A monoclonal antibody to an antigen present on the microvillous membrane of the trophectoderm of the preimplantation blastocyst of the pig

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Summary. Immunization of BALB/c mice with 14-day pig preimplantation blastocyst material followed by fusion of spleen cells with NS-0 myeloma cells resulted in a clone, SN1/38, which secreted IgG₁ which reacted specifically with the microvillous border of the pig trophectoderm and trophoblast as assessed by immunohistology. SN1/38 did not react with other fetal tissues, or with blastocysts from other animal species. It was shown by absorption studies and by enzyme-linked immunoabsorbent assay that SN1/38 was not directed against the placental form of pig alkaline phosphatase. The location of this antigen and its apparent presence throughout gestation indicate a functional role in the materno–fetal interaction.

Introduction

Placentation in mammals shows wide morphological diversity. Placentae have been categorized as varying from non-invasive (e.g. pig) to migratory (e.g. sheep) to invasive (e.g. man) (for review see Steven, 1983). In spite of this extended array of different morphologies, the immunological paradox of the survival of the demonstrably allogeneic fetus is a 'problem' surmounted by all species. It is not unreasonable to postulate that, in view of the similarity of the immune system between different mammals, there may be a common mechanism amongst species that prevents maternal immunological rejection of the fetus. Many hypotheses have been advanced as to the nature of this mechanism, but few have stood the test of time. Current opinion seems to favour a 'priming' of the maternal immune system, probably at the level of the regional lymph nodes draining the gravid uterus (Newport & Carter, 1983). Such priming involves antigen-presenting cells entering via the afferent lymphatic and/or the lymphocytes entering the node from the blood, the result being a carefully regulated response by the mother leading to tolerance. Also suggested as a means of allowing survival *in utero* is the absence of MHC (Major Histocompatibility Complex) products on the trophoblast, the site of materno–fetal contact. These gene products control self/non-self recognition reactions and in man they are HLA-A, HLA-B, HLA-C and HLA-D. They are also associated with a small molecule in the cell surface, β_2 -microglobulin. Several reports have shown that HLA and β_2 -microglobulin are absent from human villous trophoblast (Faulk & Temple, 1976; Goodfellow, Barnstable, Bodmer, Snary & Crumpton, 1976). However, human non-villous trophoblast expresses a Class I MHC molecule which is not HLA-A or HLA-B (Redman, Stirrat & Sunderland, 1983). The mouse placenta appears to have Class I but not Class II antigens on its trophoblast (Billington & Bell, 1983). For the pig, β_2 -microglobulin has been reported on pre-implantation blastocysts (Meziou, Chardon, Fléchon, Kalil & Vaiman, 1983). In spite of this relatively unclear picture of Class I and Class II MHC antigenic expression by trophoblasts of different species, there is increasing evidence that tissue-specific antigens are present on this epithelium (Searle & Jenkinson, 1978; Whyte & Loke, 1979; Johnson, Cheng, Molloy, Stern &

Slade, 1981). These antigens may be of relevance to the immunological survival of the fetus and we have chosen to study the pig in which early embryonic mortality may be as high as 40%, and the immunology of this economically important embryo loss is undefined.

Materials and Methods

Animals and recovery of blastocysts

Large White Devana sows and gilts were taken from the Institute herd and killed by captive bolt and exsanguination at known stages of pregnancy. Material from 2 animals at Day 14 of pregnancy (day of mating = Day 0) was used to raise the monoclonal antibodies. Other materials used in the immunohistology were obtained from animals at 9–18 days and on the 66th day of pregnancy. The reproductive tract was removed aseptically at laparotomy and placed in a sterile container before transportation to the laboratory. Para-aortic lymph nodes (PALN), which drain the gravid uterus, were also obtained at laparotomy. After removal of the ovaries, each horn was dissected free of the broad ligament and separated just above the cervix. A sterile glass tube was inserted into this end of the horn and secured with a ligature. Into the other end of the horn 20 ml Hank's Balanced Salts Solution (HBSS) (Flow Laboratories, Irving, U.K.) at 37°C were slowly introduced. The uterus was gently manipulated so that the bolus passed down and was collected into a sterile container. Sheep material was obtained in a similar manner, i.e. by flushing. Horse and guinea-pig material was obtained by dissecting the uterus around blastocystic swellings. Human material was obtained after delivery or following therapeutic terminations of pregnancy.

