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The glycosidic antigen recognised by a novel monoclonal antibody, 75.12, is developmentally regulated on mouse embryonal carcinoma cells

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Monoclonal antibody 75.12 raised against the human ovarian teratocarcinoma cell line PA1 detects a 'Y' or iso-le^b glycosidic structure. Using the 75.12 antibody we have established that the Y antigen is expressed on some but not all mouse embryonal carcinoma (EC) lines. The Y or 75.12 antigen-positive EC cell lines F9 and PCC4 cease to express the antigen after differentiation induced with retinoic acid and this decreased expression parallels the morphological differentiation of the EC cells. These results support not only the idea that carbohydrate structures present on embryonic cells undergo marked alteration during differentiation, but also that established mouse EC cells may differ in their differentiation states.

Key words: sugar specificity/differentiation/embryonal carcinoma/monoclonal antibody/retinoic acid

Introduction

Carbohydrate antigens on the surface of mammalian cells have excited renewed interest because their expression seems to be developmentally regulated (Stern *et al.*, 1978; Solter and Knowles, 1978; Gooi *et al.*, 1981; Kapadia *et al.*, 1981). Such studies have concentrated on the use of murine embryonal carcinoma (EC) cell lines and their derivatives. Carbohydrate structures identified on mouse EC cells including the SSEA-1 (Solter and Knowles, 1978) and Forssman antigens (Willison *et al.*, 1982), an antigen containing a Fucα1→3 Gal linkage (Miyauchi *et al.*, 1982) and a novel class of high mol. wt. fucosyl glycopeptides (Muramatsu *et al.*, 1978). Carbohydrate structures identified on human teratocarcinoma cells include a similar class of high mol. wt. fucosyl glycopeptides (Muramatsu *et al.*, 1979, 1982), and blood group antigens of the P, P^k and H series (Bono *et al.*, 1981).

The recognition and identification of such antigens has been facilitated by the isolation of monoclonal antibodies directed against them (Solter and Knowles, 1978). Amongst the most widely studied antibodies has been SSEA-1 monoclonal antibody which recognises the X or iso-le^a structure involving a Fucα1→3 Glc NAc linkage (Gooi *et al.*, 1981). Whilst the role of glycosidic antigens in differentiation and in cell:cell interactions remains unclear, the increasing number of monoclonal antibodies available to define specificities, which previously could be characterised only with difficulty on intact cells, should allow progress to be made.

Here we report the isolation of a monoclonal antibody, 75.12 raised against a human ovarian teratocarcinoma cell line PA1 which specifically recognises the Y or iso-le^b glycosidic antigenic structure involving Fucα1→2 Gal and Fucα1→3 Glc NAc linkages. The expression of this antigen on various murine embryonal carcinoma cell lines and on differentiated cell populations derived from them, either with or without retinoic acid treatment, is discussed and compared with that of the X antigen detected by SSEA-1.

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Results

Characterisation of the reactivity of the 75.12 monoclonal antibody on human cells

Monoclonal antibody 75.12 (75.12 MA) was raised against the human ovarian teratocarcinoma cell line PA1. Extensive testing against a panel of *in vitro* human cell lines (Figure 1) derived from germinal tumours has shown that the antibody binds strongly not only to the PA1 cell line, but to two choriocarcinomas (Bewo and Jar) and to some, but not all, human EC cell lines (e.g., 833KE). 75.12 MA does not react with 11 out of 16 human cell lines tested which were derived from a variety of non-germinal tissues. Amongst the positive cell lines in this latter group were HeLa, two of the four melanomas tested (Bowes and Belowski) and to a lesser extent the HL-60 and HT29F cell lines. Fluorescence tests confirmed this tissue distribution; cell lines strongly positive in the radioimmunoassay (RIA) such as PA1 or HeLa show intense labelling of between 60 and 100% of the cell population (Table I). 75.12 MA does not react with normal adult or foetal fibroblasts, group A, B, O Le^{a+b-} or O Le^{a-b+} erythrocytes, platelets or lymphocytes. The antibody is also negative on sperm preparations. From this and other studies on human tissue sections (Blaineau and Avner, in preparation), we conclude that 75.12 MA reacts selectively with a limited number of human cell types of ectodermal and endodermal origin. 75.12 MA has been shown to be an IgM isotype.

