SEROLOGICAL IDENTIFICATION AND CELLULAR DISTRIBUTION OF THREE F9 ANTIGEN COMPONENTS*

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The cell surface F9 antigens are defined by the 129/Sv anti-F9 serum (1), a syngeneic antiserum raised against irradiated cells of the murine F9 embryonal carcinoma (E.C.)[1] line (2). Through complement-mediated cytotoxicity or indirect immunofluorescence (IF) tests, the F9 antigens appear to be cell surface antigens characteristic of EC cells and their normal embryonic counterpart. They are also found on cells of the male germ line (1, 3, 4).

It has been shown recently (5) that the antibody response of 129/Sv mice against F9 cells is complex; nearly all the cytotoxic activity of the serum is associated with the IgM antibodies, whereas the binding of IgM, IgGl, and IgG2a,b anti-F9 antibodies to the surface of F9 cells can be revealed by IF.

We have isolated the anti-F9 IgM, IgG1, and IgG2a,b antibodies from the crude anti-F9 serum using an affinity chromatography technique (6). The activities of the thus isolated Ig were tested by IF on a panel of cell types. The results reported in the present paper suggest that the anti-F9 antibodies react with three independent antigenic determinants.

Materials and Methods

Sera

Anti-F9 sera. Anti-F9 sera were raised in syngeneic male 129/Sv mice against irradiated (2,000 rad) cells of the quasi-nullipotent F9, EC line, as described earlier (1, 5). Two sera prepared independently (pooled bleedings after the 4th booster injection of F9 cells) were studied and found to display identical properties. They were heat-inactivated (56°C, 30 min), centrifuged (10,000 *g*, 30 min, 4°C), and stored as aliquots at −78°C.

Normal mouse serum. Normal mouse serum was taken from male 129/Sv mice, heat-inactivated, centrifuged, and stored as above.

Rabbit anti-mouse Ig. Rabbit anti-mouse Ig, labeled with fluorescein (RAMIg-FITC) was purchased from Institut Pasteur Production, Paris. Before its use, the serum was extensively dialyzed and then absorbed with F9, PYS-2, and PCC3/A/1 cells as described (7). All batches of RAMIg-FITC used were found to react, in double diffusion tests against purified myeloma proteins, with all classes and subclasses of mouse Ig (5).

Cells. The cell lines used here have been described elsewhere (Table I) (2, 8–21). Lymphocytes were prepared from thymuses of 4-wk-old 129/Sv mice as described in (22).

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[1] *Abbreviations used in this paper:* DMEM, Dulbecco's modified Eagle's medium; E.C., embryonal carcinoma; FCS for fetal calf serum; IF, indirect immunofluorescence; PBS, phosphate-buffered saline; RAMIg-FITC, rabbit anti-mouse Ig labeled with fluorescein.

TABLE I

Description of Cell Lines Used in This Paper

Cell line	Genetic background	Origin	Cell type	In vivo differentiation	Malignancy*	Reference
F9-41	129/Sv	Induced OTT6050‡	E.C.§	Endoderm (rare)	+	2
PCC4	129/Sv	OTT6050‡	E.C.	3 germ layers' derivatives	+	8
PCC4-azaR1	129/Sv	PCC4	E.C.	3 germ layers' derivatives	+	8
PCC3	129/Sv	OTT6050‡	E.C.	3 germ layers' derivatives	+	8
PCC3/A/1	129/Sv	PCC3	E.C.	3 germ layers' derivatives	+	9
PCC8-S	129T/t^{w18}	Spontaneous, testicular	E.C.	3 germ layers' derivatives	+	‖
PSA-1	129/Sv	Induced, OTT5868¶	E.C.	3 germ layers' derivatives		10
LT-1	LT	Spontaneous, ovarian	E.C.	None	+	11
C17S1-A	C3H	Induced, C17**	E.C.	3 germ layers' derivatives	+	12
C17S1-1117	C3H	C17**	E.C.	3 germ layers' derivatives	±	12
Tera I	Human	Spontaneous, testicular		NT‡‡	+	13
PYS-2	129/Sv	OTT6050‡	Parietal endoderm	Parietal yolk sac	±	14
3/A/1/D-3	129/Sv	PCC3	Embryonal fibroblast	0	−	15
3/A/1/D-1	129/Sv	PCC3	Mesenchyme	Bone tissue	+	35
SVT2	BALB/c		Fibroblast	Fibrosarcoma	+	16
F10	BALB/c × 129/Sv	Hybrid PCC4-azaR1 × SVT2	Fibroblast	Fibrosarcoma	+	17
PCD3	129/Sv	OTT6050‡	Fibroblast	0	−	18
PCD2	129/Sv	OTT6050‡	Myoblast	0	−	19
TDM1	129/Sv	PCC3	Trophoblast	Trophoblastoma	+	20
Friend	DBA/2	F4N	Erythroid cell	Erythroleukaemia	+	21

* Malignancy, determined by injection of 2.5 10^6 cells into syngeneic mice; + malignant; − nonmalignant.

