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Surface antigen in early differentiation

(preimplantation embryo/mouse/embryonic development/teratocarcinoma)

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ABSTRACT **Addition of Fab fragments from rabbit antiserum to surface antigen F9 to 2-cell stage mouse embryos in culture does not alter cleavage; however, the addition prevents the formation of compact morulae and blastocysts. A similar effect is observed when Fab fragments are added to already compact 8-cell stage or even older morulae, but disappears at the beginning of blastocoel formation. This effect is reversible: uncompact 30-cell embryos washed free of Fab become compact in a few hours, produce blastocysts, and upon reimplantation into pseudopregnant mothers can produce mice. Development is not altered by divalent anti-F9 antibodies, by Fab fragments from sera directed against other embryo surface antigens, or by succinyl concanavalin A.**

There are reasons to assume that the cell surface plays an important role in the development of the embryo (1). We have investigated this point by examining the effect of several antisera reacting with surface structures of embryonic cells on the *in vitro* development of the mouse preimplantation embryo (2). Among these sera, one which proved effective in blocking morula compaction and blastocyst formation without inhibiting cleavage was directed against the nullipotent, embryonal carcinoma (EC) line F9. This line carries the "F9 antigen," which has been shown to be expressed on EC cells, germ line cells, and preimplantation embryos (3, 4).

MATERIALS AND METHODS

Mice. The following stocks were used: 129/Sv, an inbred subline of the original 129 strain; C57Bl/6 × CBA, an F_1 hybrid of C57Bl/6 and CBA. All mice were produced at the Institut Pasteur.

Cells. Two cultured cell lines were used: F9-41, a nullipotent EC cell (5); PYS-2, a parietal yolk sac cell (6) devoid of F9 antigen.

Mouse Anti-F9 Serum was produced in syngeneic male 129/Sv mice by hyperimmunization with irradiated F9-41 cells (3). Before use anti-F9 serum was absorbed with PYS-2 cells (vol/vol, serum dilution 1:5 in Hank's medium containing 4% heat-inactivated, gamma globulin-free, fetal calf serum, 1 hr at 4°). The absorbed serum retained most of the original activity against F9 cells.

Rabbit Anti-F9 Serum. Rabbits were immunized with 5×10^7 F9-41 cells in complete Freund's adjuvant. After 1 month the animals received four booster injections at 2-week intervals with 5×10^7 cells each. Serum was collected and heat inactivated 1 week after the last injection. The cytotoxic titer of the unabsorbed serum against F9-41 cells was 1/6000. Rabbit anti-F9 was massively absorbed against lymphocytes (twice, vol/vol, serum dilution 1:5, 45 min at 4°) and PYS-2 cells (twice, vol/vol, serum dilution 1:10, 45 min at 4°) until there was no activity left against lymphocytes and PYS-2. The cytotoxic titer on F9-41 cells was then 1/160 to 1/320.

Rabbit Anti-Embryonic Liver Cells Serum. Rabbits were immunized by the same procedure as above with isolated liver cells of 14-day-old 129/Sv fetuses. By indirect fluorescence the unabsorbed antiserum labeled 8-cell embryos at dilutions up to 1/320–1/640. The cytotoxic titer on F9 cells was 1/680.

Rabbit Anti-Mouse Brain Serum was a gift from M. Stanislavsky. Rabbits were immunized with mouse brain homogenate. In the fluorescence test, the unabsorbed antiserum labeled 8-cell embryos at dilutions up to 1/160–1/320. The cytotoxic titer on F9 cells was 1/800.

Anti-Ig Sera. Rabbit anti-mouse IgG serum labeled with fluorescein (FITC-RaMIg) was a product of Institut Pasteur Production, as well as a sheep anti-rabbit IgG serum conjugated with fluorescein (FITC-SaRIg). Before use, both sera were repeatedly and massively absorbed with F9-41 and PYS-2 cells. The absorbed conjugates were sampled and stored frozen at −80°. They were used at a 1/25 dilution.