Characterisation of the biochemical specificity of the 75.12 MA

The rather extended specificity of 75.12 MA suggested to us that it might be recognising a sugar specificity. To test this, batch adsorptions of the antibody with immunoadsorbents bearing a variety of sugar structures were carried out (Tables II and III). Only the immunoadsorbent bearing the Y determinant adsorbed 75.12 antibody activity (Table II). After adsorption with all the other adsorbents (Table II) the specific activity of the supernatants remained unchanged and equivalent to that obtained with the non-adsorbed hybridoma supernatant, or with supernatants adsorbed with silica beads alone.

This result was indicative of the carbohydrate specificity of the antibody but obviously did not allow its quantitation. This was achieved by the purification of 75.12 antibody on the Y-immunoadsorbent and use of the purified antibody in a competition RIA with a series of artificial glycosidic antigens and iodinated Y-bovine serum albumin (BSA) antigen (see Figure 2). None of the artificial antigens tested (see Table III) other than Y-BSA and X-BSA showed any significant inhibition even when concentrations as high as 100 μg/ml were us-

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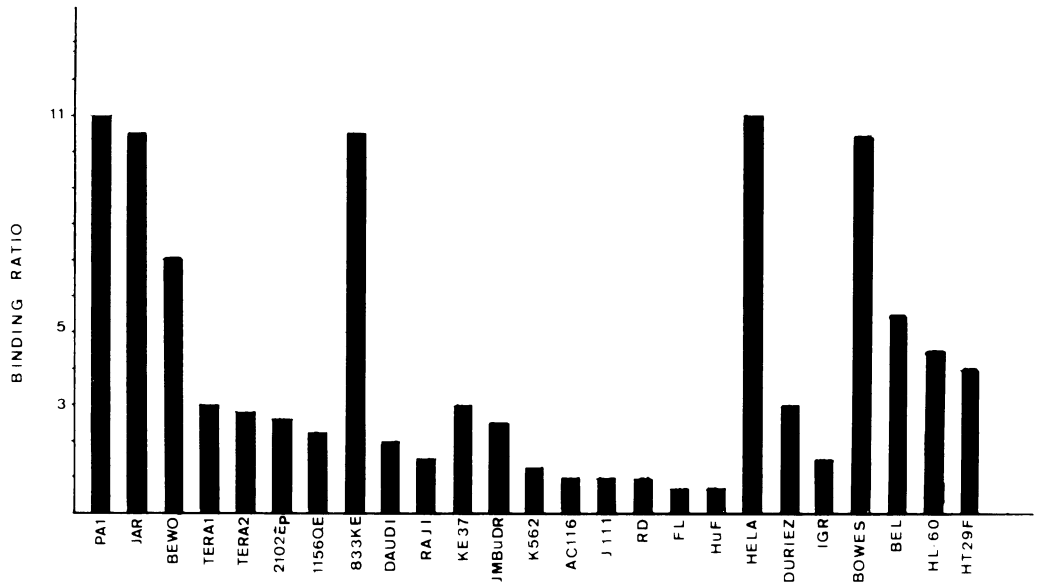

Fig. 1. Histogram showing binding ratios of 75.12 MA to various human cell lines (for origins see Table V).

Table I. Immunofluorescence testing of the 75.12 MA on human cell lines

Cell lines	IF%[a]
PA1	60
Tera2[b]	2
833 KE[b]	77
1156QE[b]	2
HT 39/7	1
HeLa	90
Raji	3
Daudi	4
HL-60	15

[a]IF%: percentage of fluorescent cells.
[b]Results obtained by cytofluorometry. In these tests supernatants from the X63 myeloma secreting an IgG ($\gamma_1\kappa$) marked between 1 and 3% of the cells. Similar results were obtained with irrelevant IgM monoclonal antibodies.

ed. 0.4 μg/ml of cold Y-BSA antigen on the other hand was sufficient to give 50% inhibition. The X-BSA antigen also inhibited, but 160 μg/ml were necessary to obtain a 50% inhibition. The Y-BSA antigen is therefore a 400 times better inhibitor than the X-BSA antigen in this system and we conclude that the 75.12 antibody is preferentially directed against the Y oligosaccharidic structure:

$$\begin{array}{l} \alpha\ \text{L Fuc} \qquad \alpha\ \text{L Fuc} \\ \quad\downarrow\ 1,2 \qquad\ \downarrow\ 1,3 \\ \beta\ \text{D Gal}(1\rightarrow4)\ \beta\ \text{D Glc NAc-O-R}_{1,2} \end{array}$$

Expression of the Y antigen detected by the 75.12 MA on mouse EC cell lines and their derivatives

