudiments. Given these laboratory devices, one can dissect apart the two tissue types of salivary gland rudiments and reassemble them so that either the epithelial or the mesenchymal component is karyotypically marked. This manipulation creates an exception to one of the limitations cited by Ford (*1*) in delineating the uses of chromosome markers: ". . . present methods of preparation do not permit the assignation of mitotic cells to different histological or hematological classes." Blastomeric fusion as performed by Mintz (*3*) offers another way to obtain tissues and organs with mixed karyotype, but does not permit precisely controlled segregation of a karyotypic marker within mesenchyme or epithelium of salivary rudiments.

Polyoma-free mice of strains C_3H/Bi and CBA/H-T_6T_6 were bred in our isolated colonies. Embryos were taken at 13 and 14 days of gestation, to supply submandibular gland rudiments at stages 2 to 5 of Borghese (*4*). Rudiments excised from embryos of both strains on the same morning were separately dis-

sected into epithelial and mesenchymal components by the trypsinization method of Grobstein (*2*). The T_6T_6-marked epithelial components, usually in groups of three or four, were then placed in close contact with comparable quantities of unmarked mesenchyme. Reciprocal reassemblies were similarly prepared. After a culture period of 18 hours at 36°C, during which mesenchyme reenveloped the epithelium, the reassembled rudiments were infected with passage 3 of polyoma virus (strain 2 PTA) (*5*). The reassemblies were implanted subcutaneously at the dorsal base of the tail in newborn F_1 hybrid mice of $C_3H/Bi \times CBA/H\text{-}T_6T_6$ and the reciprocal cross. Virus in a dose sufficient to induce indigenous salivary tumors in 30 percent of the recipients accompanied the transplants. Controls consisted of intact rudiments from each mouse strain, infected and transplanted as were the reassembled test rudiments.

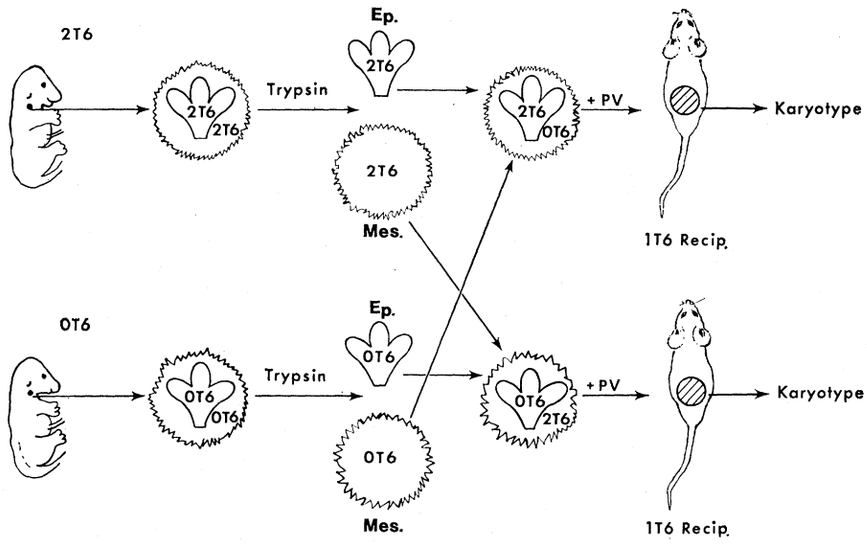

Fig. 1. Diagram of the experimental design. Salivary gland rudiments with either epithelium or mesenchyme marked by two T_6 chromosomes are constructed by recombination procedure. Tumors induced in these recombined rudiments are then karyotyped to determine whether tumors arise from epithelium or mescenchyme. See text for details.

Tumors appeared in the glands developed from reassembled rudiments in 8 of 100 recipients, after a latent period of 2 to 12 months. In the controls, tumors appeared in 6 of 37 recipients. Tumors induced in the intact or reassembled transplants were karyotyped, examined histologically, and transplanted serially to mice of F_1 hybrid and parental strains. These strains are histocompatible for the H^2 antigens. Cells from the spleen of each host were karyotyped to confirm the chromosomal status of the hosts.

A direct squash method for preparing chromosome spreads (*6*) was used because the utilization of air-dried suspensions yielded numerous host cells in mitosis, but too few tumor cells. The dividing host cells were seen in histologic sections and imprints to be part of the lymphoid infiltrate that is prominent in these tumors and probably represents local cellular immune response to polyoma-specific transplantation antigens. In squash preparations, an average of 20 percent of metaphases were in host lymphoid cells.