IgG and Fab Preparation. The immunoglobulin (IgG) fraction of an unabsorbed rabbit anti-F9 serum was isolated by precipitation with 40% saturated ammonium sulfate, pH 7.0, followed by a chromatography on Whatman DEAE-cellulose (DE52) according to Levy and Sober (7). For Fab preparation, the method of Porter (8) was used. IgG (100 mg) was digested with 1 mg of papain (Worthington). Undigested IgG was separated by Sephadex G-100 chromatography. Crystallized Fc fragments were removed by high-speed centrifugation. The Fab preparation (3.4 mg of protein per ml) was characterized by immunoelectrophoresis and a fluorescence test on F9-41 cells. Final absorptions with lymphocytes and PYS-2 cells were done as described previously. Fab fragments of rabbit sera against mouse embryonic liver cells and against mouse brain homogenate were isolated similarly. Both preparations were adjusted to 3.4 mg of protein per ml.

Rabbit Anti-Lymphocyte Serum. Nonspecific anti-mouse activity of the unabsorbed rabbit anti-F9 serum or its Fab preparation was recovered by an absorption acid-elution technique as described by Colombani *et al.* (9), using mouse lymphocytes as an immunoadsorbent. A pure lymphocyte preparation (10^9 cells) was incubated with 100 μl of an Fab solution at 3.4 mg/ml diluted 1:5 in Hanks' salt solution with immunoprecipitin-tested fetal-calf serum (IPT) for 10 min at 37° and 30 min at 4°. After three washings with phosphate-buffered saline/IPT at 4°, 0.1 M HCl was added to the cell pellet to lower the pH to 2.3 (140 μl). After 10 min at 4°, the cells were removed by centrifugation (10 min, 500 rpm) and the eluate was neutralized with 0.04 M NaOH (120 μl) at 4°. The solution was cleared of salt precipitation and particles by

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Abbreviations: EC, embryonal carcinoma; IgG, immunoglobulin G; Fab, monovalent antibody binding fragment of IgG; Suc-Con A, succinyl concanavalin A; IPT, immunoprecipitin-tested fetal calf serum.

Table 1. Summary of indirect immunofluorescence tests*

Antisera	Indirect immunofluorescence test on: F9-cells	8-cell embryos
Mouse anti-F9	1/800	1/320
Rabbit anti-F9	1/160–1/320	1/80
Rabbit anti-F9 Fab†	1/80	1/40–1/80
Rabbit anti-lymphocyte Fab†	n.t.	1/160
Rabbit anti-liver Fab†	n.t.	1/160–1/320
Rabbit anti-brain Fab†	n.t.	1/160
Rabbit anti-F9 absorbed on F9	Negative (1/5)	Negative (1/5)
Suc-Con A	n.t.	5–50 μg/ml

n.t., not tested.
* The figures represent the greatest dilution giving unambiguous labeling of the target cells.
† All Fab preparations have been adjusted to 3.4 mg of protein per ml.

high-speed centrifugation, adjusted at 4% IPT, and stored in small aliquots. Such an Fab preparation is referred to further on as "anti-lymphocyte Fab." With the same method, rabbit anti-F9 Fab was purified on F9 cells and subsequently absorbed on PYS cells and lymphocytes.

Succinyl Concanavalin A (Suc-Con A) was prepared as described by Cunningham *et al.* (10). Rabbit antiserum was prepared by two intradermal injections of 1 mg of Suc-Con A at 6-week intervals. The IgG fraction of this serum was prepared by the method of Harboe and Ingild (11) and conjugated with rhodamine by the method of Lamelin *et al.* (12).

Cytotoxicity Tests were done as described in ref. 3.

Indirect Immunofluorescence Tests were carried out as described in ref. 13.

Embryo Recovery and Culture. C57Bl/6 × CBA virgin female mice, 3–5 weeks old, were injected with 5 international units of pregnant mare serum (Endopancrine, Paris) and 42–46 hr later with 5 international units of human chorionic gonadotropin (Endopancrine, Paris). They were then mated separately with C57 × CBA males. Fertilized females were selected by the appearance of a vaginal plug (day 1 of pregnancy). Ovulation and mating were assumed to have occurred 12 hr after gonadotropin injection. The age of embryos is given in hours after ovulation. Except where otherwise indicated, 32- to 36-hr-old embryos, mostly two cells, were flushed from the Fallopian tubes, put into culture in microdrops of Whitten's medium (14) under paraffin oil, and incubated at 37° in an atmosphere of 5% O_2/5% CO_2/90% N_2 (15). In the first experiments where different antibody fractions were tested, the zona pellucida was removed by Pronase (16) after flushing and the antibody-containing medium was renewed every day. Later it was observed that this was unnecessary and the eggs were directly put in culture and the medium was not renewed. Results were scored after about 66 hr of culture (equivalent time of gestation: 102 hr).