The Y antigen recognised by 75.12 is structurally related to the X antigen known to be expressed by all mouse EC cell lines (Solter and Knowles, 1978). We looked, therefore, for expression of the Y antigen on murine EC cells and their derivatives. The Y antigen was found to be present on the F9 (F9-41) and PCC4 (PCC4/Aza) EC cell lines as well as on the EK-1-129 EK cell line (Table IV). It was not expressed by two other EC cell lines tested (PCC3/A/1 and PCC7-S-1009) nor

Table II. Adsorption of 75.12 MA activity by oligosaccharidic immunoadsorbents

Adsorbent used[a]	Experiment 1		Experiment 2	
	c.p.m.[b]	BR[d]	c.p.m.[c]	BR
–	12 725	20	10 117	27
Silica unlinked	12 760	20	NT	NT
X	12 414	19	9721	26
Y	2032	3	1656	4
H type 2	12 231	19	9882	26
Type 2 precursor	12 654	20	NT	NT
Le[a]	13 350	21	NT	NT
Le[b]	12 191	19	10 919	29
H type 1	12 048	19	NT	NT
Type 1 precursor	12 004	19	NT	NT
Le disaccharide	12 650	20	NT	NT
T	12 655	20	NT	NT
Forssman	11 922	19	NT	NT
I pentasaccharide	12 600	20	NT	NT

[a]For the structure of the oligosaccharide immunoadsorbents, see Table III.
[b]Values are the average of three determinations. X63 = 640 c.p.m. Similar values to X63 were obtained using an irrelevant IgM monoclonal antibody.
[c]Values are the average of three determinations. X63 = 375 c.p.m. Similar values to X63 were obtained using an irrelevant IgM monoclonal antibody.
[d]BR = binding ratio.

by the yolk sac endoderm line PYS-2 and the trophoblastoma cell line TDM-1. The RIA results have been confirmed by immunofluorescence studies on the F9 and PCC4 cell lines, 50–100% of the cell populations being 75.12 positive (Table IV). Treatment of the PCC3/A/1 cell line with retinoic acid did not induce Y antigen expression.

Expression of the Y antigen on differentiating cultures of the Y antigen-positive EC cell line F9 and PCC4

Both the F9 and PCC4 cell lines can be induced to differentiate by retinoic acid, though in the case of PCC4 this process is accompanied by marked cell lysis. Figure 3a shows that the Y antigen detected by 75.12 MA is progressively lost from F9 cell populations during the course of retinoic acid-induced differentiation. Few 75.12-positive cells remain in 4-day

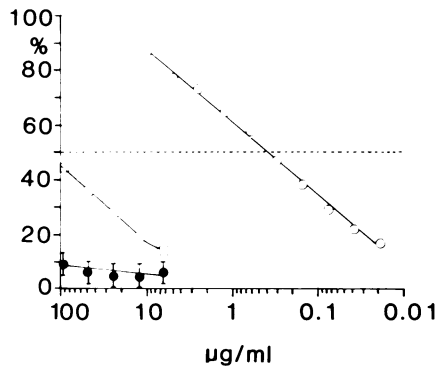

Fig. 2. Inhibition of the binding of [^{125}I]Y-BSA to purified 75.12 antibody by artificial antigens. The artificial antigens were: Y-BSA (○); X-BSA (□); remaining other antigens tested (see Table III) ± standard deviation ⊕. Vertical axis: % binding inhibition. Horizontal axis: antigen concentration in μg/ml.

Table III. Structure of the synthetic oligosaccharides used to determine the sugar specificity of the 75.12 MA

Trivial name	Structure[a]
Y	α L Fuc (1,2) / α L Fuc (1,3) / β D Gal (1→4) β D Glc NAc-O-$R_{1,2}$
X	β D Gal (1→4) β D Glc NAc-O-$R_{1,2}$ / α L Fuc (1,3)
H type 2	β D Gal (1→4) β D Glc NAc-O-$R_{1,2}$ / α L Fuc (1,2)
Type 2 precursor	β D Gal (1→4) β D Glc NAc-O-$R_{1,2}$
Lewis[b]	α L Fuc (1,2) / α L Fuc (1,4) / β D Gal (1→3) β D Glc NAc-O-$R_{1,2}$
Lewis[a]	α L Fuc (1,4) / β D Gal (1→3) β D Glc NAc-O-$R_{1,2}$ / α L Fuc (1,2)
H type 1	β D Gal (1→3) β D Glc NAc-O-$R_{1,2}$
Type 1 precursor	β D Gal (1→3) β D Glc NAc-O-$R_{1,2}$
Lewis-disaccharide	α L Fuc (1→4) β D Glc NAc-O-$R_{1,2}$ / α L Fuc (1,2)
B	α D Gal (1→3) β D Glc-O-R_1 / α L Fuc (1,2)
A type 2	α D Gal NAc (1→3) β D Gal (1→4) β D GlcNAc-O-R_1 / α L Fuc (1,2) / α L Fuc (1,4)
A Lewis[b]	α D Gal NAc (1→3) β D Gal (1→3) β D Glc NAc-O-R_1 / α L Fuc (1,2) / α L Fuc (1,3)
AY	α D Gal NAc (1→3) β D Gal (1→4) β D Glc NAc-O-R_2
I	β D Gal (1→4) β D Glc NAc (1,3) / β D Gal-O-R_2 / β D Gal (1→4) β D Glc NAc (1,6)
T	β D Gal (1→3) α D Gal NAc-O-R_2
Forssman	α D Gal NAc (1→3) β D Gal NAc-O-R_2

