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Monoclonal Antibodies as Probes for Differentiation and Tumor-Associated Antigens: a Forssman Specificity on Teratocarcinoma Stem Cells

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Summary

A set of monoclonal antibodies derived by fusing P3-NS1/1-Ag4-1 myeloma cells with spleen cells from a rat immunized with mouse spleen were screened for activity against a tumor cell panel. One of these antibodies was found to react only with mouse embryonal carcinoma cells and no other tumor cell type tested, including differentiated derivatives of teratocarcinomas. In the adult mouse, this antigen is expressed by subpopulations of cells in the spleen, bone marrow, lymph node, brain, kidney and testes, although not in liver and thymus. This antigen has a species and tissue distribution consistent with that of Forssman antigen. The molecules which carry this specificity on the embryonal carcinoma cells appear to be glycolipids.

Introduction

Differentiation antigens are characteristic of particular cell types and are often expressed during the differentiation of those cell types. They have been defined by allogeneic and xenogeneic antisera, particularly with lymphoid cells (Greaves, Owen and Raff, 1973; Cantor et al., 1975; Williams, 1977). Such surface antigenic markers have been used for cell identification (Raff, 1969), cell separation (Edelman, Rutishauser and Millette, 1971) and the characterization of the molecules bearing these antigenic determinants (Silver and Hood, 1976). There are drawbacks to such antisera, however, which have been discussed previously (Williams, Galfrè and Milstein, 1977; Pearson et al., 1977). Briefly, with xenogeneic immunization, where most molecules are potentially antigenic, the problem is to remove the antibody specificities not of interest. This can be achieved to some extent by extensive absorptions (Brown and Greaves, 1974) or by using highly purified immunogens (Williams, 1977). Congenic immunizations genetically restrict the immune response, but the antisera may be weak and recognize only polymorphic antigens. In both cases, even when operationally specific antisera have been defined, they are still very complex mixtures of antibodies of different specificities, affinities and classes.

The production of monoclonal antibodies by the myeloma-hybrid technique overcomes many of these problems (Köhler and Milstein, 1975, 1976; Williams et al., 1977). By their very nature, the monoclonal antibodies dissect a complex array of antigens without the necessity for first purifying them. Antigens present only on minor subpopulations of cells are difficult to detect because of the insensitivity of screening procedures. One way around this problem is to use tumor cells derived from particular cell types, both as immunogens and as targets for screening activities of monoclonal antibodies. Another way might be to use as immunogen the mixture of cells whose subpopulations are to be dissected, and to screen the activities of the myeloma-hybrid clones on an assortment of tumor cell lines. We show that screening of a group of monoclonal antibodies on a panel of tumor cells allows the easy detection of antigens carried only by minor subpopulations of the cells used as immunogen.

A set of ten antibody-forming clones was derived by fusing P3-NS1/1-Ag4-1 myeloma cells with spleen cells from a rat immunized with mouse spleen cells. These monoclonal antibodies exhibited different patterns of reactivity with mouse leukocytes and leukocyte cell lines (Springer et al., 1978). The binding activity of these antibodies to a panel of about 20 tumors of various types was further analyzed to learn more about their specificities. It was observed that two of the monoclonal antibodies did not bind to any of the tumor cells tested except teratocarcinomas. Binding to them was very strong, while binding to spleen was very weak.

These antibodies are of special interest because several antigens have been described using conventional antisera which are relatively specific for teratocarcinoma stem cells, cells of the early mouse embryo and spermatozoa, but not for other cell types (Artzt et al., 1973; Gachelin et al., 1977). Others, in addition, react with cells in brain and kidney (Stern, Martin and Evans, 1975; Chaffee and Schachner, 1978). One of the clones reactive with embryonal carcinoma, M1/22.25 HL, was further characterized. It reacts with cells of the preimplantation embryo, testicular cells, spermatozoa, cells in brain and kidney, and 2% of the cells in spleen. The determinant recognized is a Forssman specificity and is apparently carried on a glycolipid molecule.

Results

Preliminary Characterization of M1/22.25.8 (HL)

The production and preliminary characterization of

the monoclonal M1/22.25 will be described elsewhere (Springer et al., 1978). It is a rat immunoglobulin of μ class (IgM), and the clone does not retain the myeloma light chains. It reacts weakly with mouse spleen cells in an indirect binding assay with rabbit $(F(ab')_2$ anti-rat Fab-labeled with ^{125}I. The antibody, however, also binds strongly to sheep erythrocytes, and has a tissue and species distribution which is characteristic of Forssman antigen (see below).

Screening against Mouse Cell Lines

The data in Table 1 show the screening of three monoclonal antibodies against a tumor cell panel by ^{125}I binding. M1/9.3 and M1/69.16 culture supernatants bind to several of the tumor cell lines. In contrast, M1/22.25 does not bind to any of the variety of cell types in the panel except mouse embryonal carcinoma and sheep red blood cells (RBC). These results are confirmed and extended by immunofluorescence (Table 1).

