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In vivo and *in vitro* cultured mouse preimplantation embryos differ in their display of a teratocarcinoma cell surface antigen: possible binding of an oviduct factor

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SUMMARY

By use of a monoclonal antibody, 2B5, in indirect immunofluorescence experiments, it was found that both fertilized and unfertilized mouse eggs obtained directly from the oviduct commenced expression of a cell surface antigen at about 5 h after ovulation. Surface labelling became intense by 16 h after ovulation and persisted over all blastomeres throughout preimplantation development. In contrast, embryos cultured *in vitro* did not show appearance of 2B5 antigen until about 48 h after ovulation, at which time they were at the 2- to 4-cell stage. Antigen expression *in vitro* commonly began on a single blastomere and did not appear consistently over all blastomeres until the 8-cell stage (72 h after ovulation). Unfertilized eggs maintained for 72 h in culture did not acquire 2B5 antigen. It is postulated that the absence of 2B5 antigen on 1-cell eggs cultured *in vitro* may be due either to a failure of normal synthesis by eggs as a result of a deficiency in the culture medium, or alternatively, to absence of a soluble oviduct factor which carries the 2B5 antigen, and which normally becomes bound to the surface of eggs after ovulation. The second of these two possibilities was supported by egg transfer experiments which showed that unfertilized eggs within the oviduct became 2B5 antigen-positive even after their prior fixation in glutaraldehyde. By the 2- to 4-cell stage, however, embryos developed their own capacity for synthesis of 2B5 antigen-positive cell surface molecules. This synthesis was inhibited by tunicamycin, suggesting that the antigenic site involved the sugar component of glycoprotein. The range of tissues within the postimplantation embryo and adult reproductive tracts which labelled with 2B5 antibody was found to be very similar to that known for SSEA-1 monoclonal antibody (Solter & Knowles, 1978; Fox *et al.* 1981; Fox, Damjanov, Knowles & Solter, 1982), and as further evidence of a relationship between 2B5 and SSEA-1 antigens it was found that ^{125}I SSEA-1 antibody could be blocked in its binding to teratocarcinoma cells by preincubation in 2B5 monoclonal antibody.

INTRODUCTION

Monoclonal antibodies to cell surface antigens provide useful markers of development in preimplantation mouse embryos. Thus, for example, 2C5 antigen is first expressed at the late 1-cell stage (Randle, 1982), SSEA-1 antigen at the 8-cell stage (Solter & Knowles, 1978), and M1/22.25 (Forssman) antigen at the late morula stage (Willison & Stern, 1978). In addition to their appearance on

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preimplantation embryos, all three of these antigens are also expressed on teratocarcinoma cells and on certain pluripotential cell types in the mouse embryo, such as inner cell masses, embryonic ectoderm, and primordial germ cells.

This paper now describes a new monoclonal antibody, 2B5, which was raised to teratocarcinoma cells, and which has many similarities in its embryonic tissue distribution to antibodies 2C5 and SSEA-1. It is shown that for embryos developing *in vivo*, the 2B5 antigen first appears on the surface of the 1-cell stage, but for embryos developing *in vitro* antigen expression is delayed until the 2-, 4-, or even 8-cell stage. It is usually assumed that antigens appear on the cell surface due to synthesis by the embryo of new membrane components. However, observations now presented suggest that appearance of 2B5 antigen on the 1-cell stage *in vivo* may be due to binding of an antigen-positive factor from the oviduct, and that it is only at later stages that 2B5 antigen is synthesized by the embryo.

MATERIALS AND METHODS

Embryos and embryo culture

Unless otherwise indicated, mouse embryos were obtained from F_1 (CBA × C57Bl/6) females mated with F_1 males. To obtain preimplantation embryos, females 24 to 30 days old were superovulated by intraperitoneal injection of 5 i.u. PMS (Paines and Byrne, Greenford) followed after 48 h by 5 i.u. hCG (Intervet, Cambridge). Ovulation was assumed to occur 12 h after hCG. For accurate timing of matings, females were caged with males 12 h after hCG and then checked for plugs at 1 h intervals thereafter. Natural matings were used for postimplantation embryos (the morning of the plug being designated day $\frac{1}{2}$ of pregnancy).

Preimplantation embryos were dissected or flushed from oviducts into PBl/BSA (phosphate-buffered medium containing 4 mg ml^{-1} bovine serum albumen, Whittingham & Wales, 1969). Unless otherwise indicated, embryos for culture were flushed 16 h post hCG. They were cultured in Whittingham's medium (Whittingham, 1971) which was prepared weekly and the pyruvate supplement added fresh on the day of use. Culture was in small drops of medium under paraffin oil (Boots Pharmaceuticals Ltd) in an atmosphere of 5% CO_2 in air. In some experiments, embryo culture medium was supplemented with monosaccharide sugars (L-fucose, D-glucosamine, D-galactose and D-mannose, all at 0·5 mg ml^{-1} and obtained from Sigma), or with tunicamycin (Sigma), 2 μg ml^{-1}.

For fertilization *in vitro*, eggs were obtained from F_1 females 14 h after hCG, and spermatozoa were obtained from F_1 males after mincing cauda epididymides in Whittingham's medium. Eggs in cumulus masses were cocultured for 4 h with approximately 10^7 spermatozoa in 2 ml of Whittingham's medium. The eggs were then washed prior to culture in small drops of medium under paraffin oil.

Day $4\frac{1}{2}$ blastocysts delayed in their implantation were flushed from the uteri of mice which had been ovariectomized on the afternoon of day $2\frac{1}{2}$ of pregnancy and injected subcutaneously with 1 mg Depo-Provera (Upjohn), as described by Evans & Kaufman (1981).

Inner cell masses were isolated from day $3\frac{1}{2}$ blastocysts by immunosurgery (Solter & Knowles, 1975) and were cultured in Alpha medium (Gibco) supplemented with 10% (v/v) foetal calf serum on feeder layers of mitomycin C innactivated STO cells (Martin & Evans, 1975).

Monoclonal antibodies

Monoclonal antibody 2B5 was prepared by fusion of NS1 myeloma cells (Kohler & Milstein, 1976) with the spleen cells of a Wistar rat which had been immunized with PC13 teratocarcinoma cells (Bernstine, Hooper, Grandchamp & Ephrussi, 1973). The rat had received three immunizations, using live cells and allowing 4-week intervals between injections. The first two immunizations (2×10^7 cells) were given intraperitoneally, and the third injection was given

intravenously. The spleen cells were used for fusion 4 days after the *i.v.* injection. The clone 2B5 was identified by testing culture supernatants in indirect immunofluorescence assays on PC13 cells, and was cloned by picking single cells (Gaunt, Brown & Jones, 1983). The 2B5 antibody was identified as IgM by immunodiffusion against immunoglobulin class- and subclass-specific antisera (Miles Ltd). 2B5 ascites fluid was prepared by culture of 2B5 hybridoma cells as an ascites tumour in an F344 nude rat (Olac Ltd). The rat was primed with 1 ml of pristane (McMaster & Williams, 1979) 2 weeks before injection of $5 \cdot 10^7$ live 2B5 hybridoma cells.

Hybridoma cells producing 2H9 antibody (Stern *et al.* 1984) were prepared as above in collaboration with K. Chada. Hybridoma cells producing 2C5 antibody (Randle, 1982, 1983) were a gift from B. Randle. SSEA-1 ascites fluid (Solter & Knowles, 1978) and M1/22.25 culture supernatant (Stern *et al.* 1978) were gifts from P. Goodfellow and P. Stern respectively. Monoclonal antibody 2D6 (Gaunt *et al.* 1983) is a mouse IgM which binds to rat spermatozoa but not to mouse teratocarcinoma cells.

Fixation, embedding and sectioning of embryos and other tissues for immunofluorescence

Embryos and tissues were fixed in 95 % ethanol at 4 °C for 15 h. They were then dehydrated for 3 to 4 h in two changes of absolute alcohol at 4 °C, and cleared for 1 to 2 h in two changes of xylene at 4 °C. Material was then embedded in paraffin wax (56 °C melting point). For ease of handling, preimplantation stage embryos, embryoid bodies and isolated genital ridges were first embedded in 2 % (w/v) agar in 0·9 % (w/v) NaCl. The live tissues were added to the molten agar at 40 °C. After setting, blocks of agar containing the tissues were fixed and processed as above. Postimplantation-stage embryos from the 6th to 8th day of pregnancy were fixed within the decidual tissues. Paraffin blocks were sectioned at 10 μm. Sections for immunofluorescence were rehydrated through xylenes and alcohols, washed in water, and then rinsed in PBS (Oxoid, Dulbecco A) ready for use.

Indirect immunofluorescence on live and fixed, sectioned material

Zonae were removed from preimplantation-stage embryos by a brief incubation in acid Tyrodes solution, pH 2·5 (Nicolson, Yanagimachi & Yanagimachi, 1975).

After washing in PB1/BSA, embryos or tissue sections were incubated in 2B5 culture supernatant at 4 °C for 30 min. They were then washed twice in PB1/BSA, incubated for 30 min in fluorescein-labelled rabbit anti-rat IgG antiserum, (1/20 dilution, Miles), and then given a final three washes in PB1/BSA. Control experiments to assess any non-specific labelling were carried out by incubating samples in fresh HAT medium (Gaunt, 1982) instead of hybridoma supernatant, and then repeating the remainder of the labelling procedure given above. In early experiments, embryos were labelled in the presence of 0·1 % azide but this did not affect the pattern of surface labelling obtained. For fluorescence microscopy, embryos were mounted as previously described (Gaunt, 1983), and labelled sections were mounted under coverslips in PBS/glycerol (2:1, v/v). Labelled material was examined using a Zeiss photomicroscope fitted with epifluorescent illumination.

In some experiments, embryos were labelled instead with 2C5, SSEA-1, or M1/22.25 antibodies. Labelling procedures were as given for 2B5, except that for SSEA-1 a 1/100 dilution of ascites fluid was used as first layer, and for 2C5 and SSEA-1 fluorescein-labelled rabbit anti-mouse IgG antiserum (Miles) was used as the second layer reagent.

After photography of sections under u.v. illumination, the coverslips were removed and the sections were stained with haematoxylin and eosin. After dehydration, clearing and mounting in DPX, the sections were re-photographed using phase-contrast illumination.

Glutaraldehyde fixation and transfer of eggs to oviducts

Freshly ovulated eggs were flushed from F_1 mice 14 h after hCG. After removal of zonae using acid Tyrodes solution, the eggs were washed in PBS and then fixed for 30 min in glutaraldehyde (0·1 % solution in PBS). The fixed eggs were washed twice in PBS and were then allowed to stand overnight in PB1/BSA at 4 °C. Next day the fixed eggs were transferred to the oviducts of

day 1 pregnant F_1 mice. The fixed eggs and live embryos were flushed from the oviducts after 24 h and were examined for surface 2B5 antigen by indirect immunofluorescence.