[a]R_1: $(CH_2)_8$--CO-NH-BSA (BSA).
R_2: $(CH_2)_8$--CO-NH-Chromosorb (Crystalline silica).
Gal: galactose, Glc NAc: N-acetylglucosamine, Fuc: fucose, Gal NAc: N-acetylgalactosamine.

Table IV. Expression of the 75.12 antigen on murine EC and EK cell lines

Cell line	RIA BR[a]	IF%[b]
F9	14	50–100
PCC4/Aza	10	83
PCC3/A/1	1	NT
PCC3 + retinoic acid	1	NT
PCC7-S-1009	1	NT
EK-1-129	6	NT

[a]BR: binding ratio. Average of two or more individual cultures.
[b]IF%: percentage of fluorescent cells.

retinoic acid-treated F9 cultures and this loss of reactivity is associated with the virtually complete disappearance of cells having EC morphology from the population. The disappearance of EC morphology from such treated cultures has been shown previously to be associated with loss of the X antigenic structure (Figure 3b) (Solter *et al.*, 1979). Examination of incompletely differentiated F9 cultures (2 days) by immunofluorescence double labelling suggests that disappearance of the X and Y antigens is not necessarily concomitant, since Y^+X^- cells have been observed in such experiments.

In one such experiment, 60% of the partially differentiated F9 cell population was found to express both the X and Y antigens (Y^+X^+), 30% neither of the antigens (Y^-X^-) and ~10% of the cells to express the Y but not the X antigen (Y^+X^-). Y^-X^+ cells have not on the other hand, been unambiguously identified in such experiments. PCC4 cell populations induced by retinoic acid, like F9, lose the Y antigen as detected by 75.12 MA binding in RIA. The binding ratio drops from ~8.0 or 9.0 in undifferentiated cultures to 1.0 in retinoic acid-treated cultures (results not shown).

Expression of the Y antigen on mouse embryonic and foetal tissues

Eight and 16 celled morulas as well as intact blastocysts failed to show specific binding of the 75.12 MA in immunofluorescence tests. A 129/Sv anti-F9 serum used as a positive control, on the other hand, marked 70% of the morulas and blastocysts, >60% of blastomeres being positive. Post-implantation foetuses were examined using the immunoperoxidase technique (Schlom *et al.*, 1980). Testing with the 75.12 MA of serial sections of 10-, 12- and 14-day foetal mouse tissue failed to detect any significant binding of the antibody to any tissue. Serial sections of 16-day foetal tissue on the other hand showed Y antigen expression on the olfactory cavity and rhinopharyngeal regions as well as on certain mucus epithelia of the lower digestive tract.

Discussion

75.12 MA raised against the ovarian teratocarcinoma cell line PA1 has been shown to recognise a carbohydrate antigen having the trivial name of Y or iso-le[b] and consisting of an α1→3, α1→2 fucosylated type 2 blood group chain. The antibody is highly specific and has a 400-fold lower affinity for the 'X' glycosidic structure, consisting of a type 2 blood group chain bearing only an α1→3 fucosyl. The latter, on the other hand, is the structure defined by the SSEA-1 monoclonal (Solter and Knowles, 1978) which reciprocally does not recognise the 'Y' structure.

