The presence of F9 antigen on the surface of mouse embryonic cells until day 8 of embryogenesis

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SUMMARY

The fate of F9, a surface antigen common to embryonal carcinoma cells and cleavage embryos, as well as to male germ line cells has been studied in post-implantation mouse embryos. The antigen is readily detected on the surface of 7-day and 8-day, but not on 9-day embryonic cells, using anti-F9 serum absorption experiments and indirect immunofluorescence staining. In addition, it is shown that an anti-F9 serum absorbed with 8-day (but not with 9-day) embryonic cells does not react with cleavage embryos (morulae). It is concluded that the antigenic determinants, which are recognized on the surface of morulae by the anti-F9 serum, persist until day 8 of embryogenesis, but are not detected on day 9, either due to their absence, masking or quantitative reduction.

INTRODUCTION

The F9 antigen has been identified at the surface of a nullipotent embryonal carcinoma cell line (F9), using a hyperimmune serum raised against the F9 cell line in syngeneic 129 mice (Artzt et al. 1973). The presence of this antigen at the surface of multipotent embryonal carcinoma cells, derived from both male and female teratocarcinomas, is well documented. F9 has also been shown to appear in increasing amounts during the first three divisions of the embryo and is present throughout the whole preimplantation period of embryogenesis, including the blastocyst stage where it is present on both inner cell mass and trophoblast cells. In contrast, the F9 antigen has not been found on any other tumorigenic cells so far tested, including a variety of virus transformed lines, nor has it been possible to detect it on several differentiated cell lines derived from embryonal carcinoma, or on any adult tissue, with the notable exception of the whole male germ line (for recent reviews, see Jacob, 1977; Gachelin, 1978). The F9 antigen thus appears to be associated with early embryonic states and its presence at the surface of embryonal carcinoma cells would point to some equivalence between these cells and normal early embryonic cells. Recent experiments have shown that embryonal carcinoma cells can indeed participate in embryogenesis (Brinster, 1974; Papaioannou et al. 1975, 1978), leading eventually to the development of an apparently completely normal
embryo (Mintz & Illmensee, 1975; Illmensee & Mintz, 1976) after they have been incorporated inside the inner cell mass of a blastocyst.

The problem is thus to determine when and how the F9 antigen disappears during normal embryogenesis. We have attempted to answer this question by performing anti-F9 serum absorption experiments with cell suspensions obtained from post-implantation embryos of various developmental stages, and also by examining the staining properties of these cells in indirect immunofluorescence tests. Our results indicate that the F9 antigen ceases to be detectable on the surface of embryonic cells on day 9 of pregnancy, while it remains present on about 50% of the cells of the embryo up to this point. In addition, we show that the antigenic determinants recognized by an anti-F9 serum on 8-day embryonic cells are identical with determinants recognized by this serum on the surface of morulae.

MATERIALS AND METHODS

1. Animals

Mice were strain 129/Sv produced in the animal colony of the Institut Pasteur. In order to obtain embryos, harems of four to five females were penned with individual males and checked daily for the presence of a vaginal plug; the day when a plug was detected was considered as day 0 of pregnancy.

2. Dissection of embryos

Pregnant females were killed by cervical disruption on day 7, 8 or 9 of pregnancy. Uterine horns were dissected and decidua, freed from the uterine wall, were placed in a phosphate buffer medium (PBS). Dissection was carried out in PBS, under a binocular, using forceps. Eight-day embryos typically had 8–12 somites and had not yet achieved their turning (see Theiler, 1972). Occasional embryos whose turning had been completed by that time were considered as being in advance and discarded from the preparation. Conversely, typical 9–day embryos had achieved their turning, and those which had not were considered as being late and discarded. On day 9, the separation of trophoblast, yolk sac and embryo was accomplished with minimal contamination. On day 8, however, the ventral part of the embryo was usually contaminated with some remnants of the yolk sac. In both cases (day 8 and day 9) most of the amnion and of the allantois was taken with the embryo. Seven-day embryos freed from yolk sac were cut in two, along the line which divides embryonic from extra-embryonic ectoderm. Immediately after dissection, embryos were placed in a modified Whitten’s medium (Whitten & Biggers, 1968) containing 4% γ-globulin free foetal calf serum.

3. Embryonic cell suspensions

Embryos were dissociated at room temperature by gentle pipetting in a Ca^{2+}-Mg^{2+} free Earle’s medium using siliconed pipettes. Several successive centri-
fugations for a few seconds at very low speed in a Beckman microfuge separate single cells from as yet undissociated aggregates. After complete dissociation, cells were counted in a haemocytometer. About $3 \times 10^4$ cells per day-8 embryo and $2 \times 10^5$ cells per day-9 embryo (embryo proper) were recovered by this method.