Inhibition of ^{125}I SSEA-1 binding

SSEA-1 antibody was radio-labelled with ^{125}Iodine using the chloramine T method (Jensenius & Williams, 1974). Ascites fluid was labelled without prior fractionation (to 5 μl of SSEA-1 ascites fluid was added 10 μl chloramine T, 2 mg ml^{-1}, and 0·5 mCi ^{125}Iodine). Labelled proteins were first separated from non-incorporated iodine by gel filtration in G-50 sephadex (Pharmacia), using NaCl/Tris buffer (0·5 M-NaCl, 0·1 M-Tris, pH 8·0). A ^{125}I-labelled IgM fraction, containing SSEA-1 activity, was then isolated from other labelled proteins by gel filtration in G-200 sephadex using NaCl/Tris buffer. The labelled SSEA-1 activity, which eluted from the column as the first peak of radioactivity, was supplemented to 0·4 % with BSA and then dialysed against PBS.

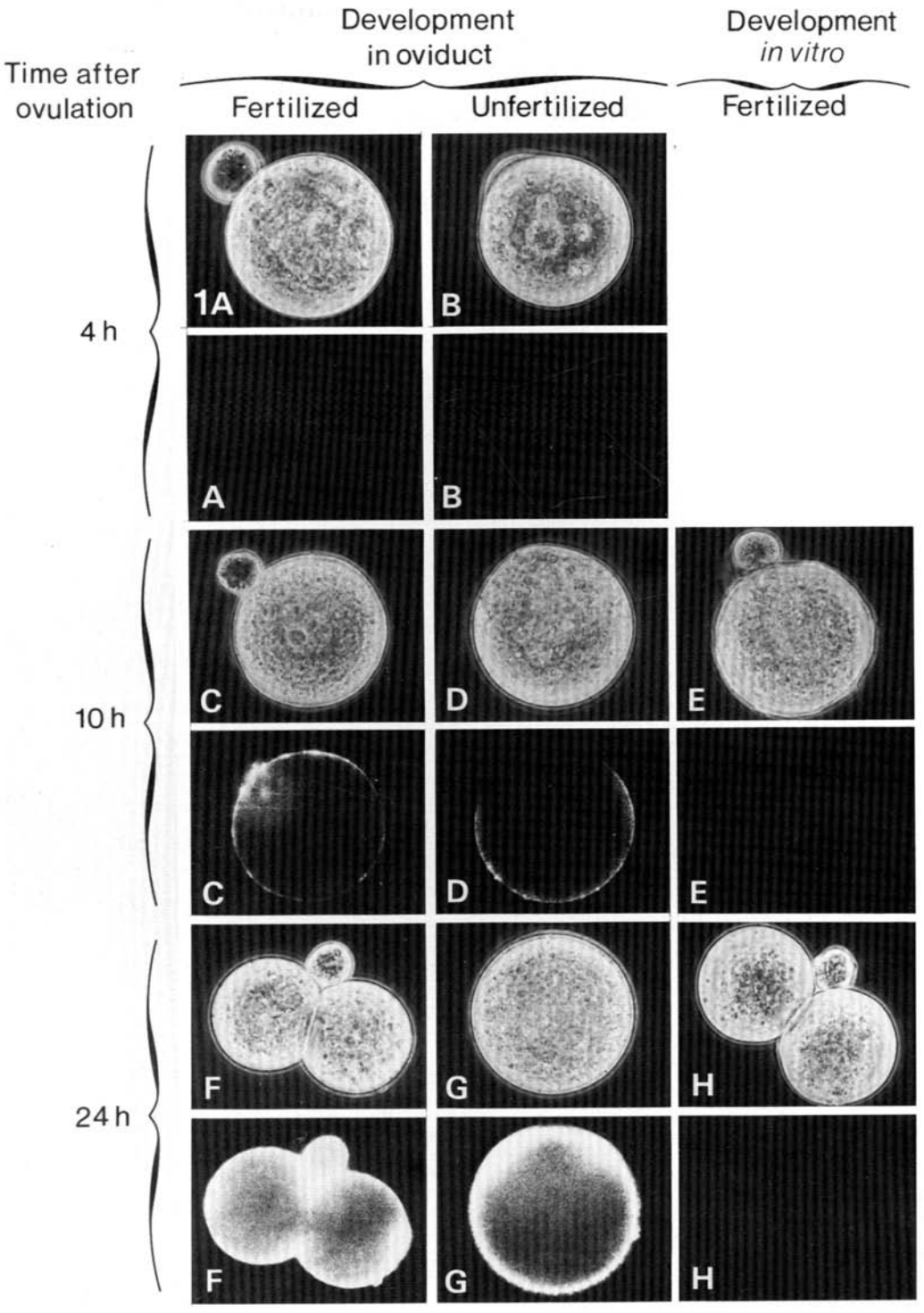
PC13TG8 teratocarcinoma cell monolayers (Hooper & Slack, 1977) were prepared by seeding 3×10^4 cells into each well of a gelatin-coated, flat-bottomed, 96-well culture plate (Linbro). After 6 h, monolayers were rinsed once in PBS at 37 °C, and they were then fixed in 0·1 % glutaraldehyde in PBS (45 min at room temperature). The fixed monolayers were washed three times in PBS, and then HAT medium (200 μl) was added to each well and the plates allowed to stand overnight. To measure binding of ^{125}I SSEA-1 to the fixed cells, each monolayer was incubated at 4 °C for 45 min in 5×10^4 c.p.m. of ^{125}I SSEA-1 in PBS/BSA, then washed three times in PBS/BSA before air drying and cutting the plastic well from the plate for counting in a gamma counter. To assess inhibition of ^{125}I SSEA-1 binding by unlabelled monoclonal antibodies, the fixed monolayers were preincubated for 45 min at 4 °C in 2B5, 2C5, SSEA-1, M1/22.25, 2H9, or 2D6 antibodies. Incubation in HAT medium served as control. After removal of these unlabelled antibodies, followed by a single wash in PBS/BSA, the binding of ^{125}I SSEA-1 antibody was measured as above. Unfixed teratocarcinoma cells were considered less satisfactory for these inhibition assays due to the agglutination which was produced by the first incubation in monoclonal antibody. As judged by indirect immunofluorescence, the method of glutaraldehyde fixation did not destroy the SSEA-1, 2B5, 2C5, 2H9, or M1/22.25 antigens.

RESULTS

2B5 Antigen on preimplantation embryos and unfertilized eggs

By indirect immunofluorescence, 2B5 antigen was not detectable on fertilized or unfertilized eggs 16 h post hCG (about 4 h postovulation; Fig. 1A,B). Weak immunofluorescence was first detected on both fertilized and unfertilized eggs flushed from the oviduct at 17 to 18 h post hCG, and fluorescence had become more intense by 22 h post hCG (about 10 h postovulation; Fig. 1C,D). In contrast, fertilized eggs of the same age which had been cultured *in vitro* from 16 h post hCG were not labelled (Fig. 1E). At 36 h post hCG, strong fluorescence was observed on both 2-cell-stage embryos and unfertilized eggs flushed from the oviduct (Fig. 1F,G), but embryos of similar age which had been cultured *in vitro* were not labelled (Fig. 1H). The second polar bodies of late 1-cell and 2-cell embryos

Fig. 1. Indirect immunofluorescence to detect binding of 2B5 monoclonal antibody to zona-free eggs after flushing from F_1 mice at 4 h, 10 h, and 24 h after ovulation (16 h, 22 h, and 36 h post hCG). The 2B5 antigen appeared at a similar rate on fertilized (Fig. 1A,C,F) and unfertilized (Fig. 1B,D,G) eggs developing in the oviduct, but antigen was not detected on eggs of similar age cultured *in vitro* (Fig. 1E,H). Eggs for *in vitro* culture were flushed from the oviduct 16 h post hCG. The same exposure times were used in preparation of all fluorescence photographs. Upper panel of each figure: phase contrast; lower panel: u.v. illumination.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in oviduct
Development
in vitro
Time after
ovulation
Fertilized
Unfertilized
Fertilized
4 h
10 h
24 h
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C
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flushed from the oviduct were brightly labelled (Fig. 1F), but antigen seemed to be acquired more slowly here than on the eggs themselves (Fig. 1C). Control experiments (not shown) indicated that none of the immunofluorescent labelling described was attributable to non-specific binding of fluorescent second-layer reagent.

After flushing from oviducts, followed by indirect immunofluorescence, 4-cell, 8-cell, and morula-stage embryos showed intense labelling over all cells (not shown, but as indicated for the 2-cell stage in Fig. 1F). Unhatched blastocysts, flushed from the uterus 84 to 92 h post hCG, were brightly labelled over all trophoblast cells.

Embryos cultured *in vitro* did not show appearance of surface 2B5 antigen until about 44 h after transfer to culture (60 h post hCG; Table 1). At this time about 60 % of embryos had cleaved to the 4-cell stage, and about half of the total group of embryos showed expression of 2B5 antigen. Appearance of antigen did not depend upon stage of cleavage: labelled embryos were found amongst 2-cell, 3-cell, and 4-cell stages. Commonly, only a single blastomere was labelled (Fig. 2A,B) although for 4-cell stages it was more usual for all blastomeres to be either labelled or unlabelled. Embryos which acquired 2B5 antigen *in vitro* never showed labelling of the second polar body.

Embryos cultured *in vitro* for 68 h had reached the 8-cell stage, and many had undergone compaction. After indirect immunofluorescence using 2B5 antibody, all embryos at this stage showed strong labelling over all blastomeres (Fig. 2C). Strong labelling persisted over all embryos until the blastocyst stage, which was reached by *in vitro* embryos 80 to 92 h after transfer to culture (96 to 108 h post hCG). Unfertilized eggs maintained in culture for 72 h failed to acquire surface 2B5 antigen (not shown).

In a separate set of experiments, eggs from F_1 females were fertilized *in vitro* (as described in Materials and Methods) and after various intervals of culture the embryos were tested for surface 2B5 antigen by indirect immunofluorescence. Appearance of antigen was the same as already described for eggs fertilized *in vivo* but cultured *in vitro*.

Embryos from CFLP mice, flushed from the oviduct 16 h post hCG and then transferred to culture, underwent cleavage and developed the 2B5 antigen more

Table 1. *Stage of cleavage and expression of 2B5 antigen shown by embryos flushed at 16 h post hCG from F_1 mice, then cultured* in vitro *for 44 h. Detection of 2B5 antigen was by indirect immunofluorescence*

Cleavage stage	Number of embryos (% of total)	2B5 antigen expression on embryos		
		None	Some blastomeres labelled	All blastomeres labelled
2-cell	15 (25 %)	6	6	3
3-cell	10 (17 %)	5	5	0
4-cell	35 (58 %)	20	5	10