Five lines of embryonal carcinoma cells, which are the stem cells of teratocarcinoma, were found to be positive (Table 2). One is derived from a spontaneous testicular teratocarcinoma (Nulli-SCC.1), three are embryo-derived and the other is derived from an ovarian teratocarcinoma (LT/A-B). The observation that the proportion of fluorescent cells from cultures of teratocarcinomas is never 100% (Table 2) has been noted with other antisera to embryonal carcinoma (Artzt et al., 1973; Stern et al., 1975). This is despite the fact that the cells are morphologically homogeneous embryonal carcinoma when tested, and titration of M1/22.25 against Nulli-SCC.1 (shown in Figure 1) gives plateau labeling. The proportion of positive cells var-

Table 1. Labeling of Various Cell Lines with Monoclonal Antibodies

			Culture Supernatant			
			M1/9. 3	M 1/69.16	M1/22.25	M1/22.25
Cell Line	Strain	Cell type	(Indirect ^{125}I Binding)[a]			(Indirect Immunofluorescence)[b]
EL4	C5 7BL/6	Thymoma	+	±	−	−
P815	DBA/2	Mastocytoma	+	−	−	−
X63Ag8	Balb/c	Myeloma	−	±	−	−
BW51 47	AKR	Thymoma	+	+	−	−
B10A/A2/P5	B10A	Abelson lymphomas	+	+	−	−
5R/A3/P2	B10A(5R)		+	+	−	NT[c]
(2 × 5R)F1/A2/P2	B10A(2R) × B10A(5R)F_1		+	+	−	NT
MBL-2	C57BL/6	Moloney lymphomas	+	±	−	NT
LSTRA	Balb/c		+	−	−	NT
Eveline	Balb/c	Friend leukemia line	−	−	−	−
Eveline DMSO-induced	Balb/c		NT	NT	NT	−
N115	A/J	C1300 neuroblastoma clone	NT	NT	NT	−
STO	Sim	Fibroblast	−	NT	−	−
SV-3T3	Swiss	Transformed fibroblast	NT	NT	NT	−
Py-3T3	Swiss		NT	NT	NT	−
B10MC6A	B10	Methylcholanthrene-induced sarcoma	−	−	−	NT
B10MC6B	B10		−	−	−	NT
Nulli-SCC.1	129/J	Embryonal carcinoma	−	−	+	+
Sheep RBC	Sheep	Red blood cells	−	−	+	+

[a] (−) Binding ratio <1.5; (+) binding ratio >8; (±) between 2–5.
[b] Second layer is rabbit anti-rat.Ig conjugated to fluorescein (R.anti-rat.Ig-F1); (−) no positive cells; mt500 counted in at least two experiments.
[c] NT = not tested.

Table 2. Labeling of Teratocarcinoma-Derived Cells with Monoclonal Antibody M1/22.25

Cell Line	Cell Type	Strain	Positive Cells by Indirect Immunofluorescence[a]	Indirect ^{125}I Binding Ratio[b]
Nulli-SCC.1	Nullipotent embryonal carcinoma	129/J	54.0 ± 2.9 (11)	13.5 (2)
PSA2	Pluripotent embryonal carcinoma (ovarian)	129/Sv	31.4 ± 2.4 (10)	6 (1)
PSA4		129/Sv	56 (2)	NT
C3H/H		C3H/H	NT	8.5 (2)
LT/A-B		LT	70 (1)	NT
Nulli-SCC.1	Pseudo-embryoid bodies	129/J	+[c] (3)	NT
PSA2	Embryoid body endoderm	129/SV	−[d] (3)	NT
PSA5E	Endoderm	129/SV	0 (2)	<1.1

[a] Second layer is R.anti-rat.Ig-F1. >500 cells were counted. Mean ± SE. Number of experiments in parentheses.
[b] 2.5×10^5 cells with neat M1/22.25 supernatant.
[c] 75% had fluorescence covering virtually the entire surface of the pseudo-embryoid body.
[d] 98% had only a few positive cells over the entire surface of the embryoid body.