4. Cell lines

The characteristics and culture conditions of F9, a nullipotent embryonal carcinoma, PCC4/Aza, an azaguanine-resistant multipotent carcinoma and PYS, an endodermal embryonal carcinoma, have been described before (Bernstine, Hooper, Grandchamp & Ephrussi, 1973; Jakob et al. 1973; Lehman, Speers, Swartzendruber & Pierce, 1974).

5. Sera

(a) **Anti-F9 serum**, raised in syngeneic male 129 mice by hyperimmunization with irradiated F9 cells, was heat inactivated, pooled, and stored as described by Artzt et al. (1973). All the experiments were performed after absorption of the antiserum onto PYS cells (vol/vol, 1 h, 0 °C, at a one to five dilution in Hanks' medium containing 4% γ-globulin free foetal calf serum). After absorption, the serum was titrated in the complement-dependent cytotoxic reaction, aliquoted and kept frozen at −80 °C. The titre was 1/2000 on F9 cells and 1/240 on PCC4/Aza cells.

(b) **Anti-H-2b serum.** An antiserum active against both H-2b and minor histocompatibility determinants produced by hyperimmunizing A/Orl mice with EL4, a C57B1/6 leukaemic line, was a gift of Dr K. Artzt. The antiserum was absorbed massively onto F9 cells (vol/vol, 1 h, 0 °C at a one to five dilution in Hanks' medium containing 4% γ-globulin free foetal calf serum). The cytotoxic titre on 129 lymphocytes after absorption was 1/2000.

(c) **Anti-H-2d serum.** A gift of Dr Motta, this serum was obtained by hyperimmunizing C57B1/6 mice with DBA/2 lymphocytes. The unabsorbed serum had a cytotoxic titre of 1/320 on DBA/2 lymphocytes.

(d) **Normal mouse serum** was always used after absorption onto PYS cells (same conditions as for the anti-F9 serum).

(e) **Anti-mouse Ig serum.** Fluorescein conjugated rabbit anti-mouse Ig serum was used as described in Kemler et al. (1976).

(f) **Complement.** Rabbit complement was used as in Artzt et al. (1973).

6. Immunological tests

(a) **Absorption of sera with embryonic cells.** Embryonic cell suspensions prepared as described above can be tested for their ability to absorb the activity of antisera in the following way (see legends to Figs. 1–3): cells were placed in 40 μl of a suitable dilution of antiserum. After incubation for 1 h at 0 °C, cells were eliminated by slow centrifugation and supernatants were again centrifuged
at maximal speed in a refrigerated Beckman microfuge, collected and used immediately in cytotoxicity or indirect immunofluorescence tests.

(b) Cytotoxicity tests. All tests performed with anti-F9 or anti-EL4 sera were made following the protocol in Artzt et al. (1973). The only change consisted in the volume of the assay: 15 μl of serum were mixed with an equal volume of cell suspension and of suitably absorbed complement.

(c) Indirect immunofluorescence tests. Anti-F9 serum was applied in indirect immunofluorescence tests on strain 129 morulae from spontaneously ovulated females according to the method of Kemler et al. (1976).

The same test was applied to embryonic cells in the following way. Dissociated embryonic cells were resuspended in 30 μl of anti-F9 serum diluted 1/30 into Eagle’s medium containing 4% γ-globulin free foetal calf serum and incubated for 45 min at 0 °C with occasional mixing. Cells were then transferred without previous washing on top of a stepwise gradient of serum made according to a modification of the method of Koo et al. (1973): briefly, cells were allowed to settle at unit gravity, in a 1 ml plastic pipette, through successive layers of increasing concentrations of inactivated γ-globulin free foetal calf serum diluted in Eagle’s medium. From top to bottom: 50 μl of 15% foetal calf serum, 75 μl of 25% foetal calf serum added with rabbit anti-mouse immunoglobulins coupled with fluorescein (dilution 1/20), and 75 μl of 35% foetal calf serum. After 3 h at 4 °C, droplets were collected at the bottom of the column and cells observed without fixation in a Zeiss fluorescence microscope.

