Europ. J. Cancer Vol. 17, No. 6, pp. 609–616, 1981
Printed in Great Britain

0014-2964/81/060609-08 $02.00/0

Immunological Properties of Rat Embryonal Carcinoma Cells*

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Abstract—*The reactivity of mouse anti-*F9 *serum on rat embryonal carcinoma cells* (F3/1) *and xenogeneic as well as syngeneic host immune responses against* F3/1 *cells were studied. Mouse anti-*F9 *serum was reactive on the cell surface of* F3/1 *cells by immunofluorescence. The same antiserum, however, was not cytotoxic against* F3/1 *cells. The* F3/1 *cells were not immunogenic in syngeneic hosts as tested by* in vivo *and* in vitro *assays. Thus, in the present study,* F3/1 *antigen(s) could not be defined with syngeneic antiserum but only defined with non-cytotoxic, surface-binding antibodies (IgG) of xenogeneic anti-*F3/1 *serum.*

INTRODUCTION

THE AUTHORS have previously described an oncofetal antigen(s) expressed on rat embryonal carcinoma cell (ECC). This antigen(s) defined by xenogeneic antisera made against rat ECC is also expressed on mouse ECC and preimplantation embryos of the mouse and rat [1].

Hence in its specificity of expression the antigen(s) shows a remarkable analogy to the cell surface antigens of mouse ECC and more precisely to the F9 antigen that has been studied in much detail. Mouse teratocarcinomas originate from germ cells or multipotential embryonic cells [2–5] and are composed of a variety of differentiated cell types and of malignant ECC which are the multipotential stem cells of these tumors [1, 6, 7]. The F9 antigen was initially described in one of the mouse ECC lines cultivated *in vitro* which lost its differentiation potential [8, 9].

The rat ECC antigen (called F3/1 antigen) we described is also found on ECC that had lost their potential to differentiate and not on other tumor lines or on adult differentiated tissues [1]. In the present study, we report on the similarities and differences between both F9 and F3/1 antigens and how the latter can be related to the differences in the pathogenesis of mouse versus rat embryonal carcinomas.

Accepted 30 December 1980.
*This work was supported by the Belgian Algemene Spaar- en Lijfrentekas (ASLK) Kanker Fonds and by the National Fonds voor Wetenschappelijk Onderzoek (NFWO)
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MATERIALS AND METHODS

Tumor lines

All rat tumor lines used in the present study were induced in the inbred strain of R rats (AG-B^2). F3/1 is a line of rat ECC which lost the ability to differentiate *in vitro* as well as *in vivo*. F40 is a line of rat yolk sac carcinoma. Both tumors were derived from displaced extraembryonic fetal membranes after inoculation of murine sarcoma virus (MSV, Moloney) into fetectomized uterus [10]. TR/DMBA is a rat fibrosarcoma induced by dimethylbenzanthracene (DMBA). One mouse line consisting of MSV-transformed C3H-fibroblasts (MO-4) was also used [11]. All the cell lines were grown in MEM supplemented with glutamine and 8% fetal calf serum at 37°C in humidified atmosphere containing 5% CO_2.

Antisera

*Anti-*F9 *serum.* Mouse anti-F9 serum produced against nullipotent mouse ECC (line F9) was kindly provided by Dr. G. Gachelin, Pasteur Institute, Paris. The serum had a cytotoxic titer of 1:2500 on F9 cells (personal communication from Dr. Gachelin). The antibodies are known to react with mouse ECC, preimplantation embryos and male germ cells of mouse [12]. Before use in our experiments mouse anti-F9 serum was absorbed on normal R rat spleen cells.

Anti-F3/1 *serum.* Xenogeneic anti-F3/1 sera were produced in rabbits by immunization with an ascitic form of F3/1 cells as described elsewhere [1]. Three adult male rabbits were first immunized i.v. (intravenously) with 10^8 viable ascitic cells taken from inbred R rats, followed by booster immunization of 2×10^8 cells injected i.p. (intraperitoneally), 1 and 2 weeks afterwards. The animals were bled 1 week after the final immunization and the individual sera were heat-inactivated and stored at −70°C. Rabbit anti-F3/1 sera were absorbed *in vivo* in R rats and *in vitro* on different lines of rat and mouse tumor cells. These absorbed antisera were reactive only on F3/1 cells, mouse ECC, and on mouse and rat preimplantation embryos [1].