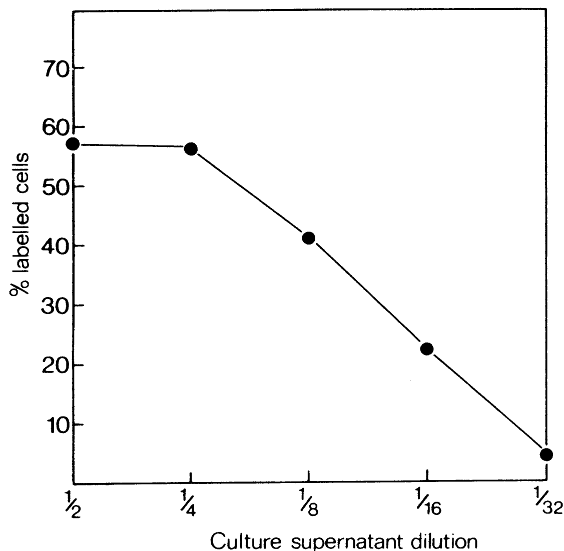

Figure 1. Titration of M1/22.25 by Indirect Immunofluorescence against Nulli-SCC.1 Embryonal Carcinoma Cells

ies among different cell lines (Table 2).

With the exception of Nulli-SCC.1, which has lost the ability to differentiate, these lines will form embryoid bodies under appropriate culture conditions. These embryoid bodies are considered to be equivalent to the embryonic portion of the mouse embryo around the time of implantation. They have an outer layer of endodermal cells and an inner core of embryonal carcinoma (see Martin, 1975). The great majority of the outer endodermal cells of PSA2 simple embryoid bodies do not label with M1/22.25 by indirect immunofluorescence. Nullipotential cell aggregates, which do not have the outer layer of endodermal cells, show bright ring fluorescence staining (Table 2). This lack of expression of the antigen on endodermal cells is confirmed by the failure to label an endodermal cell line derived from embryoid bodies. If embryoid bodies are allowed to attach to a substratum they will differentiate further, and produce dense and multilayered cultures with a variety of differentiated cell types. PSA2 cells made to differentiate in this manner have <1% of the total cells positive after about 2 weeks of growth and differentiation. It therefore seems that most of the differentiated derivatives of the teratocarcinoma stem cells do not express this antigen.

Distribution in Lymphoid Tissues

The data in Table 3 show that by indirect immunofluorescence, M1/22.25 detects a subpopulation of leukocytes which are found mostly in spleen (2.2 ± 0.45%) and bone marrow (2.0 ± 0.28%), although there are some positive cells in lymph node (0.3 ± 0.58%). No positives were found in thymus cell suspensions. Since M1/22.25 will lyse sheep RBC in the presence of guinea pig complement (GPC), this reaction can be used as a quantitative assay for the amount of antigen by absorption. A dilution of the antibody was absorbed with varying numbers of different cells, and the residual cytotoxicity was assayed on sheep RBC labeled with ^{51}chromium. Thymocytes barely absorb cytotoxicity of M1/22.25 for sheep RBC, in contrast to spleen and lymph node cells (Figure 2). The degree of absorption by spleen and lymph node cells compared with embryonal carcinoma cells is consistent with the proportion of positive cells found by immunofluorescence.

The cell type recognized in spleen is probably not a small lymphocyte; it has a granular cytoplasm

Table 3. Labeling of Lymphoid Cells with Monoclonal M1/22.25 by Indirect Immunofluorescence

Cells	First Layer[a]	% Cells Labeled[b]
Spleen	M1/22.25	2.2 ± 0.45 (7)
	–	<0.1
Lymph Node	M1/22.25	0.3 ± 0.58 (3)
	–	<0.1
Bone Marrow	M1/22.25	2.0 ± 0.56 (4)
	–	<0.1
Thymus	M1/22.25	<0.1 (3)
	–	<0.1

[a] 5 medium as first layer.
[b] Second layer is R.anti-rat.Ig-F1 (absorbed X3 mouse spleen). Between 1000–10,000 cells were counted. Mean ± SE. Number of experiments in parentheses.

and will ingest carbonyl iron. Double fluorochrome immunofluorescence experiments using rabbit anti-mouse immunoglobulin conjugated to rhodamine (absorbed with rat spleen) and M1/22.25 with rabbit anti-rat immunoglobulin conjugated to fluorescein, R.anti-Rat.Ig-F1 (absorbed with mouse spleen), show no rhodamine plus fluorescein-positive cells; this cell type does not express surface immunoglobulin. The exact cell type(s) remain to be elucidated.

Distribution in Other Tissues

The expression of the antigen in various mouse tissues was tested by absorption of M1/22.25 cytotoxicity for sheep RBC. Liver does not remove significant activity. When compared with liver on a volume/volume of tissue basis, testes have 128 times, and brain and kidney 8 times as much antigen (Figure 3a). Spermatozoa prepared from the vas deferens and the epidydimus were also able to absorb this activity, although less well than suspensions of testicular cells (Figure 3b). STO fibroblasts do not absorb any activity. These results have been confirmed by indirect immunofluorescence on trypsinized cell suspensions obtained from adult and newborn brain, kidney, testes and liver. Liver cells are negative; there is a small, variable percentage of positive cells in brain and kidney, and up to 10% in testes. Cultures of adult and newborn testes yielded an increased percentage of antigen-positive cells after 2–3 days in culture—40, 17 and 37, 34, respectively, of the surviving cells.

