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Autologous Monoclonal Antibodies Recognize Tumour-associated Antigens in X-irradiated C57BL/6 Mice

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SUMMARY

X-irradiation of C57BL/6 mice induces thymic lymphosarcomas which sometimes contain retroviruses which upon injection into normal mice mimic the effect of the irradiation. We examined whether specific antigenicities, viral or cellular, were expressed by tumour cells that could be recognized by antibodies from the irradiated animals. We developed monoclonal antibodies (MAbs) using splenocytes of the diseased animal. The reactivity of such MAbs towards thymoma cell lines established *in vitro* was investigated by means of an ELISA. At least 10 antibody specificities were detected on the 13 tumours investigated, allowing separation of the MAbs into three classes: (i) those recognizing the autologous tumour, heterologous tumours as well as normal thymic tissue, (ii) those specific for the autologous tumour, and (iii) those specific for one tumour, but not ones of autologous origin. The last two classes corresponded to specific tumour-associated antigens. Our panel of MAbs defined each tumour by the particular pattern of antigens harboured. It is striking that most of the antigens were present in the normal thymus and that only two tumours had additional antigenicities. Additionally, quantitative variations were observed in the levels of expression of these antigens.

INTRODUCTION

X-irradiation of C57BL/6 mice induces thymic lymphosarcomas (TLS) within a few months (Kaplan & Brown, 1952). Although this phenomenon was extensively investigated, the mechanism by which irradiation induces neoplastic transformation remains unclear. Several groups demonstrated the presence of retroviruses (termed radiation leukaemia viruses or RadLVs) in thymoma cell-free extracts which initiate the disease upon injection into normal non-irradiated mice (Lieberman & Kaplan, 1959; Latarjet & Duplan, 1962; Decleve *et al.*, 1975; Haran-Ghera & Peled, 1979; Benade & Ihle, 1980; Sankar-Mistry & Jolicoeur, 1980). Furthermore, immunization of C57BL mice with a RadLV-associated B-ecotropic retrovirus significantly reduced thymoma induction by X-rays (Lieberman & Kaplan, 1977). The hypothesis became accepted that radiation (at the dose used) was not tumourigenic by itself but acted via the activation of endogenous retrovirus sequences (Kaplan, 1977).

Because the otherwise non-expressed endogenous non-pathogenic retroviruses (N-ecotropic and xenotropic) as well as recombinant B-ecotropic retroviruses were often encountered in radiation-induced thymomas, it was logical to assume that RadLVs also arose by recombination between two endogenous retroviruses. This recombinational origin of RadLVs was indeed confirmed by several authors (Benade *et al.*, 1978; Gautsch *et al.*, 1978; Guillemain *et al.*, 1980; Astier *et al.*, 1982; Rassart *et al.*, 1983). However, RadLV-like agents are detected in only a minority of tumours (Ihle *et al.*, 1976; Erfle *et al.*, 1981). A number of reasons could explain this lack of detection of RadLVs in thymomas; among them, it is conceivable that such agents may

be produced in amounts too low to be detectable or that they express only part of their genetic information. It is also possible that the maintenance of the tumoural state does not depend on the permanent expression of the virus that initiated cell transformation or even does not require the continuous presence of the provirus, as has been described with the Abelson murine leukaemia virus (Grunwald *et al.*, 1982); if such was the case, then RadLV could only be detected at a preleukaemic stage.

Whatever the mechanism leading from irradiation to lymphosarcomagenesis, it seems that the eventual recombination event generating RadLV is experimentally difficult to demonstrate. Thus, it seemed that a different approach had to be envisaged. With this prospect, we attempted to show that after X-irradiation one or several genes (whether viral or cellular) are expressed or that their normal expression is amplified. We report here experiments aimed at the detection of new or superabundant antigens harboured by radiation-induced thymoma cells. It was assumed that tumour-bearing mice might develop antibodies to tumour antigens. To obtain monospecific immunoglobulins in relatively large amounts, we generated monoclonal antibodies (MAbs) by hybridizing spleen lymphocytes of diseased animals with myeloma cells.