‡ OTT6050, derived from a 6.5-d-old 129/Sv embryo grafted into the testis of an F_1 (A/He × 129/Sv) host.

§ This cell line undergoes differentiation under in vitro culture conditions.

¶ OTT5568, derived from a 3.5-d-old 129/Sv embryo grafted into the testis of an F_1 (A/He × 129/Sv) host.

‖ H. Jakob. Unpublished observations.

** C17, derived from a 7.5-d-old C3H embryo grafted in a C3H host.

‡‡ NT, not tested.

Embryos. The procedures for the preparation and the immunolabeling of preimplantation embryos were essentially as described elsewhere (7). Blastocyst inner cell masses were isolated following the technique of Solter and Knowles (23). Most of the embryos used here were produced by hormonally primed 4- to 5-wk-old 129/Sv mice mated with syngeneic males. Embryos from spontaneously ovulated females were used occasionally and gave identical results.

Fractionation of Immunoglobulins. IgM, IgG1, and IgG2a,b antibodies were separated by affinity chromatograhy through a column of Protein-A sepharose (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.), following Ey et al. procedure (6). Briefly, 1 ml of serum was mixed with 1 ml of 0.1 M phosphate buffer, pH 8.0, and adjusted to pH 8.0. This mixture was then applied onto a column of Protein-A-Sepharose (3 ml) preequilibrated with 0.1 M phosphate buffer, pH 8.0. The column was washed with the same buffer and the effluent (referred below as IgM) collected. The column was then washed with several volumes of citrate buffer 0.1 M, pH 6.0; the eluted material (IgG1) was saved. After extensive washing with the same buffer, pH 5.6, the still bound IgG2a and IgG2b were eluted simultaneously with citrate buffer pH 3.0, 0.1 M. The effluent was saved and is referred to below as IgG2a,b. The three fractions were neutralized, concentrated through an Amicon PM10 membrane (Amicon Corp., Scientific Systems Div., Lexington, Mass.), adjusted to 4% fetal calf serum (FCS), and dialyzed overnight against 100 vol of phosphate-buffered saline (PBS). The separated Ig were stored at −78°C as aliquots. To be able to compare the various preparations used, the volume of each fraction is referred to a dilution of the unfractionated serum, i.e., when 8 ml of IgM are recovered from 1 ml of crude serum, the IgM preparation is taken as diluted 1/8.

The purity of each fraction was checked by double diffusion of the separated Ig (~1 mg/ml) against antibodies raised against purified myeloma proteins (5). Material unbound at pH 8.0 contained IgM and no contaminating IgG. Because IgA and IgE antibodies are essentially unreactive onto F9 cells (5), IgM were not further purified. Material eluted at pH 6.0 reacted only with anti-γ_1 chain sera. Material eluted at pH 3.0 reacted with both anti-γ_{2a} and anti-γ_{2b} chains sera, but contained trace amounts of material reacting with anti-γ_1 chain serum.

Immunofluorescence Test. All experiments were conducted on suspensions of living cells using the double sandwich technique (22). Washed cells (10^6) were suspended in 50 μl of immunoglobulin at the appropriate dilution. After 45 min at 4°C, the unbound Ig were removed by three washings with Dulbecco's modified Eagle's medium (DMEM) containing 4% FCS. The washed cells were suspended in a 1/25 dilution of RAMIg-FITC and incubated for 30 min at 4°C. The cells were then washed three times in DMEM + 4% FCS and examined under a Zeiss epi-illumination fluorescent microscope (Carl Zeiss, Inc., New York). Controls, using Ig prepared from normal mouse serum or anti-F9 Ig absorbed with F9 cells, were run in parallel. The fraction of labeled cells, out of a total of 200 cells examined, was noted. The intensity of the immunostaining was scored from 0, no labeling to +++, very intense labeling.

The immunofluorescence test on preimplantation embryos was performed as described previously (7).

Cytotoxicity Test. The cytotoxicity test on F9 cells was performed according to published procedures (1).