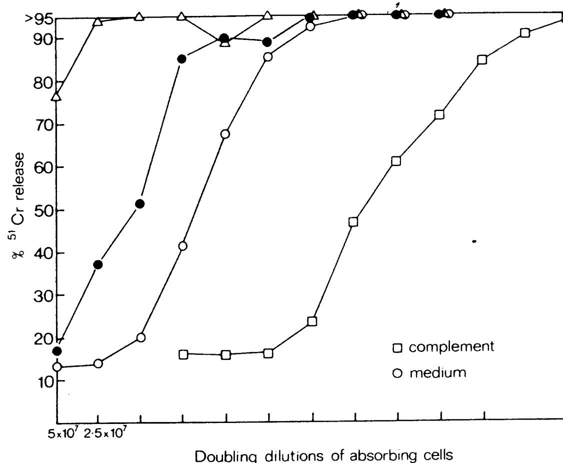

Figure 2. Absorption of M1/22.25 Cytotoxicity for Sheep RBC by Lymphoid Cells

Absorbing cells: (□——□) Nulli-SCC.1; (○——○) spleen cells; (●——●) lymph node cells; (△——△) thymocytes.

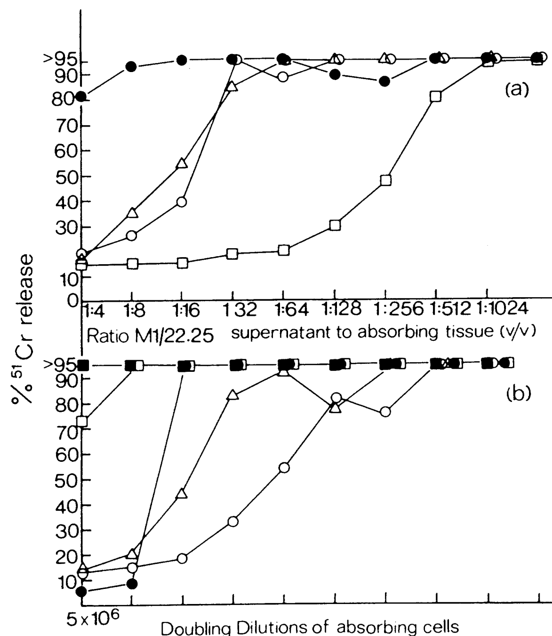

Figure 3. Absorption of M1/22.25 Cytotoxicity by Various Tissues and Cells

(a) Absorbing tissues: (●——●) liver; (△——△) brain; (○——○) kidney; (□——□) testes.
(b) Absorbing cells: (■——■) STO; (○——○) Nulli-SCC.1; (□——□) vas deferens spermatozoa; (●——●) epidydimal spermatozoa; (△——△) testicular cells prepared by trypsinization.

Characterization of the Antigen

The expression of the antigen on other species of red cells was investigated. M1/22.25 is not absorbed by human, horse or mouse RBC, but is completely absorbed by sheep and goat RBC. In addition, absorption of the antibodies by autoclaved guinea pig kidney suggests that the specificity recognized is like that on Forssman antigen.

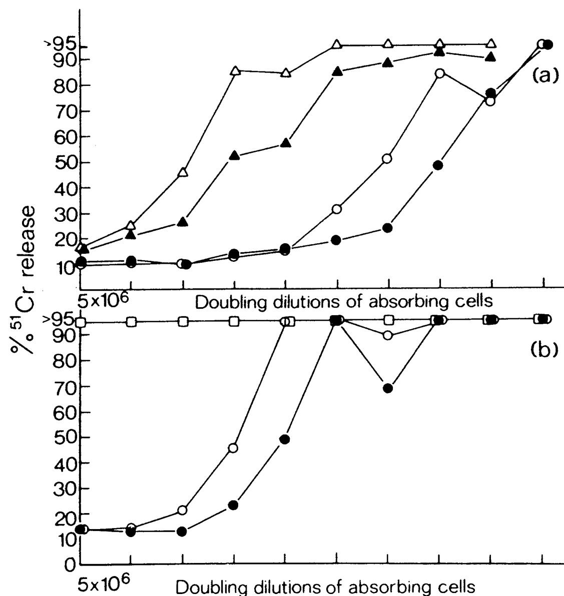

Figure 4. Absorption of M1/22.25 Cytotoxicity by Various Cells
(a) (○——○) boiled Nulli-Scc.1 cells (100°C, 5 min); (●——●) untreated Nulli-SCC.1 cells; (△——△) boiled sheep RBC (100°C, 5 min); (▲——▲) untreated sheep RBC.
(b) (○——○) 1% glutaldehyde (in PBS)-fixed Nulli-SCC.1 (0°C, 30 min); (□——□) 100% methanol-fixed Nulli-SCC.1 (0°C, 30 min); (●——●) untreated Nulli-SCC.1.