In view of the closely related antigenic structures they

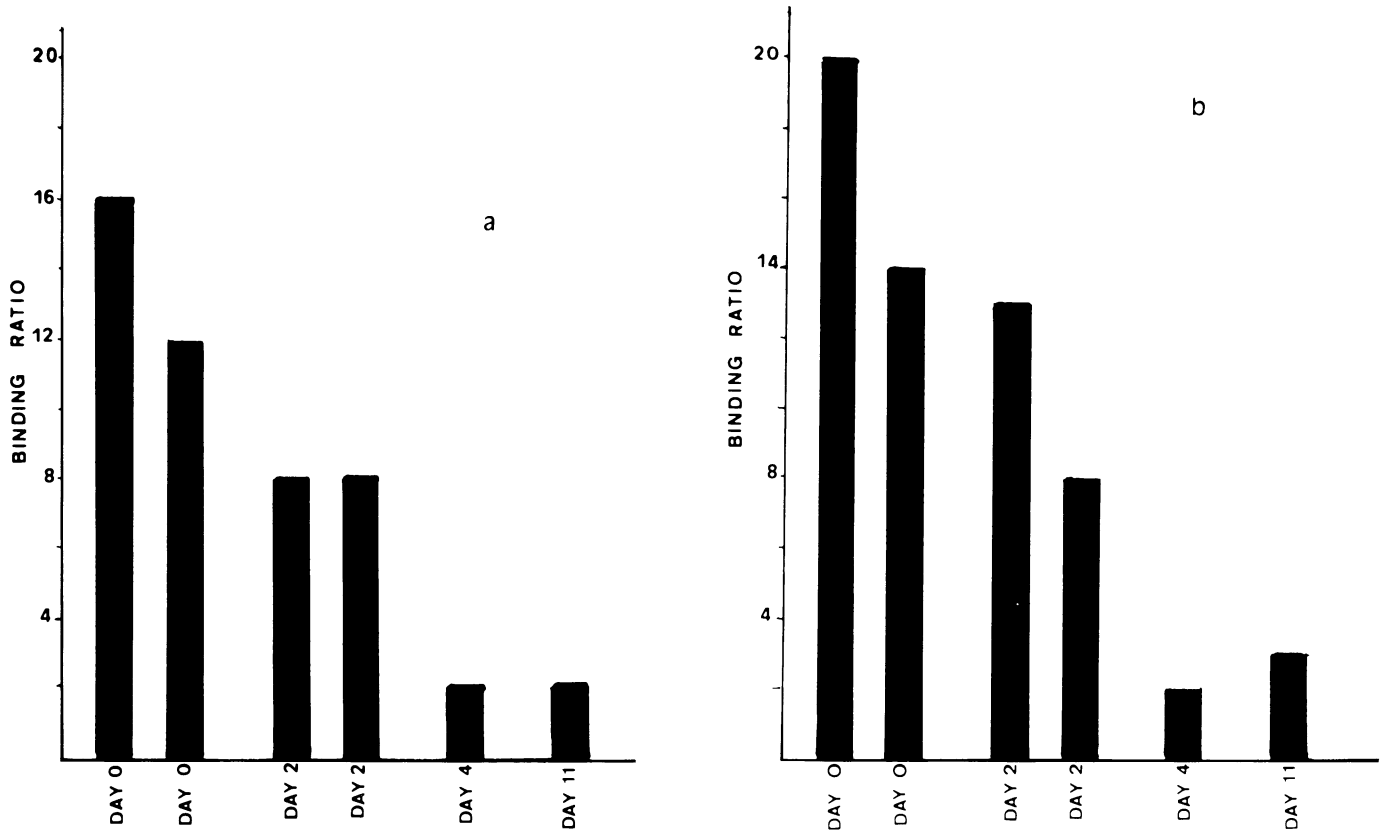

Fig. 3. Binding ratios of F9 cells treated with retinoic acid for various periods with monoclonal antibodies 75.12 and SSEA-1. **(a)** 75.12; **(b)** SSEA-1.

recognise, it is of interest to note the very different binding distributions these two antibodies have on *in vitro* cell lines. SSEA-1, for example, reacts with all mouse EC and EK cell lines, whilst the 75.12 MA reacts with only a subset of such strains. SSEA-1 is clearly positive on the Tera1 and Tera2 human EC cell lines on which 75.12 is negative. Other differences relate not only to the absence of 75.12 MA binding to either mouse sperm or human erythrocytes but above all to its tissular distribution *in vivo* (Blaineau and Avner, in preparation). Whilst some of the differences in 75.12 binding patterns to similar cell types, such as that seen for the human melanoma cells where two of the four strains are positive (Figure 1), may be due to genetic differences in the donor origin of these strains, the finding that the 75.12 MA binds to only subsets of EC cells cannot be attributed to such genetic diversity. Both F9 and PCC4 cell lines which are positive for the 75.12 antigen, and PCC3 which is negative were all derived from the same initial tumour OTT 6050, and are syngeneic in 129/Sv mice. In view of the finding that 75.12 antigenic expression disappears from the F9 and PCC4 cell lines after retinoic acid-induced differentiation, and the common genetic background of these three strains, it is tempting to imagine that the reactivity pattern corresponds to a difference in the equivalent developmental stage of these mouse EC cell lines. Although a complete analysis of the reactivity of the 75.12 MA on mouse embryonic tissue has not yet been carried out, it is clear that it is absent from 8 and 16 cell morulas and from the trophectoderm surrounding the intact blastocyst. Analysis of serial sections of 10-, 12- and 14-day old foetuses has similarly failed to reveal the presence of the 75.12 antigen. Expression of the 75.12 antigen is however found on certain epithelia in 16-day old foetal tissue. It seems probable therefore that the 75.12 antigen appears later in embryonic development than the SSEA-1 antigen, being expressed at some point either during or after blastocyst formation, antigen expression being subsequently turned off prior to day 10 of gestation.

