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DEPRESSION OF REVERSE TRANSCRIPTASE ACTIVITY BY HYBRIDOMA SUPERNATANTS: A POTENTIAL PROBLEM IN SCREENING FOR RETROVIRAL CONTAMINATION

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SUMMARY. Murine hybridoma cell lines generally produce retroviral particles (type A and/or type C), often in large numbers. We have measured reverse transcriptase activity in the supernatant of some 30 hybridoma lines of murine origin and found that the observed activity expressed as pmoles of $[^3H]$-dGMP incorporated into an acid insoluble polymer, is frequently much lower than would be expected from the amount of retrovirus seen by electron microscopy in the corresponding cells. We demonstrate that this reduction is due to the presence of a nuclease which degrades the high molecular weight product but is not due to a change in the reverse transcriptase activity. This nuclease activity may be associated with mycoplasma contamination of the cell lines.

Recently a warning was published by Bartal et al. (1) concerning the presence of A and C type retroviral particles as observed by EM in hybridoma supernatants, and the risk to patients who might receive such materials. Weiss (2) analyses this phenomenon in greater detail and considers the risks both to patients and to the laboratory personnel involved in the purification of the clinical preparations.

Over the last three years, we have observed retroviral particles by EM in all of more than 30 hybridoma cultures examined. The estimation of RT activity of the corresponding supernatants was regularly much lower than would have been expected from the numbers of particles visible by EM. We here describe the presence of an activity, possibly

ABBREVIATIONS. EM: Electron microscopy; MW: Molecular weight; RT: Reverse transcriptase; TEN: Tris 10 mM pH 7.4, EDTA 1 mM, NaCl 100 mM; U: Uracil; UdD: Uridine.

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related to mycoplasma contamination, which degrades the newly-formed high MW products of reverse transcription and thereby reduces the estimate of RT activity by 70 to 99.9 %.

MATERIALS AND METHODS

Cells : Hybridoma cultures produced in our laboratory or contributed by Dr Brochier (INSERM, U.80, Lyon France), Dr Le Borgne de Kaouel (Immunotech, Marseille France) and Dr Feldman (Institut d'Immunologie, Luminy, Marseille France) were harvested during exponential growth 72 hrs after seeding. After being counted, the cells were recovered by centrifugation at 2,000 rpm for 5 min. The cell pellet was processed for EM, and the supernatants were tested for RT activity.

Electron microscopy : Fragments of cell pellets were fixed in 2 % glutaraldehyde and 2 % osmium tetra-oxide in cacodylate buffer, sectionned and stained with 5 % uranyl acetate and lead citrate in Reynolds' solution. Thin sections were examined at various magnifications under a Hitachi H300 electron microscope.

Reverse transcriptase assay : Thirthy ml aliquots of cell culture supernatants were layered onto a 5 ml cushion of 20 % sucrose in TEN buffer (Tris pH 7.4 - 10 mM, EDTA - 1 mM, NaCl - 100 mM), and centrifuged at 25,000 rpm for 90 min in a Kontron rotor. The pellets were suspended in reaction buffer (60 μl/10^7 cells in the original culture). This material will be referred to as "culture supernatant concentrate". The RT activity was assayed, as described by Chen et al. (3), under standard optimal conditions for mammalian retroviruses (presence of 1 mM Mn acetate). Control assays were performed simultaneously using samples of a well-characterised avian retrovirus (RSV-B77) under the same conditions. Using our RSV-B77 samples in these conditions, we established that 1 FFU is equivalent to 100 virus particles and that 1 pmole of [^{3}H]-dGMP incorporated corresponds to 2.7X10^7 particles. Liebes et al. (4) showed that the activity of murine RT is 25 times less than that of the avian viruses, therefore we adopted an equivalence of 1 pmole of [^{3}H]-dGMP incorporated 6.75X10^8 murine retrovirus particles under our experimental conditions.

Interference by hybridoma culture supernatants in RT assays : RSV-B77 (2-10X10^6 FFU) was assayed for RT activity under optimum conditions for the avian enzyme (presence of 10 mM Mg acetate) (3) in the presence of "culture supernatant concentrates" (equivalent to 2-10X10^7 cells) added either at the beginning of the reaction or after 60 min incubation.