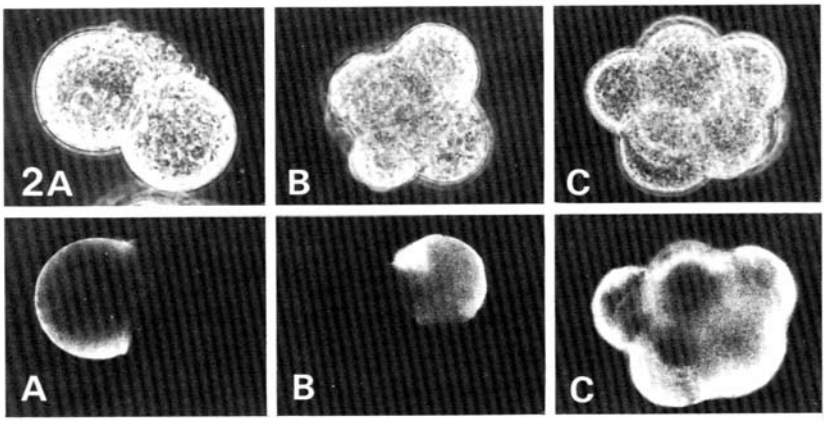


Fig. 2. Indirect immunofluorescence to show appearance of 2B5 antigen on embryos flushed from F_1 mice at 16 h post hCG, and then cultured *in vitro*. Antigen was not detected until about 44 h after transfer to culture (Fig. 2A,B) at which time many embryos showed labelling of only a single blastomere. After 68 h of culture embryos had reached the 8-cell stage and showed strong labelling over all blastomeres (Fig. 2C). Upper panel of each figure: phase contrast; lower panel: u.v. illumination.

slowly than did embryos from F_1 mice. After 68 h of culture, most embryos (15/21) were at the 4-cell stage and some (6/21) were at the 2-cell stage. Only half of the embryos, including both 2-cell and 4-cell stages, were labelled. For most (> 95 %) CFLP embryos cultured in Whittingham's medium cleavages arrested at some stage before blastocyst formation. CFLP embryos flushed directly from the oviduct or uterus prior to indirect immunofluorescence were found to be brightly labelled over all stages from mid 1-cell to blastocyst.

Appearance of the 2B5 antigen on F_1-derived embryos cultured *in vitro* from the 1-cell to the 8-cell stage was completely blocked after addition of tunicamycin ($2\ \mu g\ ml^{-1}$) to the culture medium. This observation suggests that the 2B5 antigen resides on a carbohydrate component of surface glycoprotein. The possibility was therefore considered that embryos cultured *in vitro* might be delayed in their synthesis of 2B5 antigen due to lack of exogenous monosaccharide sugars necessary for normal glycosylation. However, supplementation of embryo culture media with a variety of sugars (fucose, glucosamine, galactose, mannose; all at $0{\cdot}5\ mg\ ml^{-1}$) did not result in earlier expression of 2B5 antigen on embryos developing *in vitro*.

When 2B5 antigen-positive 2-cell-stage embryos (flushed from the oviduct 36 h after hCG) were transferred to culture, then tested for 2B5 antigen after a further 8 h, all embryos remained antigen positive although most showed weaker labelling than did control eggs examined at the beginning of the culture period (not shown).

2C5, SSEA-1, and M1/22.25 antigens on embryos developing in vitro

2C5 antigen showed a behaviour similar or identical to 2B5 antigen: embryos flushed directly from the oviduct prior to indirect immunofluorescence were

brightly labelled over all stages from late 1-cell to blastocyst, whilst embryos cultured *in vitro* first showed antigen expression at the late 2-cell, 4-cell, or 8-cell stage. For SSEA-1 and M1/22.25 antigens, no difference was detected in the stage of first expression between cultured and oviduct-derived embryos. For SSEA-1 antigen, expression was first detected by indirect immunofluorescence at the 8-cell stage, and for M1/22.25 antigen at the late morula stage.

Appearance of 2B5 antigen on glutaraldehyde-fixed eggs transferred to the oviduct

Embryos from F_1 mice, flushed 14 h post hCG and then fixed in glutaraldehyde (as described in Materials and Methods) did not show evidence of any surface 2B5 antigen when tested by indirect immunofluorescence (not shown). The fixed eggs were readily distinguishable from live eggs, however, in that they showed an overall yellow fluorescence even in the absence of any labelling with fluorescent reagents. Fixed eggs negative for 2B5 antigen were transferred to the oviducts of day-1 pregnant mice and after an interval of 24 h were flushed out together with live 2-cell embryos. Zonae were removed from the live embryos and both fixed and live embryos were examined for surface 2B5 antigen by indirect immunofluorescence. Two-cell embryos were brightly labelled, thus showing that the experimental procedures had not damaged the normal process of antigen acquisition in the oviduct. The fixed 1-cell eggs also showed specific, bright surface fluorescence (not shown). This finding demonstrates that 2B5 antigen can appear on the cell surface even without metabolic activity within the egg, and thus that 2B5 antigen must be carried by a soluble factor in the oviduct which becomes bound to the surface of the glutaraldehyde-fixed egg.

Distribution of the 2B5 antigen in embryonic and some adult tissues

Although 100% of newly formed blastocysts tested by indirect immunofluorescence were labelled over all cells on their outer surfaces, 2B5 antigen was found to disappear from the trophectoderm cells soon after blastocoele formation. Thus, blastocysts prevented from implantation (see Materials and Methods), flushed from the uterus on day $4\frac{1}{2}$ then sectioned and stained for 2B5 antigen showed intense labelling on the inner cell mass but not at all on the trophoblast or endoderm layers (Fig. 3A).

The egg cylinder on day 6 and day 7 showed labelling of the embryonic ectoderm (Fig. 3B,C) but labelling was weak in comparison to the inner cell mass tissue (Fig. 3A) from which it was derived. Extraembryonic ectoderm was not labelled (Fig. 3B). Endoderm cells were labelled either on their outer surfaces or they were unlabelled (Fig. 3B,C). Inner cell masses isolated from blastocysts by immunosurgery and then cultured for two days on STO feeder cells (see Materials and Methods) developed an outer layer of endoderm cells. After sectioning and labelling, these structures showed similar distribution of fluorescence to sectioned egg cylinders: central cells were labelled whilst outer endoderm cells were either unlabelled or were labelled only on their outer borders (Fig. 3D).