Results

Activity of IgM, IgG1, and IgG2a,b Anti-F9 Antibodies on F9 Embryonal Carcinoma Cells. Fractions containing various classes or subclasses of immunoglobulins were prepared from anti-F9 sera, and serotyped as described in Materials and Methods. They proved to have the following properties: (*a*) The IgM-containing fraction had retained cytotoxic activity: 80–100% F9 cells could be killed; the cytotoxic titer was 1/2,000–1/2,400 (compared with 1/2,000–1/3,200 for the crude serum). 70–90% F9 cells could be labeled with a high intensity in an IF test at the 1/8–1/256 dilutions. (*b*) The IgG1-containing fraction did not display any cytotoxic activity, but could label up to 100% of cells in the 1/2.5–1/20 range of dilutions. (*c*) The IgG2a,b anti-F9 antibodies were not cytotoxic, but could label up to 100% of cells in the 1/6–1/48 range of dilutions.

Controls were run with IgM, IgG1, and IgG2a,b antibodies prepared from normal mouse serum. These had no activity on F9 cells in either test, at the lowest dilutions used (1/10, 1/3, and 1/3, respectively).

Activity of IgM, IgG1, and IgG2a,b Anti-F9 Antibodies on a Panel of Cell Types. These three preparations were tested by IF on a variety of cell types: (*a*) embryonal carcinoma cells from different, eventually independent, origins, genetic backgrounds, and biological characteristics; (*b*) differentiated cells, mostly derived from embryonal carcinoma cells.

Activity of IgM anti-F9 antibodies. The results of this typing are summarized in Table II. At the 1/64 dilution, all E.C. lines were found to react strongly with IgM anti-F9 antibodies. The percentage of positive cells, however, varies widely according to the lines, some of them (PCC4-azaR1 and Tera 1, a human teratoma line) (13), showing only 15% of labeled cells. The labeling is easily detectable even at high dilutions (1/128 and 1/256), especially on cells from three lines, F9, PSA-1, and LT1 (a female teratoma line) (11), which thus appear to be the lines displaying the largest amount of IgM binding-antigens.

In contrast, anti-F9 IgM antibodies, used at the same 1/64 dilution, did not react with differentiated cells. At a lower dilution (1/32), a few positive cells (1–15%) could be observed in several cell types with a variable staining (+ to +++). The meaning of this positive response, however, is questionable; for instance, absorption of IgM anti-F9 antibodies with PYS-2 cells (diluted 1/16, vol:vol, 1 h, 4°C) did not result in

TABLE II

Activity of IgM, IgG1, and IgG2a,b Anti-F9 Antibodies on Different Cell Types

Cell type	IgM			IgG1			IgG2a,b		
	Intensity	Percent	Dilution	Intensity	Percent	Dilution	Intensity	Percent	Dilution
Undifferentiated									
F9-41	+++	70	1/64	+++	100	1/10	++	100	1/12
PCC4	++	60	1/64	++	15	1/10	0	0	1/12
PCC4-azaR1	+	15	1/64	0	0	1/10	0	0	1/12
PCC3	++	70	1/64	Traces	100	1/10	Traces	100	1/12
PCC3/A/1	++	50	1/64	0	0	1/10	0	0	1/12
PCC8-S	++	60	1/64	0	0	1/10	0	0	1/12
PSA-1	+++	100	1/64	+++	100	1/10	++	100	1/12
LT-1	+++	100	1/64	0	0	1/10	0	0	1/12
C17S1-A	++	50	1/64	0	0	1/10	0	0	1/12
C17S1-1117	+++	5	1/64	++	5	1/10	Traces	4	1/12
Tera I	+++	15	1/64	+	100	1/10	+	100	1/12
Differentiated									
PYS-2	+	15	1/32	Traces	100	1/5	0	0	1/6
PCC3/A/1*	0	0	1/32	0	0	1/5	0	0	1/6
3/A/1/D-3	0	0	1/32	Traces	100	1/5	0	0	1/6
3/A/1/D-1	0	0	1/32	Traces	50	1/5	0	0	1/6
SVT2	0	0	1/32	0	0	1/5	0	0	1/6
F10	+++	1	1/32	0	0	1/5	0	0	1/6
PCD3	0	0	1/32	+	100	1/5	+	100	1/6
PCD2	++	2	1/32	++	100	1/5	++	100	1/6
SV40-PCD2	0	0	1/32	++	100	1/5	++	100	1/6
TDM1	0	0	1/32	0	0	1/5	0	0	1/6
Friend	+	5	1/32	Traces	100	1/5	0	0	1/6
Thymocytes	0	0	1/32	0	0	1/5	0	0	1/6

* HMBA induced differentiated derivative of PCC3/A/1 (24).

any apparent decrease of activity of the reagent on F9 cells, although the activity on PYS-2 cells had disappeared.