RESULTS

1. Absorption of anti-F9 and anti-H-2b sera by day 7, 8 or 9 embryonic cells

Figure 1 shows the result of a typical experiment in which 50 μl of anti-F9 serum were absorbed with 6·10^4 8-day embryonic cells, and the cytotoxicity of absorbed and unabsorbed serum was tested on PCC4/Aza cells. It is seen that 8-day embryonic cells drastically reduce the cytotoxicity of anti-F9 serum with the value of the cytotoxic index falling from 1 to 0.25 at initial dilution. A mixture of absorbed and unabsorbed serum displays no such reduction of activity, indicating that no inhibitor of the reaction is present in the absorbed serum.

Figure 2 shows the results of quantitative absorption experiments with 8- or 9-day embryonic cells. Again, the absorbing capacity of 8-day embryonic cells is striking, with 4·10^4 cells removing 50% of the activity initially present in 25 μl of anti-F9 serum diluted 1/60 (the target cell in the cytotoxic test being PCC4/Aza). In contrast, 9-day embryonic cells leave the anti-F9 serum activity almost unaffected, with 6·10^5 cells removing 20% only of the activity under the same conditions.

Extra-embryonic cells obtained by dissociating the yolk sac of an 8-day-old embryo were tested in the same way. It is seen (Table 1) that the yolk-sac
Fig. 1. Absorption of anti-F9 serum with 8-day embryonic cells. 50 μl of anti-F9 serum (dilution 1/60) were absorbed on 6×10⁴ embryonic cells. The target cell in the cytotoxic test is PCC4/Aza.

Cytotoxic index = \frac{\% \text{ dead cells} - C'C (\% \text{ cells killed by complement})}{1 - C'C}

○, Unabsorbed serum; ○, absorbed serum; ■, mixture of absorbed and unabsorbed serum, final dilution 1/60 for both.

Fig. 2. Quantitative absorptions of anti-F9 serum with 8-day and 9-day embryonic cells. 25 μl of anti-F9 serum (dil. 1/60) were absorbed on increasing amounts of embryonic cells. The target cell in the cytotoxic test is PCC4/Aza. ○, Absorption on 9-day; ○, absorption on 8-day cells.

Cytotoxic indices are normalized to the unabsorbed serum:

\% \text{ dead cells} - C'C (\% \text{ killed by complement})

\% \text{ cells killed by unabsorbed serum} - C'C
Table 1. Absorption of anti-F9 serum with 7-day embryonic cells and 8- or 9-day yolk sac cells

<table>
<thead>
<tr>
<th>Serum absorption conditions</th>
<th>Number of absorbing cells</th>
<th>Cytotoxic test</th>
<th>Relative cytotoxic index value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (vol., dilution)</td>
<td></td>
<td>Target cell</td>
<td></td>
</tr>
<tr>
<td>Anti-F9 (30 μl; 1/750)</td>
<td></td>
<td>F9</td>
<td>0.82</td>
</tr>
<tr>
<td>5 x 10^3 9-day yolk-sac cells</td>
<td>4.5 x 10^4 9-day yolk sac cells</td>
<td>F9</td>
<td>0.58</td>
</tr>
<tr>
<td>2 x 10^4 8-day yolk sac cells</td>
<td>1.5 x 10^5 8-day yolk-sac cells</td>
<td>F9</td>
<td>0.56</td>
</tr>
<tr>
<td>1 x 10^7 7-day embryonic cells</td>
<td>3 x 10^4 7-day embryonic cells</td>
<td>F9</td>
<td>0.44</td>
</tr>
<tr>
<td>Anti-H2d (30 μl; 1/80)†</td>
<td></td>
<td>DBA/2 lymphocytes</td>
<td>1.13</td>
</tr>
<tr>
<td>7 x 10^4 7-day embryonic cells</td>
<td>1.4 x 10^7 7 day embryonic cells</td>
<td>DBA/2 lymphocytes</td>
<td>1.23</td>
</tr>
</tbody>
</table>

* Computed as: % dead cells - C'C (% cells killed by complement) - % cells killed by unabsorbed serum - C'C.
† Control absorption for a possible anti-complement effect of 7-day cell absorption. For a corresponding control with yolk-sac cells, see text.

Material removes the anti-F9 activity with a rather high efficiency. However, when a serum is first absorbed then progressively diluted for test, the titre curve presents first an increase of the anti-F9 activity, followed by a lowering as the dilutions increase. This would indicate that the decrease of anti-F9 activity in this case is due to inhibition rather than absorption.