Embryo Transfer. C57Bl/6 × CBA adult females were mated with vasectomized males and used as recipients on day 3 of pseudogestation.

RESULTS

Indirect Immunofluorescence Test. All the antisera and their IgG and Fab preparations were tested on F9 cells and 8-cells embryos by indirect immunofluorescence. The results are summarized in Table 1. Like syngeneic mouse anti-F9 serum, polyabsorbed rabbit anti-F9 sera or Fab preparations labeled F9 cells and embryos, but not PYS cells or mouse lymphocytes. Preincubation of 8-cell embryos with a syngeneic mouse anti-F9 (1/5 to 1/30 dilutions) blocked the binding of rabbit anti-F9 (1/20 to 1/40 dilutions) as measured by fluorescence.

Divalent Mouse or Rabbit Anti-F9 Antibodies Do Not Affect Blastocyst Formation. Under the conditions of culture used, most of the embryos harvested at the 2-cell stage continue their development: after 66 hr, 76% have developed and reached the blastocyst stage (Table 2, line 1).

To determine whether anti-F9 antibodies alter development, mouse or rabbit anti-F9 serum was added to the culture medium and renewed every day. No effect was observed, either with the mouse serum or with the rabbit serum, at a 1/20 dilution (Table 2, lines 2 and 3).

Rabbit Anti-F9 Fab Fragments Loosen Cell Interactions in the Morulae and Prevent Blastocyst Formation. Divalent anti-F9 antibodies have been shown to induce redistribution and capping of F9 antigen on blastomeres (4). This may result

Table 2. Effect of antisera on the *in vitro* development of 2-cell embryos

Line	Addition to Whitten's medium	Concentration	Total no. of embryos	Blastocysts after 66 hr in culture (equivalent gestation time, 102 hr) No.	%	No. of grape-like structures
1	None	—	215	167	76	0
2	Mouse anti-F9	1/20	53	35	66	0
3	Rabbit anti-F9	1/20	41	28	68	0
4	Rabbit anti-F9 Fab*	1/20	253	0	0	215†
5		1/40	20	0	0	16
6	Rabbit anti-lymphocyte Fab*	1/10	28	11	39	0
7		1/20	58	48	82	0
8	Rabbit anti-liver Fab*	1/10	59	41	69	0
9		1/20	42	33	78	0
10	Rabbit anti-brain Fab*	1/10	59	42	71	0
11		1/20	42	26	61	0
12	Rabbit anti-F9 Fab* absorbed on F9	1/20	100	68	68	0
13	Suc-Con A	50 μg/ml	60	39	65	0

* All Fab preparations have been adjusted to 3.4 mg of protein per ml.
† Early arrested and fragmented embryos account for the difference between the total number of embryos and the number of either blastocysts or grape-like structures scored in this table.

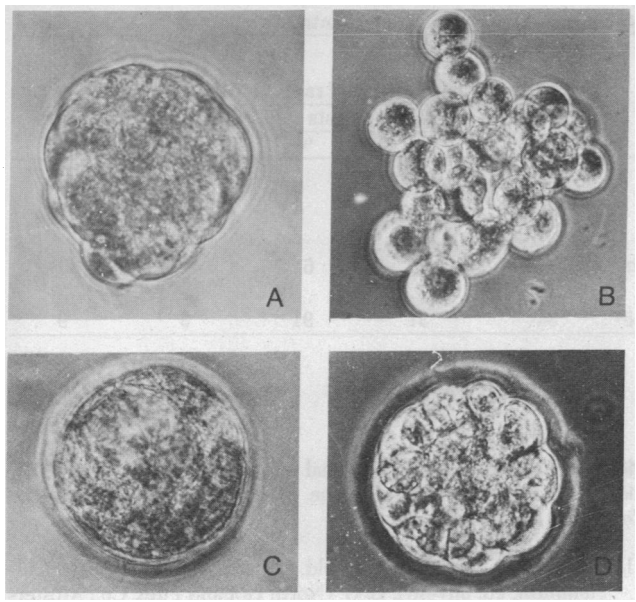

FIG. 1. Two-cell embryos with (*C* and *D*) and without (*A* and *B*) zona pellucida were grown in Whitten's medium (*A* and *C*) and Whitten's medium with rabbit anti-F9 Fab, 1/20 (*B* and *D*). (×400.)

in a permanent resynthesis of the antigen and explain the absence of effect of the serum on development. For this reason Fab fragments prepared from rabbit anti-F9 IgG were then used.

These rabbit anti-F9 Fab fragments were added to cultures of embryos. This treatment did not prevent cleavage from proceeding. However, after 48 hr, when the untreated embryos had formed compact morulae without clearly visible cellular borders, the treated embryos had a very different, grape-like structure, with well-individualized cells (Fig. 1). After 66 hr, the majority of untreated embryos had reached the blastocyst stage, while the Fab-treated embryos maintained their grape-like structure without forming blastocysts (Table 2, lines 4 and 5). Cells of the embryos began to vacuolize, and lysed after 1 or 2 more days in culture. Similar results were observed when the zona pellucida was not removed (Fig. 1) and when the Fab-containing medium was not renewed every day. As a control, the rabbit anti-F9 Fab was absorbed on F9 cells and this preparation was added to the culture. In this case no effect was observed (Table 2, line 12). In contrast, Fab first absorbed on F9 and then eluted by the acid technique retained the blocking activity.

Rabbit Fab Fragments Directed against Other Surface Antigens of the Morula Do Not Alter Blastocyst Formation. As controls, Fab fragments were prepared from unabsorbed rabbit sera directed against mouse lymphocytes, brain, and embryonic liver. By indirect immunofluorescence, these Fab preparations (3.4 mg of protein per ml) labeled the surface of morulae at dilutions of 1/160, 1/160, and 1/320, respectively (Table 1). When added to cultures of embryos, none of these preparations prevented blastocyst formation up to a dilution of 1/10 (Table 2, lines 6 and 11). At a 1/10 dilution, the anti-lymphocyte preparation exhibited some toxicity.

Similarly, Suc-Con A, which in indirect immunofluorescence tests heavily labeled embryos (5 μg/ml), had no effect on blastocyst formation up to 50 μg/ml (Table 2, line 13).

Other evidence for the specificity of the anti-F9 blocking effect comes from competition experiments in which embryos were incubated in the presence of both anti-F9 Fab (110 μg/ml) and an excess (765 μg/ml) of anti-F9 or embryonic liver IgG or of Suc-Con A (20 μg/ml). Only in the presence of anti-F9 IgG was the blocking effect of Fab suppressed: out of 29 embryos, 24 gave rise to blastocysts and only 2 to grape-like structures. In contrast in the presence of anti-liver IgG or Suc-Con A, 28 out of 28 and 30 out of 30 embryos, respectively, produced grape-like structures.

The Block Due to Anti-F9 Fab Can Be Reversed. Although the number of cell divisions occurring after the addition of rabbit anti-F9 Fab fragments was not accurately determined, it is clear that the embryos do cleave in their presence. By direct observation, one could estimate that most grape-like structures contained from 20 to 40 cells after 50 hr in culture.

Anti-F9 Fab fragments appear to act by loosening cellular interactions, thereby preventing blastocyst formation. If this is the case, this effect might be reversed by removal of the Fab. Embryos were therefore grown in the presence of rabbit anti-F9 Fab (1/20) until formation of grape-like structures. These structures were then washed, put back in culture without Fab, and assayed in two ways.

(*a*) *In vitro.* After washing, a large fraction of the grape-like structures produced blastocysts. In one experiment, for instance, 41 embryos (2-cell) were grown in the presence of rabbit anti-F9 Fab (1/20). After 53 hr, 27 embryos had 30 cells or more. Of these, 5 were washed and put in culture without Fab; 17 were treated with Pronase, then washed and put in culture without Fab. After 14 hr, the former 5 had produced 5 blastocysts; of the latter 17, 12 had produced blastocysts, the other 5 died.

(*b*) *In vivo.* After washing, short incubation *in vitro* without Fab, and reimplantation in pseudopregnant females, a significant fraction of the grape-like structures gave rise to newborns. In one experiment, for instance, out of 57 embryos (2-cell) grown for 50 hr in the presence of rabbit anti-F9 Fab, 41 produced grape-like structures with 30 cells or more. These 41 embryos were washed and put in culture without Fab. After 4 hr, they were reimplanted into four foster mothers. Of these mothers, three (33 embryos implanted) produced a progeny of 16 mice, a figure similar to that found with normal embryos.

Effect of Rabbit Anti-F9 Fab during Preimplantation Development. To determine how long during development the embryos remain sensitive to the anti-F9 Fab effect, embryos were flushed at 55, 61, and 66 hr after fertilization, put in culture with rabbit anti-F9 Fab (1/20), and examined for the production of blastocysts versus grape-like structures (Table 3). All the 55-hr embryos (8–12 cells) and the great majority of 61-hr ones (compact morulae) were still sensitive. Among the 66-hr embryos two classes can be distinguished: (*a*) The younger, which have no sign of blastocoel; (*b*) the older, which begin to form a blastocoel. The two groups were separated and treated independently. The percentages of embryos still sensitive to Fab were, respectively, 39 and 9%. It is clear, therefore, that already compact morulae are still sensitive to the Fab effect, the sensitivity disappearing only around the onset of blastocoel formation.

DISCUSSION

These results show that rabbit anti-F9 Fab fragments specifically and reversibly block the compaction of morulae and the formation of blastocysts. Although compaction is programmed to begin around the 8-cell stage, it can be reversibly altered up to the onset of blastocoel formation. Only then does some event occur that prevents the Fab effect. It is not clear whether or not this event corresponds to the establishment of a barrier to the

Table 3. Effect of rabbit anti-F9 Fab fragments on the development *in vitro* of preimplantation embryos

Hours after fertilization	Stage	Control in Whitten's medium: No. of embryos	Control in Whitten's medium: Blastocysts		Culture in the presence of rabbit anti-F9 Fab at 1/20: No. of embryos	Blastocysts: No.	Blastocysts: %	Grape-like structures: No.	Grape-like structures: %
55	8–12 cells	13	13		33	0	0	27*	82
61	Compact morulae	5	5		57	5	9	52	91
66	Onset of blastocoel formation			*a*†	107	65	61	42	39
				b‡	34	31	91	3	9

* The six other embryos containing less than 15 cells were not scored.
† Morula, with no sign of blastocoel formation.
‡ Morula, with a beginning of blastocoel formation.

penetration of antibody molecules (17). Surprisingly, divalent anti-F9 IgG exhibits no inhibitory effect. Furthermore, it specifically blocks the effect of anti-F9 Fab. At present the differential behavior of divalent and monovalent antibodies is not understood. The dominant effect of the IgG over the Fab cannot simply be due to a cross-linking of blastomeres because divalent antibodies to other cell surface components do not inhibit the action of anti-F9 Fab.

During normal development of the mouse embryo, the compaction that begins at the 8-cell stage is accompanied by the formation of tight and gap junctions. This has been shown to be reversibly inhibited *in vitro* by lowering the Ca^{2+} concentration of the medium (18) or by a treatment with cytochalasin B (18, 19). Interestingly, the effect of anti-F9 Fab is similar to that of both these treatments. It is possible that the interaction between Ca^{2+} and the antigen recognized by the anti-F9 serum is responsible for the initiation of morula compaction and junction formation. This interaction would be blocked by anti-F9 Fab. The nature of the interaction between the antigen and Ca^{2+} is not clear. The antigen could be involved in either Ca^{2+} entry into the cell or Ca^{2+} binding, thus changing the charge of the cell.

The rabbit anti-F9 serum used in these experiments detects a cell surface structure common to EC cells and blastomeres. It is not yet possible to assert the identity of this structure with the "F9 antigen" defined by syngeneic anti-F9 mouse sera, although treatment of embryos with syngeneic sera prevents the binding of rabbit anti-F9 antibodies. Nevertheless, the effect observed on development with rabbit anti-F9 Fab shows that the structure detected by this rabbit antiserum plays an important role in development.

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