No clear labelling was detected by indirect immunofluorescence on sections of 8-day or 9-day embryos, but at $10\frac{1}{2}$ and $11\frac{1}{2}$ days (Fig. 3E) primordial germ cells were brightly labelled in the genital ridges. These antigen-positive cells proliferated and by $13\frac{1}{2}$ days had segregated into cords in the male, distinguishing the testis (Fig. 3F) from the ovary (Fig. 3G).

Other tissues seen to be 2B5 antigen positive in the embryo were Rathkes pouch at 13 days, some tubules in the kidney at 15 days, and the lining cells of the bronchi at 15 days.

In the adult female, 2B5 antigen was detected on tissue sections by immunofluorescence on the epithelial cells of the oviduct (but only on the day of ovulation; not shown) and the endometrial lining of the pregnant uterus (Fig. 3B). In the adult male, epithelial cells lining a discrete region of tubules in the caput epididymidis were antigen positive and sperm acquired surface fluorescence as they progressed through this region (not shown).

Since the range of 2B5 antigen-positive tissues within the postimplantation embryo and adult reproductive tracts was clearly very similar to that known for SSEA-1 antigen (Fox *et al.* 1981, 1982), the following experiment was carried out to investigate further a possible relationship between these two antigens.

2B5 Antibody blocks the binding of ^{125}I SSEA-1 antibody to teratocarcinoma cells

Fig. 4 shows how preincubation of glutaraldehyde-fixed teratocarcinoma cells in a variety of different monoclonal antibodies affected subsequent binding of ^{125}I SSEA-1 antibody. Preincubation in 1/10 dilution of either SSEA-1 or 2B5 ascites fluids gave 95% inhibition in comparison to controls (preincubation in HAT medium). Preincubation in 2B5 and 2C5 culture supernatants gave 84% and 45% inhibition respectively. Preincubation in M1/22.25 supernatant gave a slight (about 8%) but reproducible inhibition. Binding was not inhibited by preincubation in either 2D6 or 2H9 monoclonal antibodies. 2B5 antibody (1/10 dilution of ascites fluid) also gave similar inhibition to SSEA-1 antibody (1/10 ascites fluid) in experiments using unfixed teratocarcinoma cells (not shown).

DISCUSSION

By indirect immunofluorescence, fertilized and unfertilized eggs obtained directly from the oviduct were found to commence expression of 2B5 antigen 5 to 6 h after ovulation (ovulation assumed to occur 12 h post hCG). Fluorescence on these oviduct-derived eggs became intense by 16 h after ovulation, and fluorescence persisted over all blastomeres throughout the cleavage stages of preimplantation development. In contrast, embryos cultured *in vitro* from a time 4 h after ovulation did not show appearance of 2B5 antigen until the 2-cell, 4-cell, or even 8-cell stage. This appearance of antigen *in vitro* did not occur until about 48 h after ovulation. Unfertilized eggs maintained in culture did not acquire 2B5 antigen.

Two possible sources of the 2B5 antigen which appears on the egg and 1-cell embryo in the oviduct are, first, synthesis within the egg, and second, transfer of soluble antigen from the oviducal fluid through the zona to become bound on to the egg surface. If the first of these two possibilities is correct, then failure of 2B5 antigen to appear on eggs cultured *in vitro* might most likely be due to metabolic impairment caused by lack of nutrients in the culture medium. Several observations have already shown that mouse embryo culture media do not support optimal development (for example, Bowman & McLaren, 1970*a*,*b*; Goddard & Pratt, 1983). Although this possibility of a nutritional deficiency within the egg cannot be ruled out, several observations when taken together suggest that binding of soluble antigen from the oviduct fluid is a more likely explanation of 2B5 antigen on the egg. At the time of antigen appearance on the egg the oviducal lining cells are 2B5 antigen positive. Soluble antigen might therefore be released from these cells prior to becoming bound to the egg surface. A similar mechanism apparently takes place in the caput epididymidis where spermatozoa acquire surface 2B5 and SSEA-1 (Fox *et al.* 1982) antigens as they pass through tubules lined with antigen-positive epithelial cells. Direct evidence for binding to the egg surface of an antigen-positive oviduct factor is given in the observation that eggs within the oviduct became 2B5 antigen positive even after their prior fixation in 0·1 % glutaraldehyde. Whilst this observation provides clear evidence that 2B5 antigen exists as a soluble factor within the oviduct, it remains possible that the binding of this factor to the egg is an artefact of glutaraldehyde fixation. However, since surface 2B5 antigen is absent from living eggs and 1-cell embryos maintained outside the oviduct, it is likely that 2B5 antigen on these stages within the oviduct is also acquired by binding of the antigen-positive oviduct factor. This factor has not yet been identified. It may simply be monosaccharide sugars which form 2B5 antigen on eggs by their attachment to existing cell surface carbohydrate chains. Such a mechanism would presumably require exogenous glycosyltransferase activity. Alternatively, the oviduct factor may be a soluble glycoprotein which is released from the antigen-positive lining epithelial cells, and which binds to the egg surface. The factor may be one of the proteins which have already been shown

Fig. 3. Localization of 2B5 antigen by indirect immunofluorescence on tissue sections. Left: haematoxylin and eosin stained (Fig. 3A–E); Right: u.v. illumination.