Oxen are negative; horses (and guinea pigs) are Forssman-positive, but lack this antigen on their RBC. The absorption by guinea pig kidney, but not by ox RBC, distinguishes the heterophile activity from that shown by Paul Bunnell antibodies (Boyd, 1966).

Although the Forssman hapten isolated from sheep erythrocytes and other sources had been shown to be a glycosphingolipid (see Ziolkowski, Fraser and Mallette, 1975), the specificity recognized by the clonal antibody may be on other molecules on the embryonal carcinoma cells. Thus the nature of the molecule(s) bearing the M1/22.25 antigenic determinant on these latter cells was investigated by immunoprecipitation. No ^{125}I surface (lactoperoxidase) or internally ^{35}S-methionine-labeled Nulli-SCC.1 material could be specifically precipitated. In contrast, the antigen was readily labeled when the cells were grown in the presence of ^{14}C-galactose; depending upon the second layer used, 2–10 fold more cpm were present in specific precipitates compared to controls. Specificity of cpm in the precipitates was validated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). In all cases, the specifically precipitated material ran together with the bromophenol blue front marker.

These results suggest a small glycopeptide or glycolipid, devoid of accessible tyrosines and without methionine. Since carbohydrate molecules are generally much more stable to heat than proteins, the heat stability of the antigenic target of M1/22.25 was tested with sheep RBC and embryonal carcinoma cells. As can be seen from Figure 4, the antigen on both these cells is virtually unaffected by boiling for 5 minutes at 100°C since the ability to absorb cytotoxicity for sheep RBC is not altered significantly. Although glutaraldehyde fixation does not affect the antigenicity on Nulli-SCC.1 cells (Figure 4) or sheep RBC (data not shown), methanol fixation renders the cells unable to absorb M1/22.25. This last result suggests that embryonal carcinoma cells also express M1/22.25 antigenic determinant on a glycolipid; the methanol would release a glycolipid from the membrane but probably would not affect a glycoprotein. The available data are consistent with the antigen recognized by M1/22.25 being a Forssman specificity.

Discussion

Monoclonal antibodies are especially suited for analyzing the individual components of a complex mixture of immunogens. This mixture could be the molecules of the membrane of a homogeneous cell type or those of subpopulations of cells from a heterogeneous tissue. The limitation is that the sensitivity of screening methods for analyzing the large number of antibody-forming fusion products which must be tested may not be sufficient to detect minor subpopulations. One possible approach is to use a wide variety of tumor cell lines as homogeneous probes for the activities of the clonal products. Each tumor might be expected to be a different sample of the probably limited set of differentiation antigens.

Using such a panel, we detected an unexpected activity in a group of monoclonal antibodies from a fusion of a rat spleen immunized against mouse spleen. The activity from M1/22.25 is barely detectable on whole spleen and is highly active on mouse teratocarcinoma cells, but not on a variety of other tumors tested. It had apparently been stimulated by a type of cell in the immunizing spleen forming only about 2% of the population. This approach may be generally useful since a macrophage cell line led to the identification of a macrophage-specific monoclonal antibody. In this case, the eliciting antigen was present on about 4% of the immunizing cells and was also barely detectable using the binding assay on spleen cells (Springer et al., 1978).

Several lines of evidence suggest that the monoclonal antibody M1/22.25 recognizes a specificity of the Forssman antigen. This hapten is widely distributed among animal species (and some bacteria) but not in a phylogenetically ordered way (Humphrey and White, 1963; Boyd, 1966). The

tissue and species distribution of the antigen recognized by the monoclonal antibody is identical to that of Forssman. The Forssman hapten structure has been determined from several different sources, including sheep RBC and is N-acetylgalactosaminosyl-(α1-3)-N-acetylgalactosaminosyl-(β1-3)-galactosyl-(α1-4)-galactosyl-(β1-3)-glucosyl-(β1-1)-ceramide (Ziolkowski et al., 1975). The heat stability, fixation and labeling properties of the M1/22.25 determinant are consistent with this structure. Furthermore, conventional rabbit anti-Forssman sera block the binding of M1/22.25 antibodies to sheep RBC. Anti-Forssman sera, however, may recognize several different antigenic determinants (Forssman antigen), whereas this monoclonal antibody sees only a single Forssman antigenic specificity.