Other antisera. Four fluorescein-labeled conjugates, i.e. goat anti-rabbit Ig sera (GAR/Ig/FITC), goat anti-rat IgC sera (GARa/FITC), sheep anti-rabbit IgM (Fc) sera (ShAR/IgM(Fc)/FITC) and rabbit anti-mouse Ig sera (RAM/Ig/FITC), were obtained from Nordic Immunological Lab., Tilburg, The Netherlands. All those conjugates except for GARa/FITC were absorbed on normal R rat tissue or mouse tissue before use in immunofluorescence. Goat anti-rabbit IgG serum was also obtained from Nordic.

Syngeneic immunization against F3/1 *cells*

Immunization of R rats with living F3/1 cells was tried by inoculation of F3/1 cells and subsequent removal of tumors which developed at the inoculation sites. Five female R rats (6 weeks old) were firstly inoculated s.c. (subcutaneously) in the right flank with 10^5 F3/1 cells. The subsequent tumors at the inoculation sites were surgically removed. This procedure was repeated three times (Table 2). One week after the removal of inoculation site tumors, the immunized animals were challenged with 10^5 F3/1 cells which is the minimal tumor-inducing dose in normal control rats of the same age and sex. After determination of the number of rats developing a tumor after challenge with 10^5 F3/1 cells in the back, tumors were again removed. One week after the last tumor removal, the animals were bled. The individual sera were heat-inactivated and stored at −70°C.

Indirect immunofluorescent staining (IF)

IF was carried out as described previously [13]. The trypsinized cell suspension (0.1 ml) containing 10^6 cells in PBS was incubated with 0.1 ml of absorbed antiserum (diluted up to 1:25 for anti-F9 serum, 1:2 for anti-F3/1 serum) for 30 min at 4°C. After three washes in cold PBS, the cells were stained with fluorescent conjugates (diluted up to 1:10) in PBS for 30 min at 4°C. The conjugate excess was removed by 3 washes in PBS. The cell pellets were resuspended in a drop of buffered glycerol, and placed on a microscopic slide, covered and sealed. Slides were examined using a Leitz Orthoplan fluorescent microscope with a Ploem vertical illuminator.

Serotoxicity tests

Serotoxicity tests were performed by the microdroplet technique described by Terasaki *et al.* [14] and Mittal *et al.* [15]. The target cells (F3/1 or TR/DMBA) were plated under oil [16] at 2000 cells/well in 1.0 μl of MEM mixed with an equal volume of undiluted or diluted antiserum. After 45 min incubation at 37°C a volume of 1.0 μl of guinea pig or rabbit complement (diluted up to 1:9), which was previously absorbed on normal R rat tissues, was added. The percentage of dead cells was determined after 45 min incubation at 37°C and the addition of 2 μl of 1% trypan-blue solution in 0.9% NaCl. Three controls were included for each test; one for the viability of target cells, one for the toxicity of complement and one for the toxicity of antiserum without addition of complement.

Removal of IgG class of rabbit anti-F3/1 *serum*

To remove the IgG class of antiserum, volumes of 1.0 ml of specific rabbit anti-F3/1 serum were incubated with 0.5 ml of Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals) at room temperature for 45 min [17]. The gel was then removed from the supernatant by centrifugation. As control, an equal volume (1.0 ml) of antiserum was incubated with 0.5 ml of Sepharose 4B (Pharmacia Fine Chemicals). The absorbed antisera were used in immunodiffusion and IF staining.

Double immunodiffusion

Double immunodiffusion was performed by Ouchterlony's method, in 1.0% Agarose A (Pharmacia Fine Chemicals) and 0.1% sodium azide in PBS. The plates were placed in a moist chamber at 37°C for 18 hr to obtain precipitation lines.

RESULTS

Reactivity of mouse anti-F9 *serum on rat ECC*

As summarized in Table 1, mouse anti-F9 serum at the dilution of 1:50 was reactive on the surface of F3/1 cells by indirect immuno-

Table 1. *Reactivity of mouse anti-*F9 *serum on various tumor lines by immunofluorescence*

Cell lines	Species/strain		Indirect immunofluorescence Mouse anti-F9 serum (1:50)*
M0-4	mouse/C3H	MSV-transformed fibroblast, nonproducer	—
TR/DMBA	rat/R	DMBA-induced sarcoma	—
F40	rat/R	MSV-induced yolk sac ca	—
F3/1	rat/R	MSV-induced rat ECC	+ +

*Final dilution for immunofluorescent staining.
(−):negative staining, (+ +):bright staining on most of cells; yolk sac ca:yolk sac carcinoma cells.