The control tumors confirmed the validity of the experimental design. They included four tumors that arose in rudiments transplanted intact, and one transplanted tumor that arose in the indigenous salivary gland of its F_1 hybrid host (Table 1). In addition to the five primary tumors, eight transplanted tumors in the first to fourth generations derived from this group were karyotyped, for a total of 13 tumor specimens. Metaphases acceptable for evaluation numbered 277, including 73 from three tumors in which the karyotypes of recipient and donor were identical. As expected, all of the latter 73 metaphases were consistent with both the donor and the recipient karyotypes. Among 204 metaphases examined in cases where a marker chromosome difference enabled distinction between host and donor, 32 metaphases of host cell karyotype were found. In 172 metaphases, the marker characteristics of the donor of the transplanted, intact rudiments were present, which identi-

Table 1. Karyotype identifications in test and contról tumors; pos., positive.

Tumor line	Markers in recombinant tissue		Markers in recipient	Mitoses examined (No.)	Markers recovered in tumors						Cell origin indicated
					$0T_6$		$1T_6$		$2T_6$		
	Epithelium	Mesenchyme			Cases pos./total	Mitoses (No.)	Cases pos./total	Mitoses (No.)	Cases pos./total	Mitoses (No.)	
					Control						
1	$2T_6$	$2T_6$	$0T_6$	73	2/2	8	0/2	0	2/2	65	Either epithelial or mesenchymal possible
2	$2T_6$	$2T_6$	$1T_6$	42	0/3	0	3/3	12	3/3	30	
3	$2T_6$	$2T_6$	$2T_6$	20	0/1	0	0/1	0	1/1	20	
4	$0T_6$	$0T_6$	$0T_6$	3	1/1	3	0/1	0	0/1	0	
5	$0T_6$	$0T_6$	$1T_6$	9	2/2	9	0/2	0	0/2	0	
6	$0T_6$	$0T_6$	$2T_6$	80	3/3	68	0/3	0	2/3	12	
7	$1T_6$	$1T_6$	$1T_6$	50	0/1	0	1/1	50	0/1	0	
					Test						
1	$2T_6$	$0T_6$	$1T_6$	154	0/3	0	3/3	19	3/3	135	Epithelial
2	$2T_6$	$0T_6$	$1T_6$	47	0/3	0	3/3	25	3/3	22	
3	$2T_6$	$0T_6$	$1T_6$	62	0/2	0	2/2	14	2/2	48	
4	$2T_6$	$0T_6$	$1T_6$	27	0/1	0	1/1	10	1/1	17	
5	$0T_6$	$2T_6$	$1T_6$	57	3/4	44	4/4	13	0/4	0	
6	$0T_6$	$2T_6$	$1T_6$	65	3/3	57	3/3	8	0/3	0	
7	$0T_6$	$2T_6$	$1T_6$	56	4/4	38	2/4	18	0/4	0	
8	$0T_6$	$2T_6$	$1T_6$	55	1/1	52	1/1	3	0/1	0	

fied the origin of the tumor from the graft. Aneuploidy was frequently present in cells of donor karyotype, but not in those of host karyotype.

Eight consecutive primary tumors arising in reassembled, transplanted glands comprised the test group. Four arose in reassemblies of CBA/H-T_6T_6 epithelium with C_3H/Bi mesenchyme, and the other four in the reciprocal recombination. Cytogenetic identifications (Fig. 2; A, C, and E) were made on all eight primary tumors and on 13 transplanted derivatives in their first and second generations in F_1 hosts. The results (Table 1) were similar, with certain differences to be noted.

Of 523 metaphase figures acceptable for evaluation in the 21 tumor specimens, 110 were identified as host cells on the basis of diploidy and a single T_6 chromosome. The remaining 413 were classified as tumor cells because the karyotype was that of one or the other of the donors of the two tissue types reassembled in the transplants. Further evidence of their neoplastic origin was the aneuploid condition of many cells in this category.

In every tumor of the test group, the karyotype of the tumor cells was that of the donor of the epithelial component. The karyotype of the mesenchyme donor was never recovered from the tumors.

Comparisons between tumors examined in serial transplant and primary tumors showed: (i) Aneuploidy was more prevalent in the tumor cell metaphases of transplants than in tumor cell metaphases of primaries; (ii) host cells included in the squash preparations of transplanted tumors retained their strictly euploid character; (iii) the proportion of host cells present in the serially transplanted tumors remained constant during three serial passages.

In the eight primary tumors in the test group, 14 of 186 tumor cell metaphases were aneuploid, whereas 89 of 198 tumor cell metaphases from first and second generation transplants were aneuploid. Metaphases with less than 40 chromosomes were not included as aneuploids because of the possibility of artifactual losses during handling.

In the first transplant generation of one tumor arising from a reassembly of T_6T_6-marked epithelium with unmarked mesenchyme, 42 of 49 tumor cells were hyperdiploid and contained four T_6 markers. The other seven contained two T_6 markers. In one of the control tumors, 4 of 30 tumor cells contained four T_6 markers, whereas the other 26 had only 2. Hence preferential reduplication of the markers sometimes occurred.

