Cocultivation as a Tool for the Detection of Oncoviruses in Childhood Leukemia*

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I. Introduction

In our study on the possible role of type-C oncoviruses in human leukemia we applied the technique of cocultivation of human bone marrow cells with an animal indicator cell line. The cocultivation technique proved to be very useful, for instance, for the isolation of endogenous primate viruses (Todaro et al., 1978). When bone marrow of a leukemic child was cocultivated with the rat XC cell line, a type-C virus was readily detected which was related to the simian sarcoma-virus (SiSV) (Nooter et al., 1975). The use of the XC cell line has been abandoned by us because of the pronounced cytopathic effect of primate viruses in this line and the danger of activation of the endogenous rat virus.

We changed to the dog thymus cell line A7573, which is known to be permissive for SiSV-related viruses (Teich et al., 1975), without a cytopathic effect. Till now, no retrovirus has been induced in this and any other canine cell line. We already reported detection of type-C viruses in bone marrow samples from two leukemic children in this assay system (Nooter et al., 1977). We here describe our results on a relative large scale study of cocultivation of cells and human BM samples. SiSV cross-reacting antigens could be detected in some of these cocultures by means of the indirect cytoplasmic immunofluorescence assay (IFA).

II. Characterization of Antisera

Two rabbit antisera were used in this study: one directed against the p30 of SiSV and one the p30 of Rauscher murine leukemia virus (R-MuLV).

In cytoplasmic IFA antiviral antisera, which have not been absorbed for anti-FCS activity, react with every cell line tested. After FCS absorption, the antiserum to SiSV-p30 gives a strong reaction (endpoint titer 1:320–640) with cultures infected with SiSV and a weakly positive reaction with cultures releasing R-MuLV (endpoint titer 1:20–40) (Table 1). No reaction was found with cultures producing bovine leukemia virus. The RA-MuLV-p30 antiserum gave a considerably stronger reaction with cells producing R-MuLV

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Table 1. Immunofluorescence endpoint titers of control cell cultures

Control cell cultures	Antisera	
	RA-SiSV-p30	RA-R-MuLV-p30
Virus positive:		
REF + SiSV[1]	320–640	20– 40
BALB/3T3 + R-MuLV[2]	40– 80	160–320
BoEF + BoLV[3] (N = 3)	10	10
Virus negative:		
animal:		
BALB/3T3. REF. R1–69[4]. SIRC[5]. A7573. FRhL[6]. BoEF (N = 7)	10	10
human:		
skin fibrobl. (7). lung fibrobl. (1) R970[7]. WHE[8]. A204[9]. NC37[10] (N = 12)	10	10

1 primary culture of rat embryonic fibroblasts (REF) productively infected with SiSV.
2 mouse BALB/3T3 cells productively infected with R-MuLV.
3 bovine embryonic fibroblasts (BoEF) productively infected with bovine leukemia virus (BoLV).
4 rat osteosarcoma cell line.
5 rabbit cornea cell line.
6 rhesus monkey fetal lung cell line (Flow Laboratories. Irvine. Scotland).
7 human osteosarcoma cells nonproductively infected with the Kirsten strain of the mouse sarcoma virus
8 primary culture of whole human embryo cells.
9 human rhabdomyosarcoma cell line.
10 human lymphoblastoid cell line.

than with SiSV-positive cells. Neither antiserum reacted with a variety of animal (n = 7) and human (n = 12) cell lines, which do not release a retrovirus. The virus negative lines of human origin included the NC37 cell line, in which SiSV was grown, used for p30 isolation and subsequent antiserum preparation.

III. Detection of SiSV-p30 Related Antigens in Human Bone Marrow Cocultures

After approximately one to two months of culture, in some cocultures of human bone marrow and canine A7573 cells, antigens appear which can be detected with the RA-SiSV-p30 antiserum but not with the RA-MuLV-p30 antiserum. The cytoplasmic fluorescence was of a granular nature. Maximally 60% of the cells stained positive. A few positive cocultures were followed at two-week intervals and it appeared that the positive IFA staining persisted for about 2 months but then gradually declined during the following passages and disappeared completely. The endpoint titer on the positive cocultures never exceeded 1:80.

Altogether in 8 out of 38 cocultures SiSV-p30-related antigens appeared (Table 2). Most positive cases were found in the group of leukemic donors

Table 2. Immunofluorescence endpoint titers of antisera to type-C retroviruses in cocultures of human bone marrow with dog cells

Coculture	Diagnosis	Sex	Age in years	Antisera[a]	
				RA-SiSV-p30	RA-R-MuLV-p30
	Leukemia patients:				
A1	ALL	♂	3	+ (40)	–
A2	ALL	♂	5	–	–
A3	ALL	♂	6	+ (80)	–
A4	AML	♂	8	+ (40)	–
A5	ALL	♀	5	+ (80)	–
A8	AML	♂	66	–	–
A24	CML	♂	31	–	–
A25	AML	♂	70	–	–
A26	CML	♂	76	–	–
A28	ALL	♀	5	+ (40)	–
A30	AML	♂	74	–	–
A31	AML	♂	52	+ (40)	–
A32	AML	♀	73	–	–
A35	AML	♀	46	–	–
A36	AML	♀	9	–	–
A37	AMML	♀	cong.	–	–
A38	ALL	♂	1	–	–
	Non-leukemic patients:				
A6	lymphosarcoma	♀	8	–	–
A7	renal transplantation	♀	44	–	–
A9	aplastic anemia	♂	8	–	–
A15	pyruvate kinase deficiency	♂	39	–	N.T.
A16	pure red cell aplasia	♂	18	–	N.T.
A18	myelofibrosis	♂	68	–	–
A19	aplastic anemia	♀	12	–	N.T.
A20	non-Hodgkin lymphoma	♂	8	+ (40)	–
A23	non-Hodgkin lymphoma	♂	30	–	N.T.
A27	bronchus carcinoma	♂	56	–	–
A29	myelofibrosis	♂	75	–	–
A33	myelofibrosis	♂	72	–	–
	Normal individuals:				
A10	normal	♀	14	–	–
A11	normal	♀	11	–	N.T.
A12	normal	♂	9	–	–
A13	normal	♀	62	–	–
A14	normal	♂	24	–	N.T.
A17	normal	♀	51	–	N.T.
A21	normal	♂	32	–	–
A22	normal	♂	8	+ (40)	N.T.
A34	normal	♀	14	–	–

[a] Fluorescence staining was scored as positive when the reciprocal of the endpoint titer of the antiviral antiserum was 40 or higher. The numbers in parentheses indicate endpoint titers which are expressed as the reciprocal of the highest dilution showing virus specific staining.

(6 of 17). Of these 6 positive cocultures 5 were derived from leukemic children. In the group of nonleukemic patients only 1 of 12 cocultures scored positive. The positive sample came from a child with a non-Hodgkin lymphoma. Only 1 of 9 cocultures, derived from normal bone marrow donors, contained SiSV-p30 related antigens. This sample was derived from an 8-year-old child.

Reproducibility of detection of virus-related antigens in such cocultures was demonstrated in two separate instances. Of two leukemic donors (numbers 1 and 5), which were positive in our assay, additional bone marrow samples which had been stored in liquid nitrogen, were also tested with the cocultivation technique at a later time and found to be positive as well.

IV. Blocking Tests

Specificity of the detection of SiSV-p30 related antigens in the cocultures was demonstrated by the absorption of the reactivity with purified SiSV grown in rat cells and not with purified mouse mammary tumor virus (MuMTV). The positive staining of both SiSV-producing cells and a positive culture was completely blocked after absorption of the RA-SiSV-p30 antiserum with SiSV, while absorption with MuMTV resulted in only a slight decrease in the titer of the antiserum.