Table 2. *Syngeneic immunization by surgical removal of tumors*

No. of immunization*	No. of cells for tumor development	Route of injection	Tumor incidence
1st	10^5 F3/1 cells	s.c. Rt flank	5/5†
2nd	10^5 F3/1 cells	s.c. Lf flank	5/5†
3rd	10^5 F3/1 cells	s.c. Rt flank	5/5†
Challenge	10^5 F3/1 cells	s.c. Back	5/5

*The interval between immunization depended on the time of tumor appearance of previous inoculum (5–7 days). The next immunization was done one week after the removal of tumors.
†All tumors that developed at the site of inoculation were surgically removed.

Table 3. *Determination of Ig class of R anti-*F3/1 *serum reacting on* F3/1 *cells by IF*

	R anti-F3/1 serum* abs. on F3/1 cells (GAR/Ig/FITC) 1:4†	R anti-F3/1 serum (GAR/Ig/FITC) 1:4	R anti-F3/1 serum (ShAr/IgM (Fc)FITC 1:4	R anti-F3/1 serum abs. on Sepaharose 4B (GAR/Ig/FITC) 1:4	R anti-F3/1 serum abs. on Prot. A-Seph. 413 (GAR/Ig/FITC) 1:4
F3/1 cells	−§	+ +§	−	+ +§	−

*Rabbit anti-F3/1 serum.
†Final dilution in IF.
‡No reaction.
§Bright staining on most cells.

fluorescent staining, but not reactive on the other rat and mouse cell lines including rat yolk sac carcinoma cells. The same antiserum diluted up lo 1:2, 1:10 and 1:20, however, was not cytotoxic for F3/1 cells.

*Reactivity of rabbit anti-*F3/1 *serum*

The specific rabbit anti-F3/1 serum diluted up to 1:4 was reactive on rat and mouse ECC by indirect immunofluorescence. This antiserum, however, was not cytotoxic for F3/1 cells.

Immune responses of syngeneic hosts against rat ECC

As shown in Table 2, immunization of syngeneic rats with the method of surgical removal of tumors failed to protect the host against a challenge with homologous cells. Moreover, none of those individual sera taken from immunized animals showed the presence of cytotoxic antibodies as tested by serotoxicity assays or surface-binding antibodies as tested by IF staining.

*Determination of rabbit anti-*F3/1 *Ig class reacting on* F3/1 *cells*

The rabbit anti-F3/1 Ig class reacting on the surface of F3/1 cells was first investigated by using ShAR/IgM(FC)/FITC and GAR/Ig/FITC for IF staining. F3/1 cells coated by rabbit anti-F3/1 serum showed a bright membrane fluorescence with a goat anti-rabbit Ig serum labeled with fluorescein (GAR/Ig/FITC). No fluorescence, however,

was observed with a sheep anti-rabbit IgM(Fc) sera labeled with fluorescein (ShAR/IgM/(Fc)/FITC). In order to remove IgG class of rabbit anti-F3/1 serum, antiserum was also incubated with Protein A-Sepharose CL-4B. Antiserum absorbed by this procedure did not show a precipitation line against a goat anti-rabbit IgG serum (GAR IgG) as tested by double immunodiffusion (Fig. 1). This absorbed antiserum was no longer reactive, in the presence of GAR/Ig/FITC, on the surface of F3/1 cells by IF staining (Table 3). These findings indicate that surface-binding antibodies do not belong to the IgM class but to the IgG class of rabbit anti-F3/1 serum.

DISCUSSION

Our results indicate that mouse anti-F9 serum reacts with the cell surface of rat ECC (line F3/1) as verified with indirect immunofluorescence. This finding is quite in agreement with our previous results which showed that xenogeneic (or allogeneic) anti-F3/1 sera specify the presence of a cell surface antigen(s) common to rat and mouse ECC as well as to mouse and rat preimplantation embryos [1]. Mouse anti-F9 serum (cf. Material and Methods), however, was not cytotoxic for F3/1 cells.

Since F3/1 and F9 antigens, in spite of their different origin, show a remarkable similarity in their expression on the cell surface of ECC and of early mouse and rat embryos, one would also expect a similar host immune response against both tumor lines. The F9 cells do not express H-2 antigens [18–20] and are strongly immunogenic in syngeneic host [21, 22] in which they induce the production of *in vitro* cytotoxic (IgM) and surface-binding (IgM and IgG) antibodies [12, 22]. Since the IgM component in anti-F9 serum can be selectively removed by absorption on sperm without affecting the surface-binding activity of IgG class, the F9 antigen seems to be composed of at least two specificities (F9M and F9G) [22].

On the other hand, as shown in the results, F3/1 cells are not immunogenic in syngeneic R rats as observed in *in vivo* and *in vitro* assays. The individual sera taken from immunized syngeneic rats showed neither *in vitro* cytotoxic antibodies nor surface-binding antibodies against homologous (F3/1) cells. Hence, in the present study, F3/1 antigen(s) can be defined only with xenogeneic antiserum. The rabbit antibodies reactive on the surface of F3/1 cells were exclusively confined to the IgG class, but not to IgM, as tested by immunofluorescence. Moreover, the rabbit anti-F3/1 serum was not cytotoxic against F3/1 cells. It is also interesting to point out that mouse anti-F9 serum containing both cytotoxic (IgM) and surface-binding (IgM and IgG) antibodies for F9 cells was not cytotoxic against F3/1 cells.

Taken as a whole, the F3/1 antigen(s) seems to induce in the xenogeneic host the production of surface-binding IgG antibodies but not of cytotoxic IgM and IgG. F3/1 antigen(s) may share an antigenic specificity with the F9G (IgG_1-defined F9) antigen. However, it does not possess all F9 antigenic determinants since F3/1 antigen(s) is neither immunogenic in syngeneic host nor present on the surface of rat spermatozoa as tested by absorption analysis and immunofluorescence [1].

As yet, we have no explanation for the differences in the host immune responses against F3/1 cells compared to F9 cells. One possible explanation, however, might be found in differences in origin for both tumors. F9 cells are an *in vitro* established line of mouse ECC (OTT 6050) which was derived from a 6-day 129/Sv embryo grafted into the testis of a F1 hybird (A/He × 129/Sv) host [21]. F9 cells do not express H-2 antigens [18–20]. They stimulate the immune system of the syngeneic host to produce cytotoxic and surface-binding antibodies which, besides their reactivity on early embryos and ECC of mouse and rat, are always reactive with male germ cells. On the other hand F3/1 cells are derived from displaced extra-embryonic fetal membranes after inoculation of MSV into fetectomized uterus [10, 23]. These express the major rat histocompatibility antigens (Ag-B^2). Indeed, BN (Ag-B^3) rats always reject F3/1 R rat tumor. Also the hemagglutination activity of BN anti-R spleen cells serum, on R rat red blood cells which express Ag-B^2 antigens, can be removed by absorbing the antiserum on F3/1 cells (unpublished data). Thus, F3/1 cells express both Ag-B^2 antigens and xenogeneic IgG-defined oncofetal (F3/1) antigen(s), the latter being neither immunogenic in syngeneic hosts nor present on adult germ cells. Regarding the absence of immunogenicity of the F3/1 antigen(s) in syngeneic host, one may speculate that the simultaneous presence of an oncofetal antigen(s) and the major histocompatibility antigens in the case of F3/1 cells may lead to the former antigen(s) being recognized as "self" by the

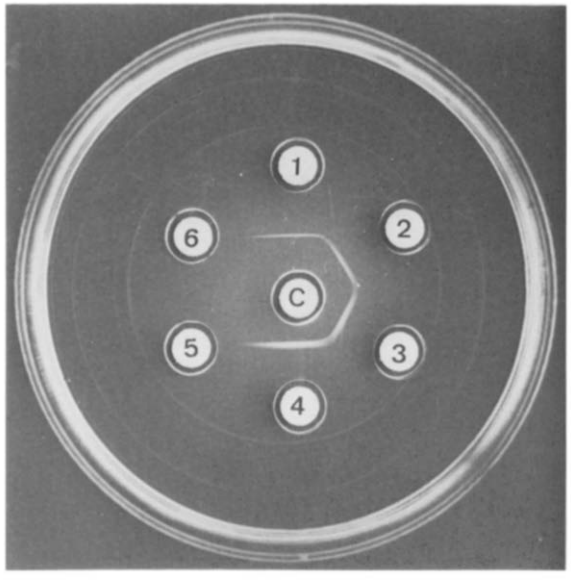

Fig. 1. *Double immunodiffusion after removal of IgG class of rabbit anti-*F3/1 *serum. C; goat anti rabbit IgG serum.*
1,2; *specific rabbit anti-*F3/1 *serum* (*unabsorbed*).
3,4; *specific rabbit anti-*F3/1 *serum* (*absorbed on Sepharose* 4B).
5,6; *specific rabbit anti-*F3/1 *serum* (*absorbed on Protein A-Sepharose* CL-4B).