In the mouse, Forssman antigen identified by the clone M1/22.25 is a differentiation antigen. It is absent from mouse thymocytes and red blood cells, but is expressed on subpopulations of cells from bone marrow and spleen, and to a lesser extent from lymph node. The type of spleen cell appears not to be a small lymphocyte and does not express surface immunoglobulin. The antigen is also expressed by subpopulations of cells in brain, kidney and testes, although not in liver. The apparent absorption by spermatozoa prepared from vas deferens and epidydimus must be treated with caution since contaminating cells might account for these results. Nonetheless, the antigen is most probably expressed on cells on the germ line since it is expressed by primordial germ cells in the 12 day genital ridge (P. Stern et al., unpublished observations).

The expression of this Forssman specificity by embryonal carcinoma cells (five different lines) is particularly interesting. The inability to label all the embryonal carcinoma cells in any of the various lines may be related to a failure of the antigen to be expressed during parts of the cell cycle (Iserksy, Metzger and Buell, 1975). Several antisera produced by immunizing mice with embryonal carcinoma cells have been shown to detect antigens common to these stem cells and cells of the early mouse embryo (for example, see Jacob, 1977). M1/22.25 antibodies also detect an antigen expressed by mouse preimplantation embryos (see Willison and Stern, 1978). Since it is known that there are naturally occurring antibodies to sheep RBC in mouse serum (Gisler, Pages and Brussard, 1975), some of which might be directed against the Forssman hapten, it is possible that some conventional antisera to embryonal carcinoma might have this additional activity.

The most studied of embryonal carcinoma-embryo antigens is F9 antigen(s). This is believed to be a glycoprotein and is not expressed by cells in adult brain and kidney. F9 antigen is not expressed by a variety of other tumor cell types and disappears if teratocarcinoma stem cells are allowed to differentiate (Jacob, 1977). The M1/22.25 Forssman specificity is also not expressed on a variety of tumor cells, and is not detected on the endoderm of simple embryoid bodies or on the differentiated cells of longer term teratocarcinoma cultures.

The screening of monoclonal antibodies against a variety of tumor cell lines was the key to the serendipitous discovery of the M1/22.25 activity against embryonal carcinoma cells and early embryo cells. Such a "shotgun" approach may be of wide application in the detection and characterization of differentiation and tumor-associated antigens. Although Forssman antigen was originally described in 1911, the availability of monoclonal antibodies recognizing this hapten will be a powerful tool in attempts to unravel its significance. Its interesting differential distribution in the early embryo (Willison and Stern, 1978) may relate it to important developmental determinative events. At the very least, it is an excellent marker of different subpopulations of cells in a variety of tissues, in differentiating teratocarcinomas and in the preimplantation embryo.

Experimental Procedures

Animals

C57BL/10ScSn (B10) mice, bred in the animal unit of the Laboratory of Molecular Biology or obtained from Olac Southern Limited (Oxon), were used throughout unless otherwise noted.

Cell Cultures

The culture medium generally used was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS). Cells were passaged by disaggregation with 0.25% trypsin in PBS-EDTA and grown in Corning tissue culture flasks.

Teratocarcinoma Cells

Pluripotent homogeneous embryonal carcinoma cell cultures PSA2, PSA4, C3H/H and LT/A-B were obtained from isolated single cells and maintained in the undifferentiated state by subculturing every 3 days and seeding the cells on a confluent layer of mitomycin C-treated STO cells. To obtain embryoid body formation, the cells were seeded at 10^7 cells per 90 mm bacteriological dish in the absence of feeder cells. The medium was changed daily, and endodermal cell layers became apparent on the aggregates 2-3 days later. Further differentiation of the cells occurred after the embryoid bodies were seeded in fresh medium on tissue culture dishes (Martin and Evans, 1975a; Evans and Martin, 1975). Nullipotent embryonal carcinoma cell cultures were obtained from isolated single cells (Martin and Evans, 1975a, 1975b). Stock cultures were maintained by subculturing these cells every third day and seeding at approximately 5×10^6 cells per 75 ml Corning flask. Nullipotent aggregates were obtained by seeding 10^7 cells per 90 mm bacteriological dish.

Other Cell Lines

The lines 5R/A3/P2, (2 ×5R)F1/A2/P2 and B10A/A2/P5 were lymphomas induced with Abelson virus, by T. Lowe in this laboratory, and were maintained in suspension culture in RPMI 1640 with 10% CS supplemented with vitamins and nonessential

amino acids. The Friend leukemia line Eveline, obtained from R. Schaffer, was grown and induced to differentiate as previously described (Friend et al., 1971). SV-3T3 and PY-3T3 were obtained from ICRF, London. B10MC6A and MC6B (Sikora, Stern and Lennox, 1977), EL4 (Gorer and Amos, 1956), P815 (Dunn and Potter, 1957), X63Ag8 (Köhler and Milstein, 1975), the M1/22.25 clone, BW5147 (Hyman and Stallings, 1974), MBL-2, LSTRA, obtained from V. Schirrmacher, PSA5E (Evans, Lovell-Badge and Stern, 1978), and N115, obtained from P. Kauffman (Amano, Richelson and Nirenberg, 1973), were grown as previously described.