METHODS

Radiation-induced thymic lymphosarcomas. One-month-old inbred C57BL/6 mice were whole-body irradiated four times at 1·75 Gy at 1 week intervals as described previously (Latarjet & Duplan, 1962). After thymomas appeared, tumour cells were transplanted into 1-month-old non-irradiated syngeneic recipients and/or cultured *in vitro*.

Cell cultures. The myeloma cell line, SP2/O-Ag-14 (Shulman *et al.*, 1978), as well as the hybridoma cell lines described below, were maintained in SFM-2, a synthetic medium defined in our laboratory (unpublished results). Adherent normal C57BL/6 thymic epithelial cells, TAC7 (Guillemain *et al.*, 1980), TAC128 and 3T3-1223/B cells, each releasing a recombinant of the endogenous retroviruses of the C57BL/6 mouse (Astier *et al.*, 1982) and XC cells (Svoboda, 1961) were grown in the same medium but supplemented with 5% heat-inactivated foetal calf serum (SFM-2 + 5% FCSI).

Radiation-induced thymoma cells were established *in vitro* as permanent cell lines using conditioned medium at the initial step of the culture. For this, dispersed tumour cells were seeded in culture vessels containing exponentially growing TAC7 cells in SFM-2 + 5% FCSI. The next day and every 3 to 4 days thereafter cells were passaged in SFM-2 alone, conditions in which TAC7 cells rapidly degenerated while tumour cells grew in suspension. All media were supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml).

Virus assays. The presence of retroviruses in the culture medium was detected using both the exogenous reverse transcriptase (RT) assay as described previously (Astier *et al.*, 1976) and the XC cell fusion assay in mixed cultures (Guillemain *et al.*, 1980). In the RT assay, values were considered positive (RT^+) or negative (RT^-) with a threshold of 0·5 pmol/h/ml [^{3}H]TMP incorporated. For the XC assay, XC^-, XC^+, XCEP or XC Int respectively refer to retroviruses devoid of syncytial effect (like the endogenous xenotropic virus), those inducing such an effect within 72 h (like the endogenous V-ecotropic virus), those inducing 'early polykaryocytosis', or those having an 'intermediate' effect with syncytia appearing between 24 and 72 h.

Neoplastic nature of cultured cells. Aliquots of lymphoma cells (2×10^5) grown *in vitro* were injected subcutaneously or intraperitoneally into newborn or 1-month-old mice.

Characterization of leukaemic T cells. The presence of Thy-1.2, Ly-1.2 and Ly-2.2 cell surface antigens was detected by an indirect immunofluorescence method (Legrand *et al.*, 1982). The anti-theta serum was prepared according to Reiff & Allen (1964) and anti-Ly sera were commercially obtained (the anti-Ly-1.2 is monoclonal whereas the Ly-2.2 is a hyperimmune serum). The direct immunofluorescence technique was used to detect surface immunoglobulins (sIgs) using a rabbit $F(ab')_2$ anti-mouse IgG (H + L).

Autologous monoclonal antibodies to thymoma cells. Microplates (96 wells) were seeded with BALB/c peritoneal macrophages (5×10^3 cells/well) in 100 μl SFM-2 + 10% FCSI and incubated for 24 h (37 °C, 5% CO_2).

C57BL/6 mice bearing radiation-induced thymomas were individually splenectomized and the splenocytes (10^7 to 10^8) were fused with 10^7 myeloma cells (SP2/0-Ag-14) using polyethylene glycol 6000 according to Nowinski *et al.* (1979). The fused cells were seeded in 10 microplates conditioned with the macrophages in 100 μl SFM-2 per well supplemented with HAT (10^{-4} M-hypoxanthine, 10^{-6} M-aminopterin, 10^{-5} M-thymidine). After 2 and 7 days incubation (37 °C, 5% CO_2), 50 μl SFM-2 supplemented with HT was added. On day 14, the presence of immunoglobulin in the culture medium was monitored by an ELISA technique using peroxidase-conjugated sheep anti-mouse IgG (H + L). After selection for specific radiation-induced antigen recognition (see below), growing colonies were expanded and cloned twice by limiting dilution in SFM-2.