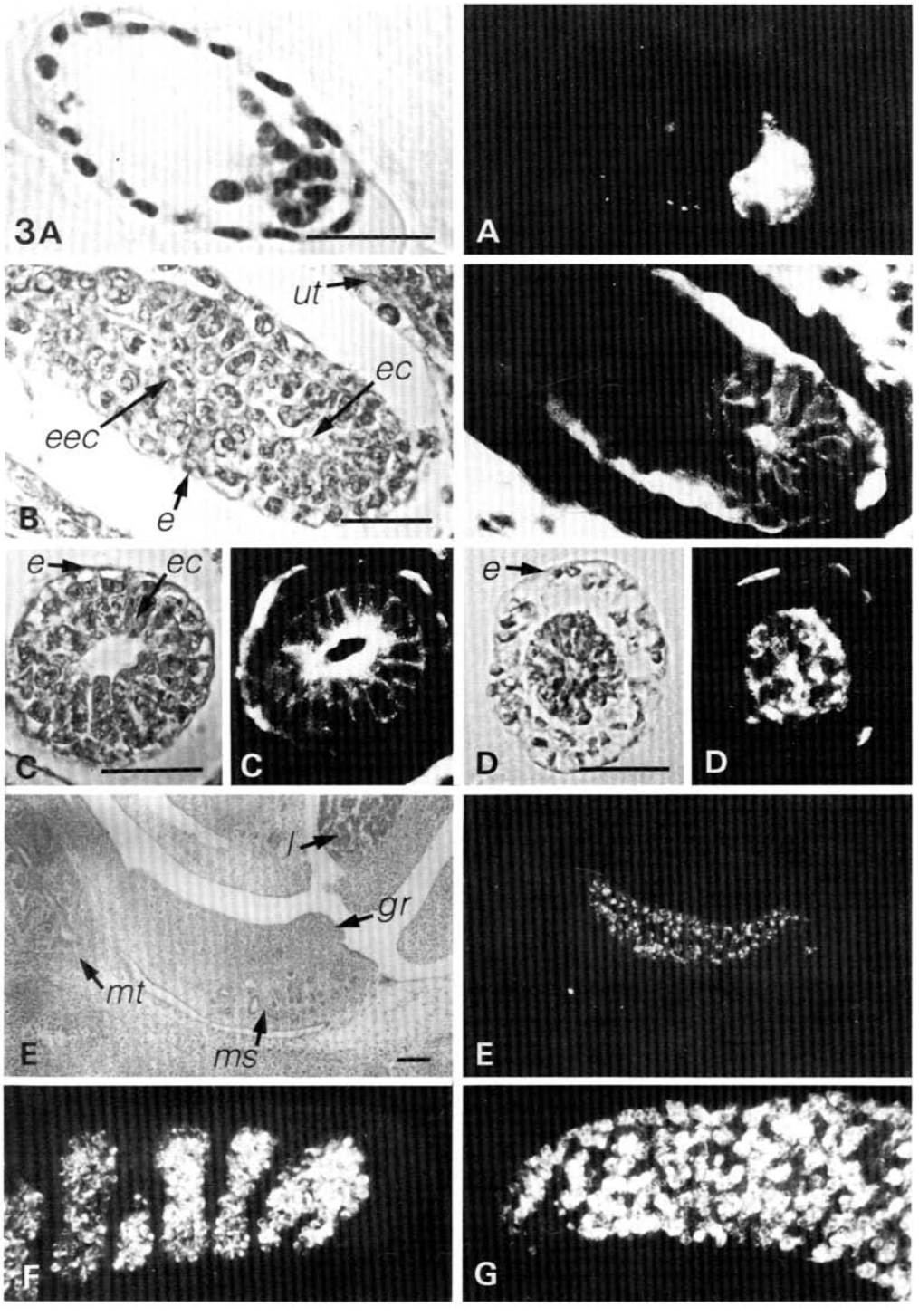
(A) Day-4½ blastocysts showed labelling only over the inner cell mass. Scale bar 50 μm.

(B,C) Day-6 egg cylinders in longitudinal (Fig. 3B) and transverse (Fig. 3C) sections showed labelling of the embryonic ectoderm (*ec*), outer surface of endoderm (*e*) and endometrial lining of the uterus (*ut*), but not of extraembryonic ectoderm (*eec*). Scale bars 50 μm.

(D) Endoderm cells (*e*) which develop around *in vitro* cultured inner cell masses showed surface labelling on some cells. Scale bar 50 μm.

(E) Longitudinal section through the posterior region of a day-11½ mouse embryo showed labelling of the primordial germ cells in the genital ridge. *gr*, genital ridge; *ms*, mesonephric kidney; *mt*, metanephric kidney; *l*, liver. Scale bar 100 μm.

(F,G) Gonads from day-13½ mouse embryos showed segregation of labelled primordial germ cells into cords in the testis (Fig. 3F) but not in the ovary (Fig. 3G). u.v. illumination.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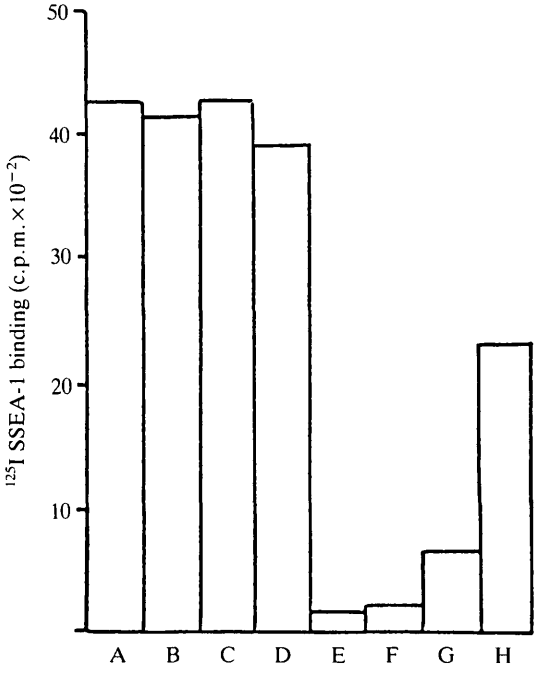

Fig. 4. Inhibition of ^{125}I SSEA-1 monoclonal antibody binding to teratocarcinoma cells by preincubation in unlabelled antibodies. (A) HAT medium (control); (B) 2D6 culture supernatant; (C) 2H9 supernatant; (D) M1/22.25 supernatant; (E) SSEA-1 ascites fluid (1/10 dilution); (F) 2B5 ascites fluid (1/10 dilution); (G) 2B5 supernatant; (H) 2C5 supernatant. Each bar represents the mean of three replicates.

to appear on the egg by use of the lactoperoxidase surface labelling technique (Johnson & Calarco, 1980).