Activity of IgG1 anti-F9 antibodies. The results are summarized in Table II. Contrasting with the comparatively simple pattern of reactivity of IgM, IgG1 anti-F9 antibodies reacted with very different cell types. At the 1/10 dilution, some E.C. lines, irrespective of their origin, did not react at all, whereas some of them (F9 and PSA-1) had 100% of their cells reacting strongly. Among differentiated cell lines, only mesodermal cell types (18, 19) were brightly labeled. Other differentiated cells tested failed to react even at a lower dilution (1/5). IgG1 anti-F9 antibodies were absorbed with mesodermal cells (vol/vol, 1 h, 4°C) and tested back on F9 cells. This absorption did not result in any significant decrease of activity of IgG1 anti-F9 antibodies on F9 cells.

Activity of IgG2a,b, anti-F9 antibodies. The results are summarized in Table II. At the 1/12 dilution, the same E.C. cell lines that reacted with IgG1 also reacted with IgG2a,b; the others did not. Among differentiated cells, again, only mesodermal derivatives reacted. The other differentiated lines were negative even at the 1/6 dilution. IgG2a,b anti-F9 antibodies were absorbed with mesodermal derivatives (vol/

vol, diluted 1/6, 1 h, 4°C) and tested back on F9 cells. As for IgG1 anti-F9 antibodies, absorption of IgG2 antibodies did not change their activity on F9 cells.

Activity of IgM, IgG1, and IgG2a,b Anti-F9 Antibodies after Trypsinization of E.C. Cells. F9 and Tera I cell lines were mildly trypsinized (0.5 mg/ml, 10 min, 22°C). After such a treatment, ~95% of the cells were alive.

The trypsinized cells were then assayed in IF test for their ability to bind IgM, IgG1, and IgG2a,b anti-F9 antibodies. With both cell lines, no significant decrease in the IgM-binding capacity of the cells was observed. In contrast, receptors to IgG1 and IgG2a,b remained barely detectable at the lowest dilution of reagent used (1/10 and 1/12 for IgG1 and IgG2a,b antibodies, respectively).

Activity of IgM, IgG1, and IgG2a,b Antibodies on Preimplantation Embryos. The normal counterpart of embryonal carcinoma cells is to be found in the multipotential cells of the early embryos (25). The F9 antigens have been found on early embryonic cells, and their expression during development is well documented (1, 4, 26, 27). The way in which the receptors of the main components of the anti-F9 serum are expressed during early embryogenesis has therefore been investigated. The results obtained are summarized in Table III.

IgM anti-F9 antibodies reacted strongly with embryos throughout their entire preimplantation development. Negative controls were run using IgM anti-F9 antibodies absorbed with F9 cells. These did not stain the embryos, irrespective of their developmental stage. Unabsorbed IgM antibodies, however, strongly stained the unfertilized ovocytes at the 1/64 dilution. The staining of embryos persisted until they reached the late morula stage. Later in development, trophectoderm cells had much less affinity for IgM anti-F9 antibodies, whereas inner cell mass cells retained the capacity to bind anti-F9 IgM.

IgG1 anti-F9 antibodies were never found to react with any cell at any stage, at any dilution of the reagent (lowest 1/2.5).

IgG2a,b anti-F9 antibodies, diluted 1/6, did not detect specific antigens on very early (one and two cell stages) preimplantation embryos. Between two and four cell stages, however, receptors begin to be expressed, which peak at the morula stage and disappear upon blastocyst formation. At this stage, these receptors are not detectable on trophectoderm cells or inner cell mass cells.

Earlier studies dealing with the expression of F9 antigens on preimplantation

TABLE III

Activity of IgM, IgG1, and IgG2a,b Anti-F9 Antibodies on Preimplantation Embryos

Antibodies	Nonfertilized egg	One-cell stage	Two-cell stage	Four-cell stage and morulae	Blastocyst	
					Trophectoderm	Inner cell mass
IgM, diluted 1/64	++*	+++	+++	+++	Traces	++
	27	8	14	24	4	18
IgG1, diluted 1/2.5	NT‡	NT	0	0	0	0
			6	8	8	4
IgG2, diluted 1/6	0	0	Traces, +	++	Traces	0
	6	9	5 10	41	9	17

* Upper line, intensity of staining; lower line, number of embryos tested.
‡ NT, not tested.