V. Reverse Transcriptase Assays

In addition to IFA, 5 of 8 positive cocultures were screened for extracellular reverse transcriptase. These cocultures have been followed for eight months (Table 3). In coculture A1 a small burst of reverse transcriptase was found during the first month. No significant activity was found in the seven following months. When a second coculture was started from the same bone marrow, a similar result was obtained.

Only in coculture A4, which was reinitiated from stored bone marrow, high polymerase activity was found, which persists already for two months.

Table 3. Reverse transcriptase activity of cocultures positive in the IFA

	RT activity after month in culture (cpm × 10^{-3})					
Coculture months	1	2	3	4	6	8
A1 I	*14,7*	0.4	0,9	1.6	1.1	1.7
A1 II	*12,1*	0,3	0.6	1,2	0,8	0,5
A3	0,9	1.8	2.3	0,4	1.7	N.T.
A4 I	1.7	0,4	0.8	1,9	2,3	N.T.
A4 II	N.T.	*116,4*	*236*			
A5	2,5	0.5	1.3	0,5	0,6	N.T.
A22	0,7	0,9	0.1	3,0	N.T.	N.T.

This isolate in which only one cell line is involved, is highly promising for further studies like bioassay as done with SKA21-3, a virus isolated from a leukemic child after a complicated procedure, involving several cell lines (Nooter et al., 1978). This SKA21-3 virus proved to be leukemogenic to rats.

VI. Discussion

In the experiments presented here we detected SiSV-p30 related antigen in 8 of 38 cocultures of human bone marrow samples and the canine cell line, A7573. Our recent results obtained by cocultivation can be explained by intimate contact between the bone marrow cells and the indicator cells, which allow transfer and subsequent propagation of the few virus particles, present in the original bone marrow samples.

Crucial for the detection of viral footprints in these cocultures is the specificity of antisera and immunofluorescence technique. After absorption of immunoglobulin fractions of the sera, no reaction was seen with any animal or human cell line, which do not produce a retrovirus. Sera of bovine origin may be a source of unwarranted contamination with bovine viruses. However, no reactions were found with the p30-antisera on bovine leukemia virus producing fibroblasts. As the SiSV has been grown in a human lymphoid cell line (NC37), it is possible that the purified SiSV-p30 preparation is contaminated with human lymphoid cell antigens. However, absorption of the RA-SiSV-p30 antiserum with SiSV grown in rat cells fully blocked the reaction with the positive cocultures.

All in all, 7 of the 8 cocultures which were positive for viral antigens are derived from children. Five of the 6 positive donors in the leukemic group are children.

From these results emerges an association between childhood leukemia and this virus-related antigen. Coincidentally the two positive cases in the control groups are also children. These results and our earlier studies suggest childhood leukemia to be a highly suitable disease entity for further virological studies.

Our results suggest a transmissible agent to be involved in childhood leukemia. Despite the lack of epidemiological evidence for the infectious nature of childhood leukemia, this virus seems to be horizontally transmitted as man does not have SiSV-related sequences in its normal cellular DNA. The possible sources of virus with regard to transmission are rather restricted: saliva, milk, urine, feces, sperm and placenta. In the cat, the saliva proves to be sole source of leukemia virus (Francis et al., 1977). In gibbons apes, however, the urine was the main source of virus excretion. Milk is the main route of transmission of exogenous leukemia viruses in mice (Law and Moloney, 1961). Obviously, the route of transmission in man is unpredictable.

So far, retroviruses have never been found by means of electron microscopy in human urine and feces. The so-called simultaneous detection test gave repeatedly positive results with regard to retroviruses in human milk (Schlom et al., 1972). In view of the presence of exogenous viruses in a few

human embryonic cell lines (Panem et al., 1977), it is tempting to speculate that either placenta or sperm are vectors for a putative human leukemia virus. Since SiSV-sequences seem difficult to detect in tumor tissues, it must be assumed that only a selected population contains complete infectious virus.

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