Histologically, all neoplasms at sites of transplant of intact and reassembled glands were indistinguishable from tumors induced by polyoma virus in indigenous salivary glands (Fig. 2, B, D, and F). They bore no resemblance to mammary tumors and occurred with equal frequency in male and female recipients.

The data are remarkably unambiguous considering that separation of epithelium from mesenchyme in this system cannot be absolute. Unquestionably a few cells of the opposite type contaminated some or all of the isolated components; the technique is not equivalent to cloning. Evidently the number of contaminating heterotypic cells was so small that the probability of their giving rise to neoplasms was reduced below detectability.

This work is relevant to the finding (*7*) that neither the epithelial nor the mesenchymal component of salivary gland can, in isolation from its opposite type, give rise to salivary tumors after infection with polyoma virus. That finding in fact necessitated the approach used here. It now appears that although mesenchyme plays an essential role during neoplastic transformation of epithelium, this role must be either permissive or inductive; mesenchyme evidently does not contribute cells to the neoplastic population.

Their epithelial origin notwithstanding, tumors of the type studied can substitute for normal mesenchyme in sustaining morphogenesis (*8*) and growth (*9*) of salivary rudiment epithelium. These and other observations

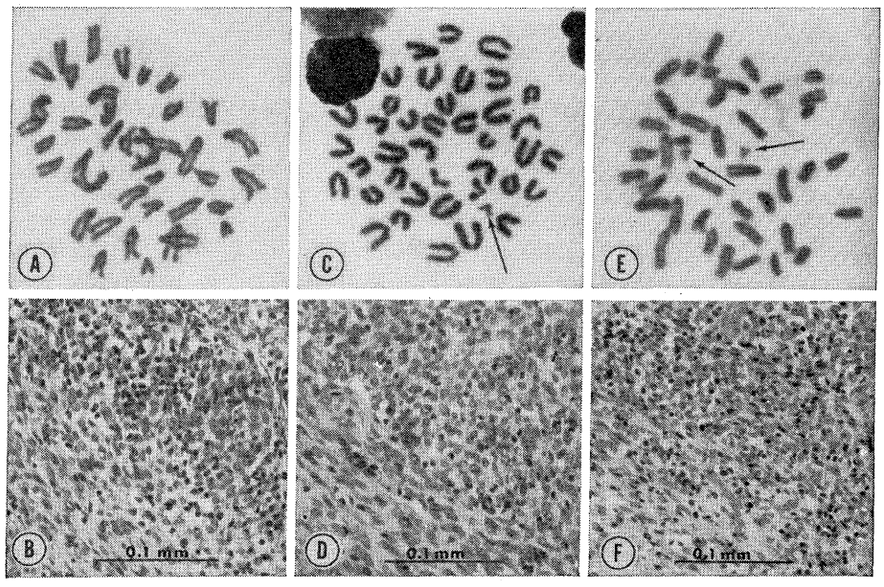

Fig. 2. Chromosome spread (A) and histologic section (B) from second transplant generation of a tumor induced in a recombination of T_6T_6-marked mesenchyme and unmarked epithelium. The metaphase figure shown is diploid ($2n = 40$) and contains no T_6 chromosome. In squash preparations from this tumor, 9 of 24 tumor cell metaphases were hyperdiploid, and none of the 24 contained any T_6 chromosomes. Three diploid metaphases each contained one T_6 chromosome, characteristic of normal cells of host origin. Histologically the tumor is typical of polyoma-induced salivary gland tumors, composed mainly of spindled and round cells in a mesenchymoid pattern. Small dark cells are lymphoid cells from which metaphase figures of host karyotype were derived. Stained with hematoxylin and eosin (× 175). Chromosome spread (C) illustrates a normal F_1 host spleen cell containing 39 normal chromosomes and one T_6 (arrow). Histologic section (D) is from a tumor in the F_1 host's indigenous salivary gland, for comparison with tumors in transplanted recombinations shown in (B) and (F). Histologically the tumors are the same, whether the cells bear one (D), two (F), or no (B) marker chromosomes. They are also the same regardless of origin from recombination experiments (B and F) or from intact, indigenous salivary gland (D). Stained with hematoxylin and eosin (× 175). Chromosome spread (E) and histologic section (F) from first transplant generation of a tumor induced in a recombination of T_6T_6-marked epithelium and unmarked mesenchyme. The metaphase figure shown is hyperdiploid ($2n + 2 = 42$) and contains two T_6 chromosomes (arrows). Among 29 metaphases from this tumor, 8 were true diploids with a single T_6 chromosome, whereas each of the remaining 21 had two T_6 chromosomes; 7 of these 21 were hyperdiploid. Histologically the tumor is identical with those illustrated in (B) and (D).

suggest a working hypothesis that oncogenesis in this system entails epithelial acquisition of the ability to produce growth-sustaining material(s) normally delivered and regulated by the supporting mesenchyme.