Preparation of M1/22.25.8 (HL)

The clone M1/22.25.8(HL) was derived from a fusion between P3-NS1/1Ag4-1 (NS1), which is resistant to 20 μg/ml 8-azaguanine and dies in HAT medium, and rat spleen cells previously immunized with spleen cells from B10 mice, enriched for T lymphocytes by passage through nylon wool and depleted of red cells by centrifugation on Ficoll-Isopaque. A detailed report on this fusion and its products will be published elsewhere (Springer et al., 1978). The clones obtained were grown in DMEM 20% fetal CS. Culture supernatant was harvested at end of log phase and centrifuged (600 × g for 10 min) and stored at 4°C with 10 mM HEPES, 0.1% NaN3. The culture supernatant from a single batch was used either neat or diluted by half in most experiments. M1/9.3.4 and M1/69.15.2 are other clones derived from the same fusion.

Preparation of Mouse Cells for Immunofluorescence and Absorption

Lymphoid Cells

Mice were killed by cervical dislocation. Their peripheral and mesenteric lymph nodes, spleens, thymic lobes and bone marrow plugs (from the femur) were removed and immediately placed in cold Earle's balanced salt solution (EBSS) containing 0.8% bovine-serum albumin (BSA), 10 mM HEPES and 0.1% azide. Cells were washed twice following teasing with 23 gauge needles and adjusted to 20 × 10^6/ml for immunofluorescence tests.

Other Mouse Tissues

Adult or newborn (4–11 days old) testes minus tunica albugea, kidneys, liver and brain were prepared by screening them through a tea strainer. The larger clumps were allowed to settle out, and the resulting suspension was washed at least 3 times with EBSS 0.8% BSA. The packed volumes were measured and a 50% suspension (v/v) was prepared.

The same tissues were prepared for immunofluorescence by finely chopping the organs with a razor blade and stirring them in Hank's BSS without Ca^{++} and Mg^{++} and containing 0.25% trypsin, 0.01% DNAase for 45 min. The clumps were allowed to settle and the supernatant was decanted into calf serum. The cells were washed extensively with EBSS medium and adjusted to approximately 10^7/ml. Cultures of testicular cells were seeded and harvested as above after 2–3 days.

Sperm were obtained by stripping the vas deferens and also by mincing the whole epidydimus in EBSS (without azide), incubating for 30 min at 37°C and passing the cells through four-ply cotton gauze.

Immunofluorescence Tests

Cultured target cells dissociated by trypsinization were washed once in DMEM 10% CS and then in EBSS 0.8% BSA, 10 mM HEPES and 0.1% azide. 25 μl of M1/22.25 were added to 25 μl of target cells at 1–2 × 10^7/ml and incubated for 30 min at room temperature. Following a single wash, 25 μl of rabbit anti-rat immunoglobulin, conjugated to fluorescein (R.anti-rat.Ig-Fl; Miles-Yeda) and diluted one fifth, were added, and the cells were resuspended by vortex. The cells were incubated for a further 30 min at room temperature, then washed twice, mounted on glass slides and sealed with a coverslip. A Zeiss photomicroscope with phase-contrast and incident fluorescence optics and an Osram H80-200 mercury lamp was used. Cells were scored positive only if they appeared healthy and intact under white light and showed clear fluorescent ring (patchy) staining.

Fluorescence experiments on mouse lymphoid tissues were performed using R.anti-rat.Ig-Fl 1/5, previously absorbed 3 times with an equal packed volume of pooled mouse spleen cells. Goat anti-mouse immunoglobulin conjugated with rhodamine (Nordic Laboratories Ltd.) was absorbed 3 times with rat spleen cells and used at one tenth dilution. All antisera used in immunofluorescence tests were routinely centrifuged at 14,000 × g for 5 min before use. Controls for the nonspecific labeling of the conjugated antiserum alone were included in every experiment.

Radioactive Binding Assay

The ^{125}I-anti-Ig binding assay was performed with ^{125}I-rabbit F $(ab')_2$ anti-rat Fab (a gift from Dr. A. F. Williams) and culture supernatants of the clones M1/22.25, M1/9.3 and M1/69.16. The iodination was performed as described by Jensenius and Williams (1974). Anti-rat.Ig was absorbed to prevent cross-reaction with mouse cell surface Ig, either after iodination by mixing with mouse serum, or before iodination by passing it through mouse serum-sepharose CL.4B. 100 μl of supernatant (neat or titration) from hybrid clone cultures were incubated with 2 × 10^5 target cells as a pellet for 30 min at 4°C. The cells, in round-bottom microtiter plates, were washed twice with EBSS medium. The cells were then suspended in 100 μl of ^{125}I.anti-Ig (100,000 cpm); after 30 min at 4°C, they were washed 3 times, suspended in 100 μl and transferred to tubes for counting. The ratio of counts bound to cells with culture supernatant or medium was calculated. A binding ratio of 1 means no specific binding; >1.5 is considered significant binding. A positive target cell at a given concentration gave a characteristic binding ratio.