embryos have been made using PYS-2 absorbed anti-F9 serum (1, 7). According to the results obtained with this reagent, F9 antigens were not detectable on unfertilized ovocytes, but did appear later, some 12 h after fertilization. Consequently, IgM and IgG2a,b anti-F9 antibodies were absorbed on PYS-2 cells before being applied onto preimplantation embryos. The pattern of expression of receptors to anti IgG2a,b antibodies was left unchanged. By contrast, unfertilized ovocytes and one-cell embryos did not react any longer with IgM anti-F9 antibodies. Receptors to these specific IgM appeared between one and two cell stages. Later expression was unchanged as compared with the one observed with unabsorbed IgM antibodies. Absorption of anti-F9 IgG2a,b antibodies with mesodermal derivatives did not result in any significant change of their activity on morulae.

Discussion

When the results reported above are summed up, it becomes apparent that the antigenic determinants detected by IgM, IgG1, and IgG2a,b antibodies are not identical; the anti-F9 serum allows the detection of at least three independently expressed antigenic determinants whether or not borne by the same molecule.

When one considers the expression of the three antigens separately, several conclusions can be reached. (*a*) The IgM anti-F9 antibody receptors are detectable on most cells displaying E.C. features, whether they are derived from spontaneous or induced murine teratocarcinomas of various genetic origins, or from human testicular tumors. They are also detected easily on preimplantation embryos. These receptors are scarcely found on differentiated cell lines. Absorption experiments carried out on total anti-F9 serum or on isolated anti-F9 IgM led to the conclusion that two types of antigenic determinants react with these IgM; one is found on E.C. cells, PYS-2, and on unfertilized ovocytes. The interaction between these IgM and their receptors does not cause a significant lysis of F9 cells. The other class of IgM receptors is found on E.C. cells and fertilized embryos only, and is detectable after absorption of anti-F9 IgM with PYS-2 cells. Therefore, this last antigen appears to be characteristic of early embryonic cells and of their tumoral counterpart. (*b*) The receptors to IgG1 anti-F9 antibodies are found on some E.C. lines and also on several differentiated cell types, all of them mesodermal derivatives. Absorption of these antibodies with PCD2, a myoblastic line, however, did not result in any significant change of their activities on F9 cells. It might, therefore, be concluded that the cross-reacting antigen common to F9 cells and the mesodermal derivatives tested is a minor component of the receptors to IgG1 displayed at the surface of F9 cells. IgG1 anti-F9 antibody receptors are not detected on preimplantation embryos. They are not merely associated with in vitro growth of cells because they have been detected at the surface of F9 cells growing as solid tumors, and the animals harboring these tumors were found to produce IgG1 anti-F9 antibodies (5). Receptors to IgG1 antibodies might be related to cell surface antigens common to E.C. cells, and various tumoral and transformed cell types identified by other authors (28–30). (*c*) The receptors to IgG2a,b anti-F9 antibodies are expressed on cultured cells (E.C. or differentiated derivatives) much the same way as IgG1 receptors. Again, absorption of IgG2a,b antibodies with PCD2 cells did not result in any significant decrease of their activity when tested back on F9 cells. IgG2a,b receptors, however, are clearly distinguished by their pattern of expression on preimplantation embryos.

It may be pointed out that F9 antigens recovered after precipitation of E.C. cell extracts with anti-F9 serum (and especially with anti-F9 IgM antibodies, data not shown) contain long saccharidic chains characteristic of early embryonic cells (31). On the other hand, the main target of IgM anti-F9 antibodies have been shown recently to be saccharidic residues (32). The absence of polymorphism of F9 antigens, which has been observed so far among various mouse inbred strains as well as among mammalian species (4), might thus reflect the preservation of these saccharidic structures.

Moreover, F9 antigens have been found to bear some relationship with t^{w32} and t^{w5} haplotypes of the T locus (7, 33). This conclusion has been drawn from studies in which IgM receptors were looked at predominantly. One might then speculate that the cell surface components altered in t^{w32} and t^{w5} homozygotes are saccharidic chains; some products of the T locus would, thus, be somehow related to carbohydrate metabolism of the embryos, a contention supported by recent evidence brought by Cheng and Bennett (34).

Summary

Using an affinity chromatography technique, IgM, IgG1, and IgG2a,b anti-F9 antibodies have been isolated from the anti-F9 serum; their activities have been analyzed by IF test on a variety of cell types, teratocarcinoma-derived cell lines, and embryos. The anti-F9 antibodies react with at least three independent antigenic determinants not expressed on the same cell types, and that appear along different time-course during embryonic development.

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