Production of antibody

Four BALB/c mice were immunized 7 days apart by two i.p. injections of mechanically dissociated 14-day blastocysts (avoiding the embryonic disc region) in sterile saline (9 g NaCl/l). After 5 days blood was collected from the tail veins and the sera tested against solubilized (1% Nonidet P-40 in PBS: phosphate-buffered saline) 14-day-old pig blastocyst material by double radial immunodiffusion. The mouse that responded with the greatest intensity of precipitin line was selected, and the spleen cells were fused with cells from the mouse myeloma cell line NS-0 (a mutant of X-63 which does not produce κ chain) using polyethylene glycol (PEG 1500) by the method of Köhler & Milstein (1975). A monoclonal antibody SN1/38 was obtained after sequential dilution cloning in the presence of irradiated (200 rad) BALB/c mouse spleen feeder cells (10^6 /ml) at a limiting dilution of one cell per flat-bottomed microtitre well. The cells were grown in RPMI 1640 medium containing 10% FCS (fetal calf serum), glutamine and HAT (hypoxanthine/aminopterin/thymidine); then weaned into hypoxanthine/thymidine and then RPMI medium alone.

Immunohistology

Small samples of tissue or flushed blastocysts were fixed in cold methanol or acetone overnight at 4°C before embedding in low melting-point wax. This method gave excellent preservation of tissue and was shown, by comparison with cryostat sections and by use of indirect immunoperoxidase methods, to retain antigenic activity. Clone supernatants were routinely screened on thin sections (7–10 μ m) of 14-day-old elongated blastocysts. The immunohistological procedure involved pretreatment with normal pig serum (2% in PBS), incubation with the clone supernatant (titrated to optimal dilution, normally 1:10 to 1:20) for 60 min at 37°C, and then addition of porcine anti-mouse-immunoglobulin-FITC (fluorescein isothiocyanate) at 1:40 in PBS for 30 min at 37°C. Between stages the sections were exhaustively washed with PBS. Controls were performed with normal mouse serum as the primary immunoglobulin stage. Sections were mounted and examined on a Leitz Diavert inverted microscope using epi-illumination fluorescence, occasionally with propidium iodide nuclear counterstain for localization of the nuclei of the cells (Ockleford *et al.*,

1981). Photographs were taken using an Olympus OM2 camera on Kodak XP-1 film rated at 1600 ASA. Fluorescent reaction was verified using an indirect immunoperoxidase method. More than 160 blastocysts at preimplantation stages of gestation were examined.

Enzyme-linked immunoassay

The enzyme-linked immunoabsorbent assay (ELISA) was performed in Nunc Immunoplates II. Wells were coated with a preparation of placental alkaline phosphatase from pig at 5 µg/ml (100 µl/well) at 4°C overnight. The optimal concentration of pig placental alkaline phosphatase (EC 3.1.3.1) was chosen by titration and the enzyme was purified, from a product obtained from Sigma (Type XVI), by column chromatography. It gave a single band after sodium dodecyl sulphate (SDS)-electrophoresis and also showed a histochemical reaction for alkaline phosphatase after agarose gel electrophoresis. The wells were rinsed three times in PBS containing 0.05% Tween with 2% normal rabbit serum (NRS) and 0.1% sodium azide. Blocking solution (200 µl PBS containing 0.5% Tween, 10% NRS and 0.1% NaN₃) was added to each well and left for 1 h at room temperature. After rinsing as before, culture supernatant SN1/38 was added in PBS with 2% NRS and 0.1% NaN₃ at 100 µl/well and left for 1 h at room temperature with shaking. After washing, rabbit anti-mouse IgG-peroxidase (from Miles Laboratories) (100 µl) was added. This was made up at 1:1000 dilution in PBS with 2% NRS and 0.1% NaN₃. This was left for 1 h at room temperature on the shaker. After washing, 200 µl substrate solution were added to each well. The substrate solution was made up freshly by mixing 18 ml distilled water, 2 ml stock solution A (1 M-sodium acetate/citrate acid buffer pH 6.0), 0.2 ml stock solution B (10 mg tetramethylbenzidine/ml dimethylsulphoxide) and 20 µl 6% H₂O₂. The colour reaction was stopped after 5 min by addition of 50 µl 3 M-H₂SO₄, and the absorbance at 450 nm was read on a Titertek Multiscan MC ELISA reader. Serial doubling dilution titrations of SN1/38 were performed to determine the optimal concentration. Samples of alkaline phosphatases from Sigma were also tested by ELISA against SN1/38. The placental forms were from man (Type XXIV) and cow (Type XV) and the intestinal forms from pig (Type IV) and calf (Type 1-S). All were tested for alkaline phosphatase enzymic activity using the protocol in Sigma Technical Bulletin 710-EP which involves a colorimetric method with 5-bromo-4-chloro-3-indolyl phosphate as substrate.