Cytotoxicity and Absorption Assays

Fresh sheep RBC (Tissue Culture Services, Slough) were washed 3 times with PBS (phosphate-buffered saline) and adjusted to 10^{10}/ml in EBSS, 0.8% BSA and 10 mM HEPES. 10^9 were labeled with 25 μl (125 μCi) Na-^{51} Cr O4 (Radiochemical Centre, Amersham) for 1 hr at 37°C. The cells were washed 3 times with EBSS medium and resuspended to 1 × 10^7/ml and kept on ice. Preliminary titration of a batch of M1/22.25 against sheep RBC in a one-stage cytotoxicity assay with unabsorbed 1/15 GPC showed 50% ^{51}Cr release at a dilution of 1/2400. The batch of culture supernatant was diluted 1/200 in EBSS medium, and 50 μl were added to 50 μl of doubling dilutions of absorbing cells or tissues. The tubes were vortexed and incubated for 45 min at room temperature. Following centrifugation at 2000 rpm for 5 min, a 50 μl aliquot of absorbed antiserum was removed and added to 50 μl of labeled sheep RBC and 50 μl of GPC (1/5); the tubes were vortexed and incubated for a further 60 min at room temperature. 2 ml of cold PBS were added, the cells were pelleted, and an aliquot was removed and counted on an LKB Wallac gamma counter. Percentage of ^{51}Cr release was calculated as:

$$\% \text{ release} = \frac{\text{counts released by test supernatants (or medium)} \times 100}{\text{counts released by detergent}}.$$

The batch of M1/22.25 culture supernatant used in fluorescence tests had a titer of >1/10^6 by this assay procedure.

Agglutination Assays

To 50 μl of neat M1/22.25 were added 25 μl of absorbing material, 6 × 10^6/ml typed human RBC, 1 mg/ml AB substance, 20 × 10^9/ml horse RBC, 11 × 10^9 OX RBC, 2 × 10^9 sheep RBC, 28 × 10^9, goat RBC, 25% autoclaved, homogenized guinea pig kidney or 5 × 10^9 mouse RBC. After 30 min, the samples (in microtiter plates) were centrifuged at 1000 × g for 5 minutes, and 10 μl aliquots were removed. The aliquots were mixed with 50 μl of 1%, sheep RBC; plates were shaken and agglutination was read several hours later.

Internal Labeling and Lactoperioxidase Iodination

Log-phase Nulli-SCC.1 were seeded at 5 × 10^6 cells per 25 ml

Corning tissue culture flask in 10 ml of DMEM 10% CS containing 50 μCi ^{14}C-galactose, or 10^7 cells per 75 ml flask in 25 ml containing 50 μCi ^{14}C-galactose. In both cases, the cells were harvested following a 24 hr labeling and washed 3 times in PBS; lysates were then prepared. Nulli-SCC.1 cells were labeled with ^{35}S-methionine at 28 μCi/ml in methionine-free DMEM with 10% dialyzed CS for 3 or 6, 12 or 24 hr. Log-phase Nulli-SCC.1 were surface-labeled with Na-^{125}I using lactoperioxidase and washed; in all cases, cells were lysed with 0.5% NP40 and processed as previously described (Ziegler and Pink, 1976). All radiochemicals were from the Radiochemical Centre, Amersham.

Immunoprecipitation and SDS-PAGE Gel Electrophoresis

Aliquots (0.2 ml) of the lysates were incubated for 1 hr at room temperature with 50 μl of M1/22.25 or control M1/70.15 culture supernatant. 10 μl of normal mouse serum or 20 μl of serum from mice bearing a MOPC 104E tumor were added. An optimal amount (determined by titration) of either sheep anti-MOPC 104E (a gift from A. Feinstein), rabbit anti-rat.IgM (Miles-Yeda) or sheep anti-MOPC 21 was then added to each tube, and the mixtures were kept at 4°C for 18 hr. The precipitates were washed 4 times with PBS containing 1 mM phenylmethanesulfonyl fluoride (PMSF) and counted in a Wallac gamma counter or a Beckman scintillation counter. The precipitates were dissolved in 50–100 μl of 10 M urea, 1 mM PMSF by boiling for 10 sec. SDS-PAGE was performed in 10% gels in a Tris-Bicine buffer system as previously described (Ziegler, Harrison and Leberman, 1974).

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