Since the SSEA-1 and 75.12 antigens differ only by a single $\alpha 1 \rightarrow 2$ fucose and both disappear from F9 EC cells after retinoic acid treatment, a similar control mechanism could be envisaged for expression of both antigens. Although this cannot be completely excluded, the finding that SSEA-1(X)$^-$ 75.12(Y)$^+$ cells are present in differentiating F9 cell populations suggests that extinction of both antigens during differentiation is not necessarily concomitant.

Control of Y and X antigen expression during differentiation could occur: (i) by changes in the availability of the immediate precursor acceptor structures for fucosylation, the H type 2 or type 2 precursor chains; (ii) by the control of transferase enzymes necessary for the $\alpha 1 \rightarrow 2$ and $\alpha 1 \rightarrow 3$ fucosylation steps; (iii) by changes in the availability of the lipid or protein molecules carrying the glycosidic specificities. Whilst it is currently impossible to rule out any of these mechanisms categorically, our finding that there is no evidence for a marked accumulation of the H type 2 or type 2 precursor chains in retinoic acid-treated cultures suggests that the disappearance of the antigens cannot be linked to a deficient terminal $\alpha 1 \rightarrow 2$ or $\alpha 1 \rightarrow 3$ fucosylation step. This result is also supported by preliminary findings of $\alpha 1 \rightarrow 2$ and $\alpha 1 \rightarrow 3$ fucosyl transferase activities in both control and retinoic acid-treated F9 cultures.

Attempts to immunoprecipitate molecules carrying the Y specificity with the 75.12 MA have so far been unsuccessful. Whilst interpretation of this type of negative result is am-

Table V. Human mouse cell lines used and their origins

Cell line	Cell type	Reference
FL	Amnion	Fogh and Lund, 1957
HeLa	Cervical carcinoma	Gey *et al.*, 1952
Bewo	Choriocarcinoma	Patillo and Gey, 1968
Jar	Choriocarcinoma	Lipinski *et al.*, 1981
HT29F	Colon carcinoma	Pinto *et al.*, 1982
AC116	Liver carcinoma	Obtained from Dr.Feldman Hôpital Beaujon, Paris
Bowes	Melanoma	Rikjen and Collen, 1981
Duriez	Melanoma	Kalil and Fellous, 1982
Belowki	Melanoma	Kalil and Fellous, 1982
IGR3937	Melanoma	Kalil and Fellous, 1982
PA1	Ovarian teratoma	Zeuthen *et al.*, 1980
RD	Rhabdomyosarcoma	McAllister, 1969
Tera1	(EC) testicular teratoma derived	Fogh and Trempe, 1975
Tera2	(EC) testicular teratoma derived	Fogh and Trempe, 1975
833KE	(EC) testicular teratoma derived	Bronson *et al.*, 1980
1156QE	(EC) testicular teratoma derived	Wang *et al.*, 1980
2102Ep	(EC) testicular teratoma derived	Wang *et al.*, 1980
HuF	Foreskin cells	Levy, 1973
Raji	Burkitt lymphoma	Pulvertaft, 1964
Daudi	Burkitt lymphoma	Klein *et al.*, 1968
K562	Erythroleukaemia	Lozzio and Lozzio, 1975
HL-60	Promyelocytic leukaemia	Collins *et al.*, 1977
Ke37	T cell leukaemia	Preud'homme *et al.*, 1979
JmBudR	T cell leukaemia	Preud'homme *et al.*, 1979
J111	Monocytic leukaemia	Osgood and Brooke, 1955
Mouse cell lines		
F9-41	Embryonal carcinoma	Bernstine *et al.*, 1973
PCC4/Aza	Embryonal carcinoma	Jakob *et al.*, 1973
PCC3/A/1	Embryonal carcinoma	Nicolas *et al.*, 1975
PCC7-S-1009	Embryonal carcinoma	Fellous *et al.*, 1978
EK-1-129	(EK) Embryonal carcinoma derived from blastocyst culture	Obtained from Drs.F.Kelly and H.Condamine
PYS-2	Yolk sac endoderm	Lehman *et al.*, 1974
TDM-1	Trophoblastoma	Nicolas *et al.*, 1976

biguous it is probable that the specificity is normally expressed, at least in part, attached to glycolipid molecules. Further studies involving characterisation of such molecules on EC cells and their early differentiated counterpart may well cast light on the problem of antigenic control.

Although it is clear that the 75.12 or Y antigen is not an exclusive germ cell-specific onco-developmental antigen, its use with both the mouse EC differentiation model and the human EC differentiation model (not shown), suggest that the antigen defined by 75.12 MA will be of great value as a differentiation marker. Clarification of the tissue and exact stage of mouse embryogenesis at which the antigen appears should enhance its value still further.

Materials and methods

Cell lines and culture conditions

Mouse myeloma cell lines P3-NS1-Ag/4 (NS1) and P3-X63-Ag/8 (X63) were obtained from G.Kohler, Basel Institute for Immunology. The origins of the other *in vitro* cell lines used in these studies are shown in Table V. All the cell lines were routinely cultivated on Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% heat-inactivated foetal calf serum (FCS) except Tera1 and Tera2 which were normally cultivated on McCoys 5A medium + 15% FCS. Cultures were routinely passaged every 2–3 days, attached cell lines being subcultured using trypsin/EDTA (0.25%, 0.1%) except in the case of PCC3 and PCC4 which were detached by gentle pipetting. Cultures were grown at 37°C in a 10% CO_2, 90% air mix. F9-41 (F9) cells were grown on gelatinised culture dishes.

Differentiation of murine EC cell cultures with retinoic acid

Cultures were treated with 2×10^{-7} M retinoic acid for periods of 2–14 days (see text). Retinoic acid-containing medium was routinely changed every 2 days and cultures replated where necessary.

Immunisation, hybridisation and selection of specific hybridomas

Two-month old male (BALB/c x C57Bl/6)F_1 mice were simultaneously injected i.p. and s.c. with 3×10^7 X-irradiated (3000 rads) cells of the ovarian teratocarcinoma PA1 which had been preincubated with a (BALB/c x C57Bl/6)F_1 mouse anti-human HeLa cell serum (v/v). In all three, injections were given at 2-week intervals, the second being i.p. and to the foot-pad, the third i.p. alone. Four days after this last injection, 1.2×10^7 spleen cells were fused with 2×10^7 NS1 myeloma cells using 50% polyethylene glycol 4000 (BDH, Poole, UK), the fused cells were dispensed into 96 well dishes (Flow Laboratories) containing 3×10^3 peritoneal macrophages as feeders (Fazekas de Saint Groth and Scheidegger, 1980) and hybrids selected in HAT medium (DMEM, low glucose formulation, supplemented with 20% FCS, 50 U/ml penicillin, 100 μg/ml streptomycin, 10^{-3} M sodium pyruvate, 2×10^{-3} M L-glutamine, 350 mg/ml D-glucose, 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, 1.6×10^{-5} M thymidine. After ~2 weeks, supernatants were tested by radioimmune binding assay against the PA1 cell line. Positive wells were cloned twice by limiting dilution using BALB/c peritoneal macrophages as feeders. Out of the initial 186 microtiter wells tested, 90 showed anti-PA1 activity. Out of the 10 hybridomas showing the strongest activity and retained for cloning and preliminary studies, one, 75.12, was chosen for detailed analysis and is described here.

Solid-phase RIA

Indirect RIAs were performed on live cells using the technique previously described by Goodfellow *et al.* (1979).

10^5 cells were pelleted in V-bottomed multiwell plates and incubated at room temperature for 1 h with 50 μl of the supernatants. After three phosphate-buffered saline (PBS) washings, 100 000–200 000 c.p.m. of ^{125}I-labelled anti-mouse IgG reagent in 50 μl PBS/BSA/Azide was added and the mixture incubated for a further hour at 4°C. After four more washings, specifically bound radioactivity in the pellet was determined using an LKB Gamma counter. The background binding to cells was given by supernatants from the IgG_1 producer X63 myeloma and binding ratios of greater than three times that of the non-specific controls were considered positive.