Results

Clones grew in 25 of the 48 wells into which the fused cells were seeded. The medium from each well was aspirated at frequent intervals and assayed for anti-pig blastocyst activity using indirect immunofluorescence on sections of 14-day blastocysts. Four clones were found to react with the blastocysts: SN1/17; SN1/20; SN1/35 and SN1/38. The last of these gave a highly specific reaction with the microvillous membrane of the trophectoderm and was consequently selected for limiting dilution cloning. It was a derivative of this original clone, SN1/38.1.1 which is reported here, but for convenience we shall call it SN1/38. The antibody produced is an IgG₁ as identified using a mouse monoclonal typing kit (Serotec).

The pattern of immunofluorescence of SN1/38 with a thin section of a blastocyst is shown in Pl. 1, Fig. 1. There was a marked reaction to the outside of the trophectoderm (that part which will form a microvillous interdigitation with the endometrium after Day 18 of pregnancy) but no reaction inside the trophectodermal cells or in the endoderm. The antigenic epitope recognized by SN1/38 was also present at Day 66 of gestation, and possibly at greater density judging by the intensity of fluorescence (Pl. 1, Fig. 2).

Cells were mechanically dissociated from the placenta of a 66-day-pregnant animal by passage through 19- and 25-gauge needles, and labelled with SN1/38 and FITC-conjugated goat anti-mouse immunoglobulin. The fluorescence was not spread evenly over the membranes of the cells, but was

located in certain regions predominantly at areas of cell-to-cell contact (Pl. 1, Fig. 3). Some cells did, however, show a more general membrane fluorescence (Pl. 1, Fig. 4), but this type of cell was only seen either singly or in association with a totally non-fluorescent cell (Pl. 1, Fig. 4, arrow).

The species and tissue specificities of the epitope recognized by SN1/38 are demonstrated in Table 1. Clone SN1/38 bound to pig trophoctoderm and trophoblast at all stages of pregnancy tested, but did not react with fetal skin, kidney and spleen, and did not recognize determinants on tissues from any of the other species tested (Table 1).

Samples of pig, human and bovine placental alkaline phosphatase and pig and bovine intestinal alkaline phosphatase were immobilized on Nunc Immunoplates and the binding of the SN1/38 supernatant to them was assayed by ELISA. Human serum albumin was also immobilized and a hyperimmune polyclonal antiserum to it was used as a positive control. Negative controls included using normal rabbit serum and BSA with the SN1/38 supernatant. Other controls involved replacing the SN1/38 with the supernatant from a fusion supernatant which was histologically negative with blastocysts, and omitting the peroxidase-conjugated antibody. The results are presented in Table 2. In no case was there significant binding of the SN1/38 supernatant, indicating that the epitope recognized was not on pig placental alkaline phosphatase or secreted into serum or urine.

To confirm the results of the ELISA, pig placental alkaline phosphatase was immobilized by conjugation to CNBr-activated Sepharose 4B using the protocol detailed by Pharmacia. After washing, the beads were added to SN1/38 supernatant and left overnight with gentle rotation. The beads were pelleted and the supernatant used in indirect immunofluorescence with 14-day pig blastocysts. There was no reduction in fluorescence compared with the supernatant before

Table 1. The reaction of the SN1/38 antiserum by indirect immunofluorescence with various pig tissues and trophoblast material of other species

Species	Tissue	Day of gestation	Intensity of fluorescence (- to +++)
Pig	Trophoctoderm	9 (pre-elongation)	+++
		11 (elongating)	+++
		14 (post-elongation)	+++
		16	+++
		17	+++
		18 (implantation)	+++
	Trophoblast§ 'Trophospheres'*	66	+++
		14 (+ 7)	+++
		14 (+ 10)	+++
	Endometrium	14	-
	Para-aortic lymph node	14	-
	Fetal skin§	66	-
Guinea-pig	Fetal kidney§	66	-
	Fetal spleen§	66	-
	Trophoblast	21	-
		44	-
Man	Trophoblast	First trimester†	-
		Full term‡	-
Sheep	Trophoctoderm	14	-
Donkey	Girdle	33	-
	Endometrial cup tissue	33 & 34	-
	Trilaminar yolk-sac	32 & 33	-
	Bilaminar yolk-sac	33	-

* These are spheres derived after several days culture of dissociated 14-day blastocysts.