Velocity gradient centrifugation of hybridoma supernatants : "Culture supernatant concentrates" corresponding to 2X10^7 cells were diluted to 1 ml with TEN buffer and layered onto a preformed 5-20 % sucrose gradient. The samples were centrifuged for 50 min at 17,500 rpm in a SW50 rotor. Successive 200 μl fractions were collected and tested for murine RT activity and for their ability to interfere with avian (RSV-B77) RT activity.

RESULTS

Figure 1 illustrates the numerous intracellular, extracellular and budding C type particles regularly observed in all the hybridoma cultures studied. In view of this result, the values for RT activity in

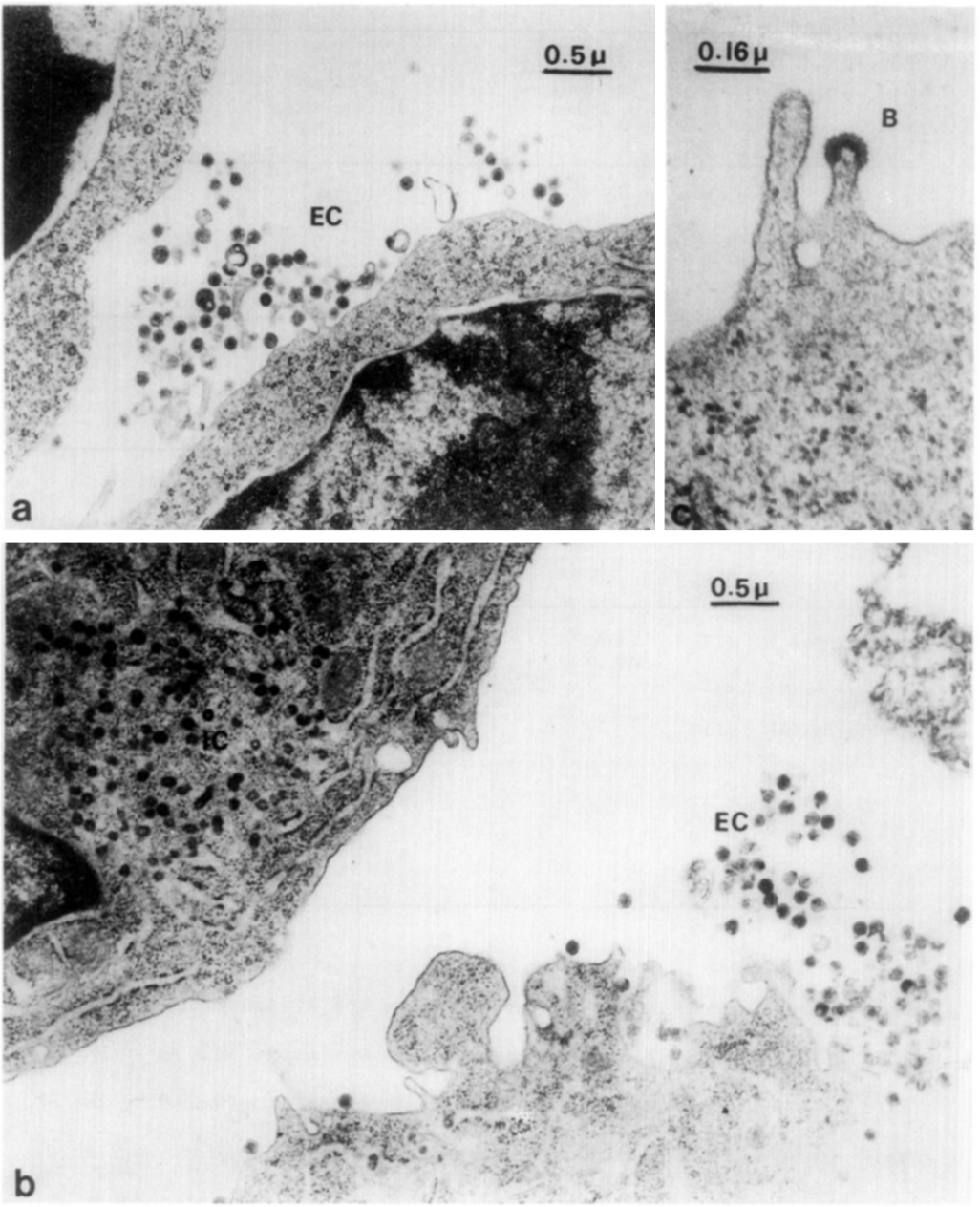

Fig. 1. C-type particles produced in murine cell lines. a-c) SP2 myeloma ; b) SP2Xspleen BALB/c hybridoma
Intra cellular (IC) budding (B) and extra cellular (EC) particles.

the culture supernatant concentrates from these cultures appeared abnormally low, in some cases less than the equivalent of one viral particle per cell (Table 1). We therefore tested the capacity of the culture

Table 1. Reverse transcriptase activity of hybridoma supernatants

Cell line or clone		Antibody produced	p moles [3H]-dGMP incorporated in 1 hr	Virus particles equivalent per cell
	SP2	0	0.208	14
Murine			0.025	1.7
	X63	+	0,177	11.9
Myeloma			0.021	1.4
	NS1	0	0.125	8.4
Hybridoma	LU1	anti	0.075	5.1
NS1		B_2 globulin		
(Luminy	LU2		0.077	5.2
Marseille)	LU3		0.066	4.5
	ME4	anti	0.002	0.1
Hybridoma		measles virus		
SP2	ME5		0.008	0.5
(U51, Lyon)			0.007	0.5
	ME6		0.039	2.6
Hybridoma	AT7	anti	0.441	29.7
NS1		ATPase		
(U51, Lyon	AT8		2.956	199
Sciences	AT9		0.277	18.6
University)	AT10		0.792	53.5
B77 control			49.3	38
			26.7	
10^7 FFU = 10^9 virus particles				

supernatant concentrates to interfere with the RT assay of a preparation of RSV-B77 under optimal conditions for avian RT. As shown in Table 2, the different culture supernatant concentrates added to the RT assay mixture depressed the incorporation of [^{3}H]-dGMP into the acid-insoluble material by 71 to 99.9 % as compared to untreated controls. This result could be due either to an inhibition of RT activity or to degradation of the high MW DNA formed. We therefore added culture supernatant to a RSV-B77 RT assay 60 min after starting the reaction. We observed that after 30 min incubation with culture supernatant concentrate, the acid-insoluble counts were reduced from 440,000 to 4,000 cpm, indicating degradation of the pre-formed DNA.

Table 2. Reverse transcriptase inhibitory activity of hybridoma supernatants

Experiment	Sample	Incorporation of [3H]-dGMP (cpm) 0 min	Incorporation of [3H]-dGMP (cpm) 60 min	% inhibition of B77-RT activity
I	Buffer control	147	233	
	B77 control	769	554 841	
	B77 + NS1	-	1 476	99.7
II	B77 control	387	162 022	
	B77 + NS1	-	7 230	95.5
	+ SP2	-	47 077	71
	+ X63	-	37 346	77
III	B77 control	496	370 075	
	B77 + LU1		35 046	90.6
	+ LU2		26 650	92.8
	+ LU3		14 861	96
IV	Buffer control	121	119	
	B77 control	302	248 167	
	B77 + ME 5		245	99.9
	+ ME 6		692	99.7
	+ ME 7		352	99.8

We attempted to separate this nuclease activity from the RT activity by velocity centrifugation of some supernatants through sucrose gradients. A typical result is shown in Fig. 2. Reverse transcriptase activity is present as a rather broad peak with shoulders, suggesting heterogeneity in the population of virus particles. The nuclease activity was present at the top of the gradient and in the lowest fractions. Sediment from the bottom fraction, which had the highest nuclease acticity, was examined under the EM and was found to comprise cell debris and structures resembling more or less degraded mycoplasma. It is interesting to note that the sum of the RT activity in the positive fractions corresponds well with the value measured for the original supernatant corrected for the observed nuclease activity (sum of the activi-

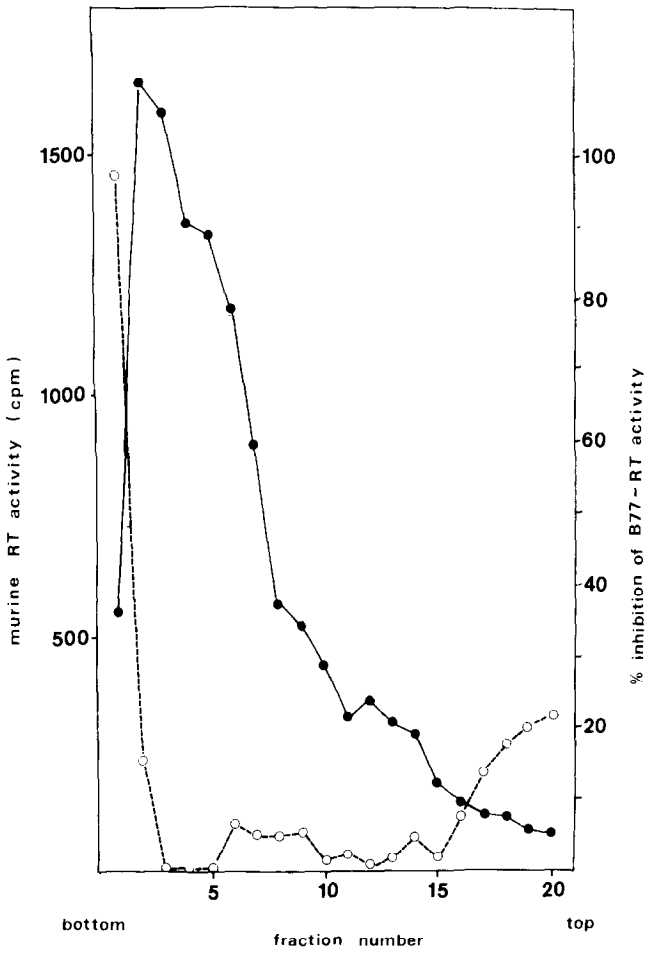

Fig. 2. Separation of reverse transcriptase activity from inhibitory activity of hybridoma fluids. Fractions processed for murine RT (Mn^{++}) (●—●) and for inhibitory activity on RSV B77 RT (Mg^{++}) (o---o).

ty for fractions 1 to 16 = 11,612 cpm, RT activity of whole supernatant = 496 cpm, inhibition of RSV-B77 RT = 96.8 %, and corrected RT activity of original supernatant = 15,500 cpm).

In order to correlate the nuclease activity with the presence of mycoplasma in the cultures, we compared the effect on B77-RT of a myeloma cell line without detectable contamination with that of a heavily contaminated one. The screening for mycoplasma was performed by $[^3H]$ -uridine- $[^3H]$ -uracil incorporation ratio (5). The results are presented in Table 3 and indicate that in the absence of detectable mycoplasma contamination, no reduction in B77-RT activity is observed.

Table 3. Inhibition of B77-RT activity and presence of mycoplasma in the cultures.

Myeloma	$\frac{[^3H]\ UrD}{[^3H]\ U}$	Mycoplasma	pmoles $[^3H]$-dGMP incorporated in 1 hr	Virus particles equivalent per cell	% inhibition of B77-RT activity
NS1	0,55	++++	0,006	0,4	99 %
SP2	152	0	0,123	8,3	0 %

DISCUSSION

Murine hybridoma cell lines appear regularly to express C type particles as well as the non-infectious intracysternal A type particles (1,2). The C type particles are frequently xenotropic although ecotropic and amphitropic viruses also occur (2). These viruses are presumably derived through induction of the endogenous proviral sequences present in one or the other of the cells used for the fusion. Such induction has been shown by Moroni and Schumann (6) and others (7,8) to occur on antigenic stimulation of lymphocytes from mice or from chickens (9,10) and to result in the production of complete viral particles or of viral antigens.

The potential therapeutic use of monoclonal antibodies will obviously require the elimination of xenotropic murine retroviruses capable of transforming human cells *in vitro*. We show here that the RT assay for the detection of retroviruses in these materials frequently underestimates the degree of viral contamination by as much as one thousand fold. This is due to the presence of a nuclease which degrades the high MW DNA formed. We have previously described the presence of such a nuclease in cells contaminated with mycoplasma (11), and in this study, mycoplasma were associated with high nuclease activity. While retroviral contamination is unlikely to alter the properties of monoclonal antibodies used for diagnostic purposes, mycoplasma contamination

may not be so inoffensive due to their production of proteases. In the light of recent studies concerning "onc" genes and LTR promotion (12,13,14) rigorous purification would appear to be indicated before human trials of monoclonal antibodies. Considering our present results we would recommend that, if a RT assay is used for the detection of retroviral contamination, it should be combined with a study of the inhibitory activity of the samples on RT by a standard retrovirus. Murine retroviruses (and mycoplasma) may not be completely harmless to humans - especially those in need of monoclonal antibody therapy.

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