Mouse Mammary Tumour Virus and Polyoma Virus Information in Mammary Tumours of Athymic Mice Inoculated with Polyoma Virus

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SUMMARY

Polyoma virus inoculation of athymic mice results in the development of mammary tumours with a much higher incidence than the development of salivary gland tumours, the latter being the most common for immunocompetent normal mice. The possibility existed that polyoma virus might act as a co-carcinogen in activating the expression of mouse mammary tumour virus (MMTV). Molecular hybridization studies, however, showed that the mammary tumour development was accompanied by neither the amplification of MMTV genomic sequences nor by their more extensive transcription. In contrast, tumour tissue contained about 60 to 100 copies of polyoma virus genome equivalents per cell and some of these sequences were apparently transcribed into RNA. While these results do not rule out the transient involvement of MMTV expression in mammary tumour development, it appeared that the mammary gland cells were directly transformed by polyoma virus. Apparently, polyoma virus displayed a tropism in athymic mice that was different from that in normal mice.

INTRODUCTION

Polyoma virus in mice induces tumours in a relatively large but limited group of organs and tissues. Some of the tumours are epithelial in origin (e.g. salivary, mammary, thymic, ameloblastic, hair follicle) while others are mesenchymal (e.g. bone, renal medulla, subcutaneous connective tissue) (Dawe et al., 1959; Stanton et al., 1959; Dawe, 1967; Gross, 1970). Although the relative incidences of the various tumour types vary with mouse and virus strain, the most common site of origin has been the salivary gland (Dawe, 1967; Gross, 1970). Initially, it was found necessary to inoculate mice in the early neonatal period to induce tumours, presumably to escape cellular immune resistance. More recently, it has been possible to induce tumours in athymic mice inoculated with polyoma virus as adults (Allison & Taylor, 1967; Allison et al., 1974; Vandeputte et al., 1963, 1974). The relative incidence of the different tumour types varied in these studies.

In an unpublished work using NIH nu/nu mice inoculated with polyoma virus (strain PTA), Dawe and co-workers found the mammary tumour to be the predominant tumour in these athymic mice. Of a group of 32 female animals inoculated at 6 weeks of age, 22 developed mammary tumours and only 2 had salivary tumours at 22 weeks of age. Furthermore, they noted that the tumours arose directly from mammary duct epithelium, without the prior appearance of hyperplastic alveolar nodules (C. J. Dawe et al., unpublished results). A question, therefore, arose whether mouse mammary tumour virus (MMTV) played any part, either augmentative or essential, in the development of mammary tumours in adult

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athymic mice. It was possible that polyoma virus acted as a co-carcinogen in inducing endogenous MMTV, such as herpesviruses do for endogenous type C retrovirus in mouse cells (Hampar et al., 1976). Alternatively, polyoma virus could directly transform mammary gland cells resulting in neoplasia. This would imply that the preferential target cell for polyoma virus in athymic mouse is mammary epithelium while in normal mouse it is salivary epithelium. We have investigated the presence and expression of MMTV as well as polyoma virus information in mammary tumours of athymic mice by molecular hybridization. We find that mammary tumour development is not accompanied by an amplification of MMTV genomic sequences or their more extensive transcription. It appeared that mammary cells were directly transformed by polyoma virus, since they contained multiple copies of polyoma virus genomic sequences with some of these sequences being apparently transcribed into RNA.

METHODS

Tissues. Primary mammary tumours were induced by subcutaneous inoculation of NIH nu/nu female mice with polyoma virus, strain PTA (0.5 × 10⁸ TCID₅₀/mouse) at 6 weeks of age. The tumours developed in about 6 to 12 weeks thereafter and were excised when 2 to 3 cm in size. For transplanted tumours, mince of tumour tissue was used in place of polyoma virus and these were serially passaged. The tumour tissues and normal tissues of involved or uninvolved mice were either used immediately or stored frozen at -80 °C until used.