† Approximately 8-12 weeks of gestation.

‡ At delivery, approximately 40 weeks of gestation.

§ Tissues derived from the same conceptus.

Fig. 1. Indirect immunofluorescent staining with the SN1/38 monoclonal antibody of a thin section through a pig blastocyst at 14 days. There was marked fluorescence of the microvillous border of the trophectoderm but no reaction inside the trophoctodermal cells or in the endoderm (E). The bar represents 40 μ m.

Fig. 2. The reaction of SN1/38 with the fetal aspect of the placenta from a 66-day-pregnant pig. There was marked fluorescence of the trophoblast membrane. In this section the fetal trophoblast had separated from the maternal endometrium. The bar represents 40 μ m.

Fig. 3. Cells mechanically dissociated from the placenta of a 66-day-pregnant animal and stained with SN1/38. Fluorescence appeared to be largely concentrated at areas of cell-cell contact. The bar represents 20 μ m.

Fig. 4. Two cells from the same placenta as shown in Fig. 3. The right-hand cells shows a general membrane fluorescence whereas the left-hand cell (arrow) is non-fluorescent. The bar represents 20 μ m.

Table 2. Results of enzyme-linked immunoabsorbent assays with SN1/38 supernatant: the wells were coated with the substances listed in the first column, the primary antibody is listed in the second column, and the second antibody was anti-rabbit or anti-mouse immunoglobulin conjugated to peroxidase

Coating	Primary antibody	E ₄₅₀ *
Human serum albumin (HSA-Sigma)	Rabbit anti-HSA (Nordic)	0.577 ± 0.012
Human serum albumin	SN1/38	0.154 ± 0.004
Bovine serum albumin (BSA-Sigma)	SN1/38	0.153 ± 0.005
Alkaline phosphatases		
Pig placental	SN1/38	0.156 ± 0.004
Pig intestinal	SN1/38	0.154 ± 0.002
Bovine placental	SN1/38	0.155 ± 0.003
Calf intestinal	SN1/38	0.156 ± 0.004
Human placental	SN1/38	0.156 ± 0.003
Pig placental	SN1/7†	0.155 ± 0.003
Pig placental	SN1/38‡	0.155 ± 0.002
Pig pregnancy urine		
4-day	SN1/38	0.157 ± 0.003
6-day	SN1/38	0.155 ± 0.004
7-day	SN1/38	0.155 ± 0.004
Pig pregnancy serum		
10-day	SN1/38	0.154 ± 0.002
14-day	SN1/38	0.155 ± 0.003

Values are mean ± s.e.m. for at least 30 observations of each.

None of the samples tested had binding significantly above background, whereas a hyperimmune polyclonal anti-human serum albumin showed positive binding.

* Absorbance at 450 nm.

† Histologically non-reactive clone supernatant.

‡ Second antibody (peroxidase conjugate) omitted.

absorption. A range of doubling dilutions of the primary antibody were used in both cases so that this test spanned the fluorescence end-point.

Discussion

The data presented here indicate that there is an antigen present on the placental trophoblast of the pig at the site of maternal-fetal contact and which is absent from the other tissues of the pig which were studied. The observations that it is located on the microvillous border of the trophectoderm, i.e. in direct contact with maternal tissue, and that it is present at all the stages of gestation studied, including preimplantation stages, suggest a role for this antigen in the materno-fetal interaction.

Although placentation in the pig is restricted to microvillous interdigitation (Steven, 1983), the trophectoderm, and later the trophoblast, must play a role in the transport of substances to the developing fetus. The fact that SN1/38 antiserum recognizes an antigen on pre-elongation (Day 9) as well as on post-elongation (> Day 12) blastocysts (Table 1) indicates that the antigen is not involved in the elongation process. SN1/38 did not react with uterine flushings indicating that it was not directed towards polypeptides released by preimplantation pig blastocysts (Godkin, Bazer, Lewis, Geisert & Roberts, 1982), although the concentration may be too low in flushings to be detectable. The absence of binding of SN1/38 to pregnant pig endometrium (Table 1) also suggests that it is not the purple protein (uteroferrin) thought to be responsible for iron transport from the mother to the fetus (Basha, Bazer & Roberts, 1979). Certainly the SN1/38 antigen does not appear to be secreted, at least in early pregnancy, because it is absent from serum and urine (Table 2). Because it was also absent from the para-aortic lymph nodes (Table 1) that drain the gravid uterus,

it appears that the antigen does not dissociate from the trophoctoderm or else it is not 'trapped' in the regional lymph nodