No Evidence for Particles
Encapsulating RNA-instructed DNA Polymerase and High Molecular Weight Virus-related RNA in Herpesvirus Induced Tumours of Non-human Primates

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SUMMARY

The simultaneous detection test gave no evidence for the presence of RNA tumour viruses in herpesvirus induced malignant lymphomas of non-human primates. The 12 tumours tested were obtained from three different monkey species inoculated with *Herpesvirus saimiri* or *Herpesvirus ateles*. Particles encapsulating RNA-instructed DNA polymerase and high mol. wt. virus-related RNA were easily demonstrated in tumours of the mouse induced by type-C or type-B oncornaviruses and in human lymphoid cells infected with simian sarcoma virus type 1 which were examined in parallel. Attempts to demonstrate partial expression of an oncornavirus genome in the herpesvirus induced tumours and attempts to detect an interspecies antigen related to monkey oncornaviruses were negative and strengthened the observations made with the simultaneous detection test.

The detection of particles with properties of RNA tumour viruses in Burkitt's tumours and in tumours of the Marek's disease was recently reported (Kufe et al. 1973; Peters et al. 1973). Since both tumours are closely linked in their etiology with herpesviruses (Klein, 1972), the detection of these particles raises the question if oncornaviruses are necessary as a co-factor in the etiology of herpesvirus induced tumours. To explore this possibility the tumorous spleens or lymph nodes from 12 non-human primates with malignant lymphoma induced by oncogenic herpesviruses (*Herpesvirus saimiri* and *H. ateles*) (Laufs & Fleckenstein, 1973; Laufs & Meléndez, 1973) were tested for the presence of particles encapsulating RNA-instructed DNA polymerase and high mol. wt. virus-related RNA.

The RNA-instructed DNA polymerase and high mol. wt. 70S RNA are two diagnostic features of the RNA tumour viruses. Their simultaneous detection is possible with a new technique (Schlom & Spiegelman, 1971; Gulati, Axel & Spiegelman, 1972). Briefly in this test the [3H]-DNA transcript synthesized *in vitro* by the reverse transcriptase is demonstrated as a complex with the virus 70S RNA template. To determine the sensitivity of this technique in our laboratory tumorous spleens of BALB/c mice infected with Rauscher leukaemia virus (RLV, kindly supplied by Dr D. Kufe, Institute of Cancer Research, Columbia University, N.Y.), mouse mammary carcinoma tissue from C3H mice infected with mouse mammary tumour virus (MMTV) and NC-37 cells carrying simian sarcoma virus type 1 (SSV-1, kindly supplied by Pfizer Inc., Maywood, New Jersey) were examined in parallel with the monkey tumours for the presence of RNA tumour viruses. The monkey tumours included: 4 induced with *Herpesvirus saimiri* (HVS) in CT-marmosets (*Saguinus oedipus*), 2 induced with *H. ateles* (HVA) in CT-marmosets,
3. Detection of 70S RNA-[\(^3\)H]-DNA (O—O) in the tumorous spleen of a mouse (a) infected with Rauscher leukaemia virus (RLV) and in a mouse mammary carcinoma (b) induced by mouse mammary tumour virus (MMTV). The deproteinized reaction mixture was sedimented in a linear glycerol velocity gradient (9 to 26% w/w in TNE) at 40,000 rev/min at 4 °C for 3 h (Spinco ultracentrifuge, rotor type 41). External marker was [\(^3\)H]-70S RNA from simian sarcoma virus. Fractions were collected from the bottom and assayed for C\(_{12}\)COOH-precipitable radioactivity. Half of the reaction mixture was incubated in the presence of RNase A (50 \(\mu\)g/ml) and RNase T1 (50 \(\mu\)g/ml) for 15 min at 37 °C before sedimentation analysis (○—○).

4. induced with HVA in CJ-marmosets (Callithrix jacchus) and 2 induced with HVS in owl monkeys (Aotus trivirgatus). In each case the oncogenic herpesvirus was re-isolated from the tumour.

A cytoplasmic extract was prepared from 1 to 2 g of finely minced tumour tissue or of 1 to 2 ml of packed cells in 10 ml TNE buffer (0.01 M-tris-HCl, pH 8.3; 0.15 M-NaCl; 0.002 M-EDTA) according to the recently described procedure (Kufe et al. 1973). Particles with a density corresponding to that of oncornaviruses (1.16 to 1.19 g/ml) were prepared from the extract in a sucrose density gradient and pelleted. To the resuspended pellet (in 300 \(\mu\)l of 0.01 M-tris-HCl, pH 8.3) were added: Nonidet P40 (NP 40) (final concentration 0.1%, Shell Chemical Co.) to break the virus particles present in the pellet, actinomycin D (100 \(\mu\)g/ml) to inhibit DNA-instructed DNA synthesis, oligo d-(pT) (0.08 mg/ml, Collaborative Research Inc., Waltham, Mass.) as a primer for the RNA-instructed DNA polymerase and dithiothreitol (DTT, final concentration 0.01 M). After incubation at 0 °C for 10 min the synthesis of RNA-instructed [\(^3\)H]-DNA was then performed by addition of 250 \(\mu\)l of a mixture containing 0.06 M-tris-HCl (pH 8.3), 1 mm-dATP, 1 mm-dGTP, 1 mm-dCTP, 0.1 mm-[\(^3\)H]-TTP (40 to 60 Ci/mmol, Amersham Buchler, Braunschweig, West Germany), 7.5 mm-MgCl\(_2\), 5 mm-DTT and 80 mm-NaCl. After 10 min of synthesis at 37 °C the nucleic acids were separated from the proteins in the reaction mixture by extraction with phenol-cresol-hydroxyquinoline. The presence of RNA-instructed [\(^3\)H]-DNA was analysed in a glycerol sedimentation velocity gradient (Kufe et al. 1973).
Fig. 2. No evidence for 70S RNA-[\textsuperscript{3}H]-DNA complexes in malignant lymphomas of non-human primates induced by oncogenic herpesviruses. Reaction mixtures of particles isolated from the monkey tumours were analysed on a glycerol sedimentation velocity gradient as described in the legend to Fig. 1. The figure shows 5 representative results: tumour tissue from CT-marmosets (Saguinus oedipus) infected with HVS (\(\triangle\) and \(\Delta\)) and with HVA (\(\circ\)); tumour tissue from a CJ-marmoset (Callithrix jacchus) infected with HVA (\(\bullet\)) and tumour tissue from an owl monkey (Aotus trivirgatus) infected with HVS (\(\square\)).

A distinct peak of acid-precipitable [\textsuperscript{3}H]-TTP was observed in the glycerol gradient with a sedimentation coefficient of 70S after examination of the mouse tumours induced with RLV and MMTV. The [\textsuperscript{3}H]-TTP peak represents complexes which contain 70S RNA molecules since prior treatment with RNase (Kufe et al. 1973) eliminates the peak (Fig. 1). The tumorous spleens or lymph nodes from non-human primates with malignant lymphoma were found to be negative for [\textsuperscript{3}H]-DNA-RNA complexes in the 70S region of the glycerol sedimentation velocity gradient (Fig. 2).

The presence of inhibitors in the monkey tumours directed against the DNA polymerase was excluded by reconstruction experiments. One half of a pellet of NC-37 cells infected with SSV-1 was mixed with an equal vol. of monkey tumour cells. This cell mixture was
Fig. 3. Detection of 70S RNA-[³H]-DNA complex in simian sarcoma virus type I-infected NC-37 cells. The nucleic acids in the reaction mixture were precipitated with ethanol and analysed in a pre-formed Cs₂SO₄ density equilibrium gradient (ρ = 1.34 to 1.79 g/ml) containing 3 mM-EDTA and 10 µg/ml yeast RNA as carrier at 59,000 rev/min at 16 °C for 24 h (Christ ultracentrifuge, rotor type 60). External markers for this type of gradient were polyribosomal RNA from NC-37 cells and DNA from Micrococcus lysodeikticus. Similar amounts of cts/min of the [³H]-DNA 70S RNA complex could be shown in the glycerol sedimentation velocity gradient of the same reaction mixture.

homogenized and examined in parallel with the other half of the NC-37 cell pellet in the simultaneous detection test. The amount of [³H]-DNA (1,400 cts/min) complexed to 70S RNA derived from the cell mixture was not significantly diminished by the presence of the monkey tumour cells in comparison with the amount of [³H]-DNA (1,790 cts/min) complexed to 70S RNA derived from the NC-37 cell pellet without monkey tumour cells (data not shown).

The [³H]-DNA product obtained from NC-37 cells infected with SSV-1 was precipitated with ethanol and analysed on a Cs₂SO₄ gradient (Kufe et al. 1973). Part of the product bands in the RNA density region (Fig. 3). This radioactivity peak consists of DNA molecules which remained complexed to large virus RNA molecules. The [³H]-DNA-RNA complex could be destroyed by incubation with RNase. The radioactivity peak in the DNA density region represents DNA product released during the manipulation. No peak with [³H]-TTP could be found in the RNA density region of the Cs₂SO₄ buoyant density gradient after examination of 6 HVS and 4 HVA tumours of CT-marmosets (Fig. 4).
Fig. 4. In HVS (○—○) and HVA (●—●) induced tumours of non-human primates no 70S RNA-[\(^{3}H\)]-DNA complex could be detected in the RNA density region in pre-formed Cs\(_{2}SO_{4}\) density equilibrium gradients as described in Fig. 3.

The absence of RNA virus particles does not exclude the presence of an integrated RNA virus genome which is non-productive but partially expressed or silent. Since no [\(^{3}H\)]-DNA product could be isolated from the herpesvirus induced malignant lymphomas the polyribosomal RNA of the tumour cells was analysed with the [\(^{3}H\)]-DNA product of SSV-1 for base sequences from the hypothetical RNA tumour virus. Assuming a base homology of the simian oncornavirus SSV-1 with the hypothetical RNA virus, which could interact in a non-productive state of activity with the oncogenic herpesviruses from non-human primates, the [\(^{3}H\)]-DNA product of SSV-1 should hybridize with the polyribosomal RNA from the monkey tumour cells.

The [\(^{3}H\)]-DNA-RNA complexes obtained in the 70S region of glycerol sedimentation velocity gradients from the NC-37 cells infected with SSV-1 were precipitated with two volumes of ethanol in the presence of 0·2 M-NaCl at −20 °C for 12 h and treated with 0·5 M-NaOH at room temperature for 4 h to hydrolyse any virus RNA present. The radioactive DNA product banded solely in the DNA region of a Cs\(_{2}SO_{4}\) gradient (Steinke, Laufs & Dörfler, 1975). The [\(^{3}H\)]-DNA was denatured by incubation at 80 °C for 15 min in 50 % formamide–50 % 5 mM-EDTA and subsequent quick chilling. The polyribosomal RNA used in the hybridizations was derived from a cytoplasmic pellet consisting of a
mixture of monosomes and polysomes according to a recently described method (Kufe, Hehlmann & Spiegelman, 1973a). Annealing reactions between [3H]-DNA of SSV-I (1000 to 2000 cpm/min) and RNA from polysomes of tumours (300 µg) were performed in 100 µl of 0.4 M NaCl-50% formamide-50% 5 mM-EDTA at 37 °C for 24 h. Annealing reactions were analysed in C$_{2}$SO$_{4}$ gradients (Kufe, Hehlmann & Spiegelmann, 1973). About 40% of the input [3H]-DNA from SSV-I hybridized to the polysomal RNA from NC-37 cells infected with SSV-1 (Steinke et al. 1975) but no [3H]-DNA hybridized to the polysomal RNA from herpesvirus induced tumours. All of the eight different herpesvirus induced tumours tested so far gave negative results in the annealing reactions with the [3H]-DNA product of SSV-I. These observations, which gave no indication for a partial expression of an oncornavirus genome, do not rule out a silent oncornavirus genome in the tumours. However, the lack of nucleic acid sequences homologous to single-stranded [3H]-DNA transcripts prepared from SSV-I and gibbon ape type-C virus in any primate tissue DNA and the lack of sequences homologous to a [3H]-DNA transcript from a baboon type-C virus in the DNA from New World monkey tissue (Benveniste et al. 1974) do not favour the involvement of a silent oncornavirus genome in the etiology of herpesvirus induced tumours in New World monkeys.

The negative findings could be due to the limited sensitivity of the simultaneous detection test. However, our observation that the herpesvirus induced tumours of New World monkeys do not contain reverse transcriptases as shown in the simultaneous detection test was strengthened by attempts to detect an interspecies antigen related to monkey oncornaviruses. Virus reverse transcriptases and intraviral gs antigens have provided the most significant markers to date for detection and specification of type-C viruses (Aoki, 1974). Three tumours induced with HVS and one tumour induced with HVA were tested in the complement fixation test with FP$_{4}$ serum for the presence of an interspecies antigen and found to be negative (W. Schäfer, personal communication). The FP$_{4}$ serum used in the test reacted with SSV-I as well as with RD 114 C-viruses (Schäfer et al. 1973).

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