Isolation of DNA and RNA. DNA was isolated essentially as described previously (Arya & Yang, 1975; Strauchen et al., 1980). Briefly, minced tissue in 0.15 M-NaCl-0.15 M-sodium citrate-0.001 M-EDTA, pH 7 (SSCE) was gently disrupted in a Polytron homogenizer (Brinkman Instruments, Westbury, N.Y., U.S.A.). The suspension was made 2% in SDS, extracted several times with buffer-saturated phenol:chloroform:isoamyl alcohol (24:24:1), and ethanol-precipitated. The precipitate was dissolved in SSCE and digested with RNase A and RNase T₁ followed by digestion with Pronase. The solution was then repeatedly extracted with phenol:chloroform:isoamyl alcohol and DNA was ethanol-precipitated. The size of the DNA was reduced to about 300 to 400 residues by controlled sonication with a Branson Sonifier (Heat-Systems, Inc.). For isolation of RNA, minced tissue in 0.1 M-NaCl-0.01 M-sodium acetate pH 5.2-0.1% diethyl pyrocarbonate-100 μg/ml polyvinyl sulphate was homogenized as described above. It was extracted with phenol:chloroform:isoamyl alcohol and ethanol-precipitated. The precipitate was dissolved in a magnesium chloride-containing buffer and digested with DNase followed by digestion with Pronase. The mixture was again extracted with phenol:chloroform:isoamyl alcohol and RNA was ethanol-precipitated. The details of this procedure have been described previously (Arya 1980; Arya et al., 1980; Arya & Gordon, 1981).

Synthesis of MMTV complementary DNA. MMTV cDNA was synthesized by transcribing virus RNA with avian myeloblastosis virus reverse transcriptase using calf thymus DNA oligomers as primers (Arya & Young, 1980; Arya, 1981). The hybridization of ³H-labelled cDNA with MMTV ¹²⁵I-labelled RNA suggested that it contained a uniform representation of about 75 to 85% of virus genome; other sequences were less frequently represented. The specific activity of cDNA was estimated to be about 2 × 10⁷ ct/min/μg.

Preparation of labelled polyoma virus DNA probe. Labelled polyoma virus DNA probe was obtained by nick-translation of a cloned DNA containing a full length polyoma virus DNA insert in plasmid pBR322 DNA. The cloned DNA was kindly provided by Dr M. Israel of the U.S. National Institutes of Health. Nick-translation was carried out essentially as described by Maniatis et al. (1976) using 2 μM each of [³H]dCTP (65.6 Ci/mmol), [³H]dTTP (69.4 Ci/mmol), dGTP and dATP. The reaction was terminated by phenol:chloroform extraction when about 20% of the input radioactivity had been incorporated into DNA. The
Fig. 1. Kinetics of hybridization of labelled MMTV cDNA with athymic mouse DNA. A mixture of MMTV cDNA (5 ng/ml) and mouse DNA (2 to 4 mg/ml) in 2 × SSCE–0.02% SLS was incubated at 65 °C. Aliquots were withdrawn at intervals, diluted and digested with S1 nuclease. Hybridization was performed with DNA from primary mammary tumour (●), from transplanted mammary tumour (○), from normal liver (□) and from MMTV-producing murine Mm5mt/c1 cells (■).

extract was chromatographed on a Sephadex G-100 column and the column flow-through fractions were ethanol-precipitated. The precipitate was dissolved and subjected to 5 to 20% (w/w) alkaline sucrose density-gradient centrifugation. The molecules of less than 80 to 100 nucleotide residues were discarded and the rest of the DNA was collected by ethanol precipitation. The specific activity of the DNA was about 3 × 10⁶ ct/min/μg.

Hybridization. For kinetic experiments, a sample of RNA or denatured DNA in 0.01 M-SSCE was heated to 95 °C for 2 min and transferred to a bath at 65 °C. A sufficient amount of labelled cDNA or nick-translated DNA in 20 × SSCE–0.02% sodium lauroyl sarcosinate (SLS) was immediately added to obtain appropriate concentrations of the reactants in 2 × SSCE–0.02% SLS or 4 × SSCE–0.04% SLS. Aliquots were withdrawn at specified times, diluted, and digested with S1 nuclease. Self-annealing of cDNA (less than 2%) was monitored by simultaneous incubation of the probe with an appropriate amount of yeast RNA or salmon sperm DNA. The amount of cDNA that was S1 nuclease-resistant at zero time was less than 1% and that of nick-translated DNA was negligible. All Rₜ or C₀t values have been reduced to standard conditions of 0.18 m-Na⁺ and 60 °C (Britten et al., 1974). Hybridization of nude mouse RNA with polyoma virus DNA probe was performed by incubating labelled probe with increasing amounts of RNA in SSCE–0.02% SLS at 65 °C for 20 h. The hybrids were scored by S1 nuclease digestion.