For 1-cell and 2-cell embryos developing in the oviduct, appearance of 2B5 antigen on the second polar body was more gradual than on the fertilized egg itself. The surface membrane of the polar body might be expected to accumulate more slowly an antigen bound from the oviduct fluid since the plasma membrane here is smooth in comparison with the highly folded membrane of the egg (Nicosia, Wolf & Mastroianni, 1978).

Although 2B5 antigen on the 1-cell and 2-cell embryo in the oviduct is probably attributable to binding of an oviduct factor, it is clear that embryos at later stages develop a capacity for synthesis of surface 2B5 antigen. Thus, embryos cultured *in vitro* acquired surface antigen by the 2-cell, 4-cell or 8-cell stage. This acquisition was blocked by tunicamycin, an inhibitor whose principal action is to block glycosylation of asparaginyl residues of glycoproteins (Waechter & Lennarz, 1976; Rothman, Katz & Lodish, 1978). It is probable, therefore, that the 2B5 antigen which is synthesised by preimplantation embryos resides on a carbohydrate component of surface glycoprotein. Although it is possible that both sources of the 2B5 antigen on the embryo, that is, both the oviduct factor and the embryo-

derived glycoprotein are the same molecule, this is not necessarily the case since monoclonal antibodies may recognize a group of only three sugar residues within a more complex carbohydrate structure (for example, Gooi *et al.* 1981).

Individual blastomeres of embryos cultured *in vitro* developed ability to synthesise 2B5 antigen at different rates so that both labelled and unlabelled blastomeres commonly occurred within the same embryo. Eventually, however, all blastomeres became labelled. It is possible that this phenomenon is an artefact attributable to the suboptimal conditions of embryo culture. However, in experiments using embryos flushed directly from the oviduct, Solter & Knowles (1978) noted a difference in the rate at which blastomeres of individual 8-cell stage embryos became positive for SSEA-1 antigen.

In addition to cells of the preimplantation embryo, several other pluripotential cell types of the mouse also bound 2B5 antibody: inner cell mass, ectoderm, primordial germ cells and teratocarcinoma cells. 2B5 antigen was not, however, completely specific to pluripotential cells and certain epithelial cell types were also labelled: endoderm and the lining epithelia of the oviduct, uterus, caput epididymidis, bronchi and some kidney tubules. The 2B5 antigen has many similarities in its distribution to SSEA-1 antigen (Solter & Knowles, 1978). Thus, SSEA-1 antibody, like 2B5, binds to teratocarcinoma cells and inner cell masses (Solter & Knowles, 1978), the cores of embryoid bodies, the external surface of the visceral endoderm, embryonic ectoderm, primordial germ cells, tubules in the developing kidney, the endometrial lining of the adult uterus, the oviducal epithelium (Fox *et al.* 1981), epithelial cells lining a discrete region of the caput epididymidis, and mature spermatozoa (Fox *et al.* 1982). As further evidence of a relationship between 2B5 and SSEA-1 antigens, glutaraldehyde-fixed monolayers of teratocarcinoma cells could be 95% inhibited from binding ^{125}I SSEA-1 antibody if they were preincubated in 2B5 antibody. The 2B5 antigen also shows many similarities in its distribution to the 2C5 antigen (Randle, 1982, 1983) and it is therefore of interest that 2C5 antibody also produced some inhibition of ^{125}I SSEA-1 binding to teratocarcinoma cells. To account for these findings it is suggested that SSEA-1, 2B5 and 2C5 antibodies bind to antigenic sites which lie in close proximity on the same cell surface molecules. The SSEA-1 antibody is known to recognize $\alpha 1 \rightarrow 3$ fucosylated type-2 blood group sugar chains (Gooi *et al.* 1981) present on teratocarcinoma cells principally on high relative molecular mass glycoproteins (Childs *et al.* 1983). In spite of the similarities between 2B5 and SSEA-1 antigens, it must be assumed that they are not identical. Thus, 2B5 antigen first appears at the 1-cell stage rather than the 8-cell stage on embryos developing within the oviduct and, moreover, it does not recognize certain other SSEA-1 positive tissues in embryos, such as neural ectoderm of the 8-day embryo (Fox *et al.* 1981).

Bird & Kimber (1984) suggested that the SSEA-1 antigen might be an important component in the mechanisms of adhesion and compaction between embryonic cells since they found that oligosaccharides which contain the SSEA-1 antigen were effective in preventing compaction of embryos. The 2B5 monoclonal

antibody apparently binds to sites on the cell surface which are related to SSEA-1 sites, at least on teratocarcinoma cells, and therefore the 2B5 antigen may also bind to sugar chains involved in cell–cell adhesion. Unlike SSEA-1 antibody, 2B5 antibody binds to the 1-cell egg in the oviduct, probably recognizing a bound oviduct factor which carries the 2B5 antigen. The importance of this finding is not yet clear. It is not known whether there is specific binding of one factor or whether there is binding of all oviduct proteins to the egg. It is hoped that further work will indicate a function for oviduct products bound to the egg, perhaps enhancement or blocking of sperm–egg binding, or perhaps a role in embryo metabolism.

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