The information that host cells undergo division within the tumors raises the possibility of host cell recruitment into the neoplastic population, as shown by Ponten (*10*) in some RNA virus–tumor systems. The evidence is against this as follows: (i) host cells were euploid in transplant generations as well as in primaries, whereas donor (tumor) cells became more frequently aneuploid in serial transplant; (ii) the proportion of host cell metaphases did not increase progressively during one to four transplant generations; (iii) the histologic structure of the tumors was invariably typical of salivary gland tumors, and salivary gland tissue of the host could not have been present at the transplant sites; (iv) the latent period for appearance of transplants was shorter (as brief as 10 days) than that for tumor induction by virus; and (v) the dividing lymphoid (host) cells in the tumors offer a simpler, more consistent explanation.

Distinguishing between epithelial and connective tissue neoplasms is not usually difficult, but a number of classical problematic examples have perennially stimulated debate among histopathologists. In man, some of the most controversial examples include the mixed tumors of salivary glands (*11*). The procedure used in this study cannot be applied to human neoplasms, but it has yielded strong evidence that certain experimental tumors with predominant connective tissue features actually arise from epithelial cells. From events during embryogenesis it is known that epithelial and mesenchymal modes of cell existence are interconvertible under appropriate conditions (*12*).

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Ammonia Production in Muscle: The Purine Nucleotide Cycle

Abstract. *Experiments are reported which throw new light on the problem of ammonia production by muscle and, probably, by other tissues.*

Muscular work is accompanied by the production of ammonia (*1–8*). The adenylate deaminase (E.C. 3.5.4.6) reaction (*9*) is the major source of ammonia in muscle (*4, 7*) (Eq. 1).

Adenosine monophosphate (AMP) + H_2O → inosine monophosphate (IMP) + NH_3 (1)

The amount of ammonia produced by frog skeletal muscle is proportional to the work done by the muscle (*2, 6*). However, the deamination of AMP is not directly involved in muscular contraction (*10*). The regeneration of AMP from IMP occurs in two steps which are catalyzed by adenylosuccinate synthetase (E.C. 6.3.4.4) (*11–13*) and adenylosuccinase (E.C. 4.3.2.2) (*11, 13*) (Eqs. 2 and 3).

IMP + aspartate + guanosine triphosphate (GTP) → adenylosuccinate + guanosine diphosphate (GDP) + P_i (2)

adenylosuccinate → AMP + fumarate (3)

We report here that extracts of rat skeletal muscle produce ammonia from aspartate in a cyclical reaction sequence under conditions that mimic muscle doing work. The cycle consists of the reactions catalyzed by adenylate deaminase, adenylosuccinate synthetase, and adenylosuccinase. Supporting this conclusion is the finding that ammonia production from aspartate shows a catalytic requirement for either IMP, AMP, or adenylosuccinate. The net effect of the "purine nucleotide cycle" is the formation of fumarate and ammonia from aspartate, and, more indirectly, from glutamate. Possible metabolic functions of the cycle are discussed.

The following comments provide a rationale for the experimental approach which was adopted. Details of the experiments are given in the legends to the figures and table.

Adenylosuccinate, AMP, and IMP have strong absorption spectra. Although they overlap somewhat, these spectra can readily be distinguished from one another (Fig. 1). Changes in the relative amounts of the three nucleotides can be followed by obtaining ultraviolet absorption spectra as a function of time. The amounts of IMP, adenylosuccinate, and AMP present are then calculated from the extinction coefficient of each substance at various wavelengths. The wavelengths used were 281, 270, and 262.5 nm. The calculation of the concentration of each of the three nucleotides is particularly

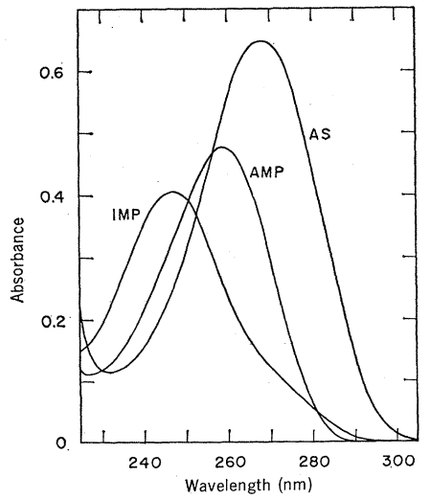

Fig. 1. Absorption spectra of IMP, AMP, and adenylosuccinate (AS). Spectra were obtained on a Cary split-beam, recording spectrophotometer with neutral solutions of 34.8 μM IMP, 32.1 μM AMP, and 34.1 μM adenylosuccinate in a cuvette with a 1-cm light path.