RESULTS

MMTV-specific genomic sequences in tumour cells

The content of MMTV-specific sequences in tissue DNAs was determined by hybridizing cellular DNAs with MMTV cDNA. The C₀t₁/₂ of hybridization of DNA from primary mammary tumour, from transplanted mammary tumour (third passage) and from normal liver was about 1000, with primary tumour DNA driving the reaction somewhat faster than other DNAs (Fig. 1). The control DNA from MMTV-producing murine cells (Mm5mt/c1) gave a C₀t of about 80. The C₀t₁/₂ of single copy mammalian DNA (2 copies/diploid genome) under these conditions was determined to be about 2400 (S. K. Arya, unpublished results). These results thus suggested that tumour tissue DNA on average contained about
five to six MMTV genome equivalents per diploid cell genome. Normal liver DNA contained almost the same amount of MMTV-specific sequences as did tumour tissue DNA. Whereas the DNA from MMTV-producing control cells hybridized to the extent of 90% with MMTV cDNA, the hybridization with tissue DNA seemed to be approaching a limiting value around 80% (Fig. 1). This suggested that some of the MMTV-specific sequences may be absent or present less frequently in tissue DNAs. Alternatively, MMTV-specific sequences in tissue DNAs may not be completely homologous with the strain of MMTV used for generating the probe.

**MMTV-specific RNA in tumour cells**

To determine if mammary tumour tissue contained MMTV-specific RNA, the kinetics of hybridization of tumour tissue as well as normal liver RNA with MMTV cDNA was determined (Fig. 2). The tissue RNA was in large mass excess over cDNA in these reactions. As controls, MMTV RNA and RNA from MMTV-producing murine cells (Mm5mt/c1) were used (Fig. 2). The \( R_{0.5} \) of homologous hybridization of virus RNA with its cDNA was about \( 1 \times 10^{-2} \). The \( R_{0.5} \) of RNA from primary mammary tumour, from transplanted mammary tumour (third passage) and from normal liver was higher than \( 2 \times 10^5 \). The results suggested that only a very small fraction of tissue RNA was MMTV-specific. Taking the cell RNA content to be 10 pg and mol. wt. of MMTV RNA to be \( 3 \times 10^6 \), we estimate that one copy of MMTV RNA per cell would give a \( R_{0.5} \) of \( 2 \times 10^4 \). Since this \( R_{0.5} \) was not achieved for any of the tissue RNA up to a \( R_{0.5} \) of \( 2 \times 10^5 \), the results suggested that the tissues contained less than one copy of MMTV RNA per cell.

**Polyoma virus-specific genomic sequences in tumour cells**

The fraction of tissue DNA that was polyoma virus-specific was evaluated by determining the acceleration in the rate of self-annealing of polyoma virus probe caused by tissue DNA (Fig. 3). As noted in Methods, polyoma virus probe, generated by \textit{in vitro} nick-translation, was composed of full length polyoma virus and plasmid \textit{pBR} 322 DNA, of which about 60%
Table 1. Tissue DNA caused acceleration in the rate of self-annealing of polyoma virus probe

<table>
<thead>
<tr>
<th>DNA</th>
<th>Observed ( C_{0.5} )</th>
<th>Polyoma DNA ( C_{0.5} )</th>
<th>Rate factor</th>
<th>No. of virus genome equivalents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe plus salmon sperm DNA</td>
<td>( 1.0 \times 10^{-2} )</td>
<td>( 6.0 \times 10^{-3} )</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Probe plus primary tumour DNA (10 ( \mu )g)</td>
<td>( 3.5 \times 10^{-3} )</td>
<td>( 2.1 \times 10^{-3} )</td>
<td>2.8</td>
<td>65</td>
</tr>
<tr>
<td>Probe plus primary tumour DNA (33.3 ( \mu )g)</td>
<td>( 1.4 \times 10^{-3} )</td>
<td>( 8.4 \times 10^{-4} )</td>
<td>7.1</td>
<td>70</td>
</tr>
<tr>
<td>Probe plus transplanted tumour DNA (33.3 ( \mu )g)</td>
<td>( 1.0 \times 10^{-3} )</td>
<td>( 6.0 \times 10^{-4} )</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Probe plus normal liver DNA (33.3 ( \mu )g)</td>
<td>( 1.1 \times 10^{-3} )</td>
<td>( 6.8 \times 10^{-4} )</td>
<td>8.8</td>
<td>88</td>
</tr>
</tbody>
</table>

was polyoma virus DNA. As shown in Fig. 3, annealing in the presence of tumour tissue DNA gave biphasic \( C_{0.5} \) curves suggesting that these DNAs accelerated the rate of self-annealing of only a part of the probe. This was anticipated because tissue DNAs were not expected to contain plasmid pBR322 DNA sequences. The \( C_{0.5, t} \) of total probe was about \( 1 \times 10^{-2} \) and about 60% of the total sequence complexity of the probe was due to polyoma virus DNA. The \( C_{0.5, t} \) of the probe if it was composed of only polyoma virus DNA would be thus \( 6 \times 10^{-3} \) (\( 1 \times 10^{-2} \times 0.6 \)). The tissue DNAs increased the \( C_{0.5, t} \) of the probe to varying degrees depending on the source and amount of tissue DNA (Table 1). The number of virus DNA copies per cell can be computed from the acceleration in the rate of self-annealing of probe caused by a given amount of added heterologous DNA (Gelb et al., 1971). These computations are presented in Table 1. The results showed that tumour DNAs contained 60 to 100 copies of polyoma virus genome per cell. The normal liver DNA apparently did not contain polyoma virus DNA sequences.

**Polyoma virus-specific RNA in tumour cells**

When polyoma virus probe was hybridized with increasing amounts of tumour cell RNA, more and more of the probe entered into hybrid form (Fig. 4). At saturating amounts of primary mammary tumour RNA or RNA from a mixture of first and third passage transplanted mammary tumours, about 25% of the polyoma virus probe was hybridized. This figure must be corrected for the fact that only about 60% of the probe was polyoma virus-specific. Thus, tumour cell RNAs appeared to contain transcripts corresponding to about 40% of the virus genome. (As noted above, tumour cells did not contain sequences corresponding to plasmid pBR322 genetic information.) In contrast, normal liver RNA failed to convert a significant amount of polyoma virus probe into hybrid form (Fig. 4), suggesting that normal liver did not contain a detectable amount of RNA transcripts of polyoma virus DNA.

**DISCUSSION**

Since polyoma virus inoculation of athymic mice resulted in a high incidence of mammary tumours, we examined the possibility that this oncogenic DNA virus might be acting as a co-carcinogen in activating endogenous MMTV, a type B retrovirus. This seemed possible because of the reported induction of endogenous type C retrovirus by herpesviruses (Hampar et al., 1976). An examination of the tumour cell DNA for MMTV-specific sequences showed that it contained about five to six copies of MMTV genome equivalents per cell genome. The number of MMTV genome equivalents in tumour cell DNA was essentially the same as in normal liver DNA. Similar results have been previously reported for other species of mice, particularly for those with low incidence of mammary tumours (Morris et al., 1977; Michalides et al., 1979). Although small differences in the virus DNA content of cells
Fig. 3. Kinetics of reannealing of labelled cloned polyoma virus plasmid pBR322 DNA in the presence and absence of athymic mouse DNA. The mixture contained 6.6 × 10⁻² µg/ml polyoma virus probe DNA and salmon sperm DNA (○, I or 3 mg/ml), primary mammary tumour DNA (■, 1 mg/ml; †, 3.33 mg/ml), transplanted mammary tumour DNA (■, 3.3 mg/ml) or normal liver DNA (○, 3.33 mg/ml). The mixture in 2 × SSCE-0.02% SLS was incubated at 65 °C, aliquots were withdrawn at intervals, diluted, and digested with S1 nuclease.

Fig. 4. Hybridization of labelled cloned polyoma virus plasmid pBR322 DNA with athymic mouse RNA. A mixture containing 1.6 × 10⁻⁴ µg polyoma virus DNA probe and varying amounts of nude mouse RNA in 100 µl SSCE-0.02% SLS was incubated at 65 °C for 20 h. The mixture was diluted and digested with S1 nuclease. Hybridization was performed with RNA from primary mammary tumour (○), from transplanted mammary tumour (●) and from normal liver (■).

may go undetected by Cot analysis, there was no indication that selective gene amplification of the MMTV genome occurred either during tumour development or serial passage of the tumour cells by transplantation. The presence and transcription of one or a few copies of MMTV genome per cell, or parts thereof, may be sufficient for the development or maintenance of the tumourigenic potential. However, investigation of the steady state levels of MMTV-specific RNA in tumour tissues showed that they contained on average less than one copy of MMTV-specific RNA per cell. Low levels of MMTV transcription in normal tissues as well as in mammary tumours of non-viral aetiologies have been reported previously (Dudley et al., 1978; Michalides et al., 1979). Furthermore, only a weak correlation between MMTV transcription in mammary tissue and incidence of mammary tumours in several strains of mice has been noted (Varmus et al., 1973). It has been recently reported that mammary tumour development in Balb/c mice, due to urethane and/or X-ray treatments, is accompanied by increased MMTV transcription (Michalides et al., 1979). Our results, on the other hand, show that the maintenance of the mammary tumourigenic potential in athymic mice as a result of polyoma virus infection is not accompanied by an amplification of MMTV genome sequences or their transcription into stable RNA species. These studies do not rule out the possibility that the initiation of mammary tumour development may have involved transient activation of MMTV expression. These negative findings, however, may be consistent with the observation that mammary tumours in these mice arose directly from mammary epithelium without the prior appearance of hyperplastic alveolar nodules, the latter generally being the case for MMTV-induced tumours.

In contrast to the case for MMTV sequences, polyoma virus DNA sequences were readily detected in mammary tumour tissues. The primary tumour tissue contained on average about 60 to 70 virus genome equivalents per cell genome and the transplanted tumour tissue appeared to contain more virus DNA sequences than the primary tumour. In the previously studied cases of polyoma virus-transformed cultured cells, the number of virus genomes per
cell was generally much smaller than noted above and rat cells transformed by polyoma virus apparently contain integrated as well as unintegrated polyoma virus DNA sequences (Westphal & Dulbecco, 1968; Kamen et al., 1974; Prasad et al., 1976; Basilico et al., 1979; Birg et al., 1979). We have recently examined the state of polyoma virus DNA in some of the tumours by restriction analysis. Preliminary results showed that tumour DNAs contain multiple tandem repeats of virus DNA integrated in the cell genome. In addition, some tumour DNAs contained unintegrated virus DNAs. Some of the molecules had deletions corresponding to the carboxy end of large T antigen gene sequences. The details of these results will be presented elsewhere. Furthermore, some of the polyoma virus DNA sequences in the mammary tumours were apparently transcribed. The polyoma virus-specific RNA sequences in tumour tissues corresponded to about 40% of the virus genome. This is more than expected if only the early region of polyoma virus genome is transcribed asymmetrically (Kamen et al., 1974). Since we used total cellular RNA instead of cytoplasmic RNA for hybridization experiments, it is probable that tumour tissue RNA contains RNA transcripts of other regions of the polyoma virus genome. The nuclear RNA of polyoma virus-transformed cultured cells apparently contains transcripts not only of early and late regions of virus genome but also of the anti-early and anti-late transcripts (Kamen et al., 1974; Kamen & Shure, 1976; Birg et al., 1977). Further studies on the identification of polyoma virus sequences that are transcribed in these tumours are now in progress. Thus, athymic mouse mammary tumour cells qualitatively resembled previously studied polyoma virus-transformed cells. Although we cannot rule out the transient involvement of MMTV expression in mammary tumour development, these epithelium-derived tumour cells showed biochemical characteristics similar to other polyoma virus-transformed cells.

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