Absorption experiments were more difficult to carry out with 7-day-old embryos because of the low number of cells recovered after dissociation. However, when the anti-F9 serum was incubated with cells obtained by dissociating whole 7-day-old conceptuses, a strong diminution of the anti-F9 activity was observed (Table 1). That this is not due to an inhibition of the cytotoxic reaction itself is shown by the fact that the same material had no effect on the activity of an anti-H-2d serum. The same type of control was used with 8-day- and 9-day-old embryos (Fig. 3a, b). In this case, an anti-EL4 (anti H-2b) serum was incubated with embryonic cells (trophoblast and yolk-sac cells excluded) obtained from 8-day- or 9-day-old embryos and tested for cytotoxic activity on H-2b lymphocytes obtained from 129 mice. While the titration curve of the serum remained unchanged after incubation with 8-day-old cells, there was a slight shift (two doubling dilutions) of the curve when the serum had been incubated...
Fig. 3. Absorption of anti-EL4 serum onto 8-day (a) and 9-day (b) embryonic cells. 25 μl of anti-EL4 serum (dil. 1/800) were absorbed onto 5·10⁴ embryonic cells. The target cells in the cytotoxic test are 129 lymphocytes. (a), (b): O, unabsorbed serum; ●, absorbed serum.
Table 2. Immunofluorescence labelling of 129 morulae with anti-F9 serum absorbed with 8-day or 9-day embryonic cells. See Materials and Methods for details concerning the absorption conditions and the immunofluorescence test

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Anti-F9 serum absorption</th>
<th>Immunofluorescence test on morulae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Volume Number</td>
<td>Dilution</td>
</tr>
<tr>
<td>1</td>
<td>20 µl 1/6 x 10^6</td>
<td>1/80</td>
</tr>
<tr>
<td></td>
<td>40 µl 1/25 x 10^6</td>
<td>1/100</td>
</tr>
<tr>
<td>2</td>
<td>20 µl 6 x 10^5</td>
<td>1/100</td>
</tr>
<tr>
<td></td>
<td>40 µl 1/25 x 10^6</td>
<td>1/100</td>
</tr>
<tr>
<td>3</td>
<td>20 µl 4 x 10^5</td>
<td>1/80</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>25 µl 7 x 10^5</td>
<td>1/80</td>
</tr>
</tbody>
</table>

with 9-day-old cells. This indicates that the presence of H-2 is demonstrable under these conditions on the 9-day cells, but not on the 8-day cells. At the same time, this confers some specificity to the absorption phenomenon of anti-F9 activity by 8-day embryonic cells.

2. Labelling in indirect immunofluorescence tests of 2-day-old embryos (morulae) with anti-F9 serum absorbed with 8- or 9-day-old embryonic cells

The results of the absorption experiments reported above suggest that 8-day-old, in contrast to 9-day-old embryonic cells strongly absorb the anti-F9 serum cytotoxic activity, as detected on embryonal carcinoma cells. Since it is known that the anti-F9 serum also reacts with determinants present on the surface of preimplantation embryo cells (Artzt et al. 1973), we examined whether the absorption of anti-F9 serum with 8-day embryonic cells would also remove its activity on morulae. Table 2 shows the results of four independent experiments in which anti-F9 serum was absorbed with various amounts of 8- or 9-day embryonic cells and tested, using an indirect immunofluorescence technique, on morulae from 129 mice. It is seen that absorption with 8-day-old embryonic
cells prevents the anti-F9 serum from labelling the morulae while absorption with 9-day-old cells leaves the serum essentially unaffected. This is especially clear when the serum is used at a 1/80 dilution, because at this dilution the unabsorbed serum stains 100% of the morulae, whereas one dilution further it only stains 60% of the morulae. In a total of nine independent experiments, in only one case was the serum absorbed with 8-day cells able to react with morulae about as strongly as the unabsorbed control (experiment not shown). The reason for this discrepancy is not known. All in all, the results of these experiments indicate that absorption of the anti-F9 serum with 8-day-old cells simultaneously removes the cytotoxic activity of the serum on F9 and PCC4/Aza cells and its capacity to label morulae.

3. Immunofluorescence labelling of 7-, 8- or 9-day-old embryonic cells with anti-F9 mouse serum

In an attempt to visualize the interaction of anti-F9 serum with embryonic cells, immunofluorescence labelling experiments were performed with suspensions of dissociated embryonic cells. As these turned out to be very sensitive to successive washings and centrifugations, a technique adapted from Koo et al. (1973) was used (see Materials and Methods) in which cells were allowed to fall through the layers of a stepwise gradient of foetal calf serum where labelling reagents alternated with washing medium.