Indirect immunofluorescence testing

10^6 cells were incubated with 50 μl of undiluted monoclonal antibody supernatant for 45 min at 4°C, then washed three times with PBS/BSA/Azide and incubated with 50 μl of fluorescein-conjugated rabbit anti-mouse immunoglobulin (Nordic RAMIG/FITC) diluted 1/100 in PBS/BSA/Azide, for a further 1 h at 4°C. After three further washings with PBS/BSA/Azide, the cells were resuspended in a drop of 80% glycerol in PBS and examined using a Leitz microscope equipped for epi-illumination. Negative controls with X63 supernatants and positive controls using a mouse anti-HeLa cell serum were routinely run with each test series. Double labelling experiments were carried out using either biotin-avidin reagents (Vector) or 75.12 directly fluoresceinated antibody and indirectly labelled SSEA-1 antibody. Control experiments included the use of SSEA-1 and 75.12 antibodies pre-adsorbed on their respective immunoadsorbents.

Flow microfluorometry

Flow microfluorometry studies were carried out on cell populations treated as for indirect immunofluorescence testing. After the final incubation with the RAMIG/FITC serum and subsequent washing, the cells were however treated to remove cell debris by centrifuging through 0.5 ml FCS, the supernatant then being removed and 0.5 ml of PBS being added to the cell pellet immediately prior to fluorimetric analysis, 3000 cells being routinely analysed. The fluorescence intensity distribution of the monoclonal antibody was compared with that obtained using the X63 myeloma supernatant as negative control.

Cell isolation from human donors

Erythrocytes. Fresh citrated blood from A, B or O donors was used to prepare erythrocytes (Davidson and Parish, 1975).

Human bone marrow cells. Ficoll purified bone marrow cells were obtained from the sternum of healthy donors using the technique described by Davidson and Parish (1975).

Human spermatozoa. Human spermatozoa were prepared according to the method of Fellous *et al.* (1974) from semen obtained from healthy donors whose spermatozoa were first of all assessed for their mobility, viability and number. Purified sperm were maintained in PBS/BSA at 37°C.

Hybridoma isotype characterisation

10-fold concentrated culture supernatants were tested by double immuno-

diffusion in agarose against commercially available rabbit anti-mouse immunoglobulin class and subclass-specific antibodies (Nordic). Such antisera were verified for their class specificity by testing against purified mouse myeloma proteins of known isotypic class (Bionetics). The 75.12 MA was shown to be an IgM.

Immunoadsorbents

Synthetic oligosaccharides coupled to insoluble matrices of crystalline silica (Lemieux, 1978) were generous gifts from Professor Lemieux (Edmonton, Canada). The synthesis of type 2 precursor disaccharide, X trisaccharide, H type 2 trisaccharide and Y tetrasaccharide were performed by Professor Lemieux's team (Hindsgaul *et al.*, 1982). Chemical pathways for the synthesis of most of the other oligosaccharides have been reviewed in Lemieux (1978). The structures of the oligosaccharides are given in Table III.

Artificial antigens

The same synthetic oligosaccharides used to prepare immunoadsorbents have been coupled to BSA at a molar hapten/BSA ratio of 15–20 to 1 (Lemieux, 1978). The Y-BSA compound was labelled with ^{125}I (10 μCi/mg) by the chloramine T method (Greenwood *et al.*, 1963). Structures are given in Table III.

Batch adsorptions

300 μl of undiluted hybridoma supernatants were incubated at room temperature with 50 mg of each immunoadsorbent. Tubes were maintained under constant gentle agitation for 1 h at room temperature. After decanting, the supernatants were tested for their antibody activity in the cellular RIA described above.

Antibody purification

4 ml of ascitic fluid was passed through a column of Y-immunoadsorbent (1.5 g). After extensive washing of the column with PBS, the bound material was desorbed with 1% NH_4OH and immediately neutralised with glycine/HCl. About 7 mg of protein were eluted from the column.

RIA with artificial antigens

1 ml polystyrene tubes were coated overnight with 50 μl of a 10 μg/ml solution of purified antibody in PBS. After washing, 50 μl of the 2-fold serial dilutions of inhibitors in 1% BSA/PBS were incubated overnight at room temperature. The tubes were washed again, 50 μl of the [^{125}I]Y-BSA (20 000 c.p.m.) in 1% BSA/PBS added and the whole incubated at room temperature for 6 h. After a final wash, bound radioactivity was counted in an LKB automatic gamma counter. In the absence of inhibitor, 30% of the radioactivity was bound to the coated tubes. All washings were performed with 1 ml of tap water and each point was done in triplicate.

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