Recognition of tumour cell antigens by MAbs. Tumour cells originating from the thymoma itself, from grafted thymoma cells or from cell lines established *in vitro* were homogenized in a lysis buffer [0·05 M-Tris–HCl pH 7·6,

Table 1. *Typing, tumoural nature and maintenance of the phenotype after* in vivo *passage of cultured cells derived from radiation-induced tumours*

Cell line	Antigen detected by specific antisera				Tumoural nature		Phenotype of cells in resulting tumour		
	sIg	Thy-1.2	Ly-1.2	Ly-2.2	No. tumourous animals/no. injected animals	Latency (days)	sIg	Thy-1.2	Ly-1.2
2945	0	93*	90	3	3/3	23	0	86	78
3302	0	86	0	0	3/3	23	0	81	9
3316	0	80	27	22	3/3	23	0	87	28
3317	0	87	+†	56	3/3	23	0	85	0
3334	0	95	—	—	3/3	23	0	97	24
3350	0	93	—	—	2/3	31	0	84	0
3438	0	98	0	85	3/3	23	0	97	28
4745	0	92	+	87	3/3	31	0	97	28
3309	0	+	9	18	ND‡	ND	ND	ND	ND
3320	0	96	—	—	ND	ND	ND	ND	ND
3324	0	98	+	5	ND	ND	ND	ND	ND
3541	0	90	—	—	ND	ND	ND	ND	ND
4072	0	13	5	0	ND	ND	ND	ND	ND
4638	0	+	17	22	ND	ND	ND	ND	ND
4701	0	100	52	46	ND	ND	ND	ND	ND
4708	0	98	+	54	ND	ND	ND	ND	ND
4721	0	52	+	44	ND	ND	ND	ND	ND

* Percent of fluorescent cells using specific antisera.

† + or − means positive or negative result obtained when the viability of the cells was too low for precise quantification

‡ ND, Not done.

0·003 M-magnesium acetate, 0·25 M-sucrose, 10^{-4} M-phenylmethylsulphonyl fluoride (PMSF), 0·2% n-octylglucoside]. The lysate was centrifuged (600 *g*, 6 min), the pellet was resuspended in the same buffer without n-octylglucoside and centrifuged a second time under the same conditions; the two supernatants were mixed and extensively dialysed against phosphate-buffered saline (PBS) containing PMSF (10^{-4} M) to remove n-octylglucoside. Finally, the dialysate was centrifuged (30000 *g*, 20 min) and the supernatant was used as a source of tumour cell antigens in an ELISA to test for the specificity of the MAbs.

In this test, tumour extracts were used to coat microplate wells (1 μg in 100 μl carbonate–bicarbonate buffer pH 9·6). After 12 to 16 h (4 °C), the plates were washed three times with PBS containing 0·05% Tween 20 (PBS-T) and 100 μl hybridoma culture medium was added and incubated (2 h, 37 °C). After three washes as above, peroxidase-conjugated sheep anti-mouse IgG (H + L) was added in 100 μl PBS-T and allowed to react for 30 min (37 °C). After three more washes, wells in which MAbs were present were detected by addition of 100 μl 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate (6)] substrate. The results were evaluated with a multichannel photometer at 414 nm. In these experiments only the MAbs giving strongly positive reactions were considered.

RESULTS

Establishment of tumour cells in vitro

The method of irradiation led to a 71% incidence of TLS with an average latency period of 210 days. The tumour cells were used in part to graft normal non-irradiated C57BL/6 mice and in part to develop permanent cell lines (see Methods). After two to three passages in SFM-2 medium, the thymoma cells started to grow exponentially in suspension and reached an average density of $2·4 \times 10^6$ cells/ml. During the establishment of such cultures, 21 cultures (86%) were obtained. If TAC7 cells were omitted, only 55% survived. Morphologically the established cells resembled lymphocytes.

Phenotypic characterization of lymphoma cells

The cells of 17 cell lines were typed with specific antibodies recognizing immunoglobulins or Thy-1.2 antigens. The results presented in Table 1 indicate the T cell origin of all lines of the cultured cells, characterized by the presence of Thy-1.2 antigen and lack of sIg on the

Table 2. *Results of reverse transcriptase and XC cell fusion assays performed on cultured thymoma cells*

Cell line	Reverse transcriptase assay*	XC cell fusion assay†	Phenotype
3324	3·49	XC$^-$	RT$^+$ XC$^-$
4065	7·29	XC$^-$	
4721	6·77	XC$^-$	
2945	2·89	XC$^+$	RT$^+$ XC$^+$
3317	3·38	XC$^+$	
3438	4·86	XC$^+$	
4638	11·63	XC$^+$	
4708	1·54	XC$^+$	
3302	4·01	XC Int	RT$^+$ XC Int
4072	1·29	XC Int	
4701	11·05	XCEP	RT$^+$ XCEP
3541	0·09	XC$^-$	RT$^-$ XC$^-$
4064	0·22	XC$^-$	
4069	0·37	XC$^-$	
4745	0·30	XC$^-$	
3316	0·09	XC Int	RT$^-$ XC Int
4073	0·42	XC Int	
TAC7	0·15	XC$^-$	Controls
TAC128	2·46	XCEP	
3T3-1223/B	2·88	XC$^+$	

* RT activity:[^{3}H]TMP incorporated (pmol/h/ml).

† XC$^-$, Lack of polykaryocytosis; XC$^+$, late polykaryocytosis; XC, Int, intermediate polykaryocytosis; XCEP, early polykaryocytosis.

cytoplasmic membrane. In addition, analysis of the differentiation antigens, Ly-1.2 and Ly-2.2, harboured by these cells revealed significant differences between the cell lines, thus suggesting that the induced tumour cells differed in their phenotype.

Neoplastic nature of cultured cells

The ability of the cultured cells to grow autonomously was monitored by grafting into normal, 30-day-old, mice. The results (Table 1) indicate that all attempts performed were successful. The tumours developed with a short latency period, suggesting that they did not arise from infection of normal cells with a tumourigenic retrovirus.

It was also shown that the original phenotypes were retained by the cells from secondary tumours. Thus, the phenotype of the cells of a given *in vitro* cell line is stable and does not evolve upon proliferation in the grafted host.

Virus expression in cultured thymoma cells

The release or expression of retroviruses in the medium of cultured thymoma cells was investigated by the exogenous RT assay and the XC cell fusion technique. There was a high degree of variation among the cell lines with regard to virus production and to the phenotypes of the viruses which were expressed. As indicated in Table 2, 11 out of 17 cell lines were found positive in the RT assay, three of these were XC$^-$. This phenotype may correspond to cell lines producing xenotropic virus or other retroviruses devoid of cell fusion-inducing ability. Among the RT$^+$ XC$^+$ cell lines two classes were observed, one corresponding to the phenotype of the endogenous N-ecotropic virus of the C57BL/6 mouse (XC$^+$), while the other (XCEP or XC Int) was indicative of the presence of a retrovirus different from the two major classes of endogenous retroviruses of the C57BL/6 mouse.

Six cell lines had a RT$^-$ phenotype but could be classified into two categories, one (XC$^-$) most probably not expressing any retrovirus and the other XC Int. The latter phenotype could result

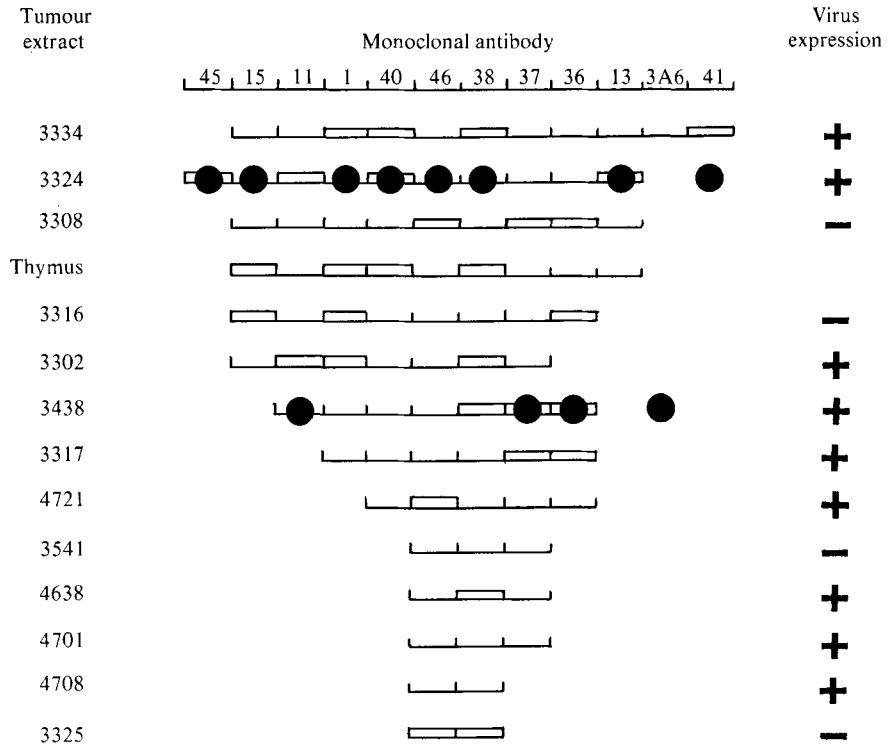

Fig. 1. Pattern of recognition of tumour extracts by MAbs. The cell lines from which tumour extracts were prepared are indicated at the left, and include a normal thymus control. Virus expression was measured in the cultured tumour cells by the RT assay. The MAbs are indicated across the top, and the ● symbols indicate the mouse from which each was isolated. The antibody specificities towards the different cell lines were measured by ELISA: no entry, little or no reactivity; horizontal line, absorbance three- to sixfold higher than control values; box, absorbance at least sixfold higher than control values.

from the presence of viral glycoprotein antigens at the cell surface without release of complete viral particles.

Production of MAbs

The counterpart to the development of permanent cell lines was the production of MAbs able to recognize specifically radiation-induced tumour-associated antigens. Splenocytes of six C57BL/6 mice with TLS were hybridized with myeloma cells. In preliminary tests the presence of Igs in the culture medium of the hybrids was monitored by ELISA; on average, 67% of the wells containing hybrids also contained Igs. The positive cultures were then tested for their content of antibodies against tumour cells. Tumour cell extracts (either from grafted tumours or from tumour cells cultured *in vitro* were used as target antigens for the screening of these antibodies by ELISA. The tumour-specific Ig-releasing hybridoma cultures were cloned twice in SFM-2.

Differential reactivities of monoclonal antibodies

Prior to the cloning of hybridoma cells, 46% of Ig-secreting cultures reacted with autologous tumour cells. Interestingly, a fraction of these were also able to recognize normal thymus tissue.

After cloning, the MAbs were reacted against different extracts prepared with tumour cells growing *in vitro*. The results of repeated experiments concerning the reactivities of 12 MAbs obtained after hybridizations of the spleen cells from two mice (3324 and 3438) toward 14 cell extracts are schematically presented in Fig. 1.

Our major findings can be summarized as follows. Three of the MAbs (45, 3A6 and 41) reacted with only one tumour, and interestingly two of them (3A6 and 41) did not react with their autologous tumour. These MAbs were also the only ones which do not give any reaction with normal thymus. All the other MAbs recognized the autologous tumour, normal thymus and a number or all of the other tumours tested. Fig. 1 also shows that the reaction of a given MAb with different tumour extracts varied in intensity. All the tumours analysed differed in the qualitative or quantitative expression of the antigens detected by our MAbs, and thus may be considered unique. Tumour 3308 exhibited the same nine antigenicities found in normal thymus. In two others (3334 and 3324) additional antigenicities were observed, whereas in all other tumour extracts tested, different thymic antigens were present. Finally, no apparent relationship was observed between retrovirus expression of cultured tumours and their pattern of antigenicity.

DISCUSSION

Despite numerous investigations, the role of retroviruses or of their recombinants in radiation-induced murine thymic lymphosarcomas is still unclear. Thus, we undertook a study to detect antigens present in such tumours which could be qualitatively or quantitatively associated with the malignant state of the thymic cells, The origin, viral or cellular, of such antigens was not investigated in this first step.

We examined whether the tumour cells expressed antigens that could be recognized by the immunoglobulins produced in the irradiated animal itself by establishing monoclonal antibodies from splenocytes of the leukaemic animals. Seventeen cell lines from tumours were established and studied in detail. On a morphological basis as well as by the presence of the differentiation antigens observed at the cell surface, these cells had the characters of thymic T lymphocytes. However, the different tumour-derived cell lines did not have the same phenotype with regard to the expression of the 'differentiation' antigens investigated.

Virus expression was also investigated in these cultured thymoma cells. The results obtained with the RT assay clearly indicate that 35% of the cell lines do not produce retroviruses at a detectable level, thus supporting the finding that RadLVs are not systematically encountered in radiation-induced tumours (Ihle *et al.*, 1976). Hypothetically, it is possible that such agents are produced in amounts too low to be detected, that they express only part of their genetic information, or that the maintenance of the tumoural state does not depend opn the expression of the virus that initiated cell transformation (Grunwald *et al.*, 1982). The XC cell fusion assay also gave results indicative of the polymorphism of the retroviruses expressed. In eight instances, the viruses have the phenotype of the endogenous ecotropic ($RT^+ XC^+$) or xenotropic ($RT^+ XC^-$) retroviruses. In the other cell lines, no virus was produced. Virus expression and/or the XC cell fusion phenotype did not parallel any Ly-1.2 and/or Ly-2.2 antigenic phenotype of the cells, thus adding further aspects to the polymorphism of the TLS as mentioned above.

Among the monoclonal antibodies obtained from the tumour-bearing animals, 12 were extensively studied for their ability to recognize cell extracts (13 derived from different tumours and one from normal thymus). We detected at least 10 antibody specificities. The first class contains MAbs that recognize autologous and heterologous tumours as well as normal thymic tissue. It may be assumed that such antibodies recognize normal antigens, the expression of which is enhanced in some lymphomas. The second class, represented by MAb 45, is specific only to the autologous tumour. This may perhaps be a new tumour-associated antigen. The third class represented by MAbs 3A6 and 41 is also specific to one tumour but not the autologous one. These could represent pre-leukaemia-associated antigens.

Because no relationship was observed between retrovirus expression in tumours and the recognition by particular MAbs, the hypothesis according to which irradiation acts in activating cryptic retroviruses which in turn become the aetiological agents of the disease is not well sustained. Nevertheless, this hypothesis is far from being ruled out for the reasons mentioned above.

It is striking that most of the MAbs recognize antigens in normal thymus to a greater or lesser extent, and that only two tumours have additional antigenicities. Apart from these two, all the

others may be characterized not only by an increased expression of one or a few antigens but also by a loss of one to seven such antigens. These observations are in accord with a model of antigenic modulation in which a normal antigen may be absent from a given tumour cell as a consequence of the selective pressure from the antibody directed against it (Fenyo *et al.*, 1968; Doig & Chesebro, 1978; Chesebro *et al.*, 1979; Ioachim & Sabbath, 1979; Lieberman *et al.*, 1979).

All these results were obtained using thymoma-derived cell lines. Therefore, it seemed important to investigate the validity of our conclusions regarding primary thymomas. Preliminary experiments (data not shown) were performed using the MAbs and 19 new X-ray-induced primary thymomas. The same type of pattern of reactivity was obtained. Noticeably, MAb 45 recognized only one tumour, and nine tumours could be characterized by a loss of one or several antigens expressed by the normal thymus.

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