After the cells had fallen for 4 h through the gradient, 30–50% of the input number was recovered at the bottom. Using the anti-F9 serum at the dilution 1/30, more than 95% of the cells from 7-day-old embryos were labelled with discrete patches of fluorescent staining, 58% of cells from 8-day embryos, and essentially none from 9-day embryos. As a control, cells were incubated with normal mouse serum or with anti-F9 serum massively absorbed with F9 cells, and processed the same way; these were not found to be stained.

DISCUSSION

While the F9 antigen has been detected on undifferentiated embryonal carcinoma cell lines and on all mammalian preimplantation embryos examined so far, its presence on any mouse adult tissue or on any differentiated cell line derived from embryonal carcinoma has never been ascertained, with the notable exception of the male germ line (Jacob, 1977; Gachelin, 1978). Its progressive disappearance from embryonal carcinoma cells grown in vitro under conditions where these cells lose their multipotentialities and differentiate into non tumorigenic tissues has been followed by Nicolas et al. (1975). However, no information on the fate of the antigen during normal embryogenesis beyond the blastocyst stage was available.

The results of the experiments reported above show that the F9 antigen is readily detected, both in absorption and in indirect fluorescence labelling
experiments on day 8 of embryogenesis but not on day 9. Cells from 7- or 8-day-old embryos absorb the activity of the anti-F9 serum but not of homologous or heterologous anti H-2 sera. This indicates that the inhibition of anti-F9 cytotoxic activity under these conditions is not due to some anti-complement effect. However, the absence of inhibition of anti-F9 activity by 9-day-old embryonic cells should not necessarily be interpreted as reflecting the absolute absence of F9 antigen on these cells. For instance, the presence of H-2 products has been ascertained by more sensitive techniques, as early as the blastocyst stage (Searle et al. 1976; Webb, Gall & Edelman, 1977; see also review by Klein, 1975), while with the technique used here they are detected on day 9 only. A minimal interpretation of our results would then be that there is a drastic reduction in the number of directly accessible F9 antigen sites on the surface of 9-day cells, as compared to 8-day cells.

This is confirmed by indirect fluorescent labelling experiments. Since these experiments have been performed on dissociated embryonic cells, they bring no information relevant to the spatial distribution of the antigen at that stage. Furthermore, 30–50% of the cells put on top of the gradient are recovered at the bottom. One could then wonder whether differential selection may influence the survival of F9 positive and negative cells. Although we have no precise data to answer this question, we feel that this is not likely. Since virtually every cell of the 7-day-old embryo, and no cell of the 9-day-old embryo is labelled in immunofluorescence experiments, it would seem that the F9 antigen disappears (or is quantitatively drastically reduced) from the whole population of embryonic cells at the same critical time, namely 24 h spanning over mid day 8. The F9 antigen would thus dwindle away as the first somites are formed and the embryo makes its turning (see Theiler, 1972). This would follow rather closely the time when embryonic ectoderm cells lose their multipotentiality, as judged from their ability to give rise to derivatives from the three germ layers when displaced into ectopic transplantation sites (Diwan & Stevens, 1976). It is tempting to speculate that F9 is present in embryogenesis as long as the bulk of the embryo comprises multipotent cells able to give rise to teratocarcinoma (Damjanov & Solter, 1974). The antigen would disappear shortly after the multipotential cells are no longer present and would be found on primordial germ cells only.

Since the anti-F9 serum absorbed with 8-day embryonic cells does not react with morulae, one can conclude that the determinants recognized by the serum on the surface of 2-day embryos are also present on 8-day embryos. There is evidence that these determinants bear some relationship with the so-called T locus of the mouse, because it has been shown that F9 is absent on the surface of embryos homozygous for certain t haplotypes such as $t^{w32}$ and possibly $t^{w5}$ which block the embryogenesis at early stages (Artzt, Bennett & Jacob, 1974; Kemler et al. 1976). t haplotypes appear to be responsible for the synthesis of antigens which, like F9, have been detected on sperm (Yanagisawa et al. 1974;
F9 antigen on mouse embryonic cells

Artzt & Bennett, 1977) and on morulae (Kemler et al. 1976). Since F9 and t antigens, such as \(t^{\omega 32}\) appear to have some genetic relationship, one might also expect the \(t^{\omega 32}\) antigen to persist on embryonic cells until day 8 of embryogenesis.

We should like to thank Dr L. C. Stevens for his help in the experiments made with the 7-day embryos, and for many stimulating comments. We are grateful for the excellent technical assistance of Miss M. T. Schebelen. We thank Dr K. Artzt for a gift of anti-EL4 serum and Dr R. Motta for a gift of anti-H-2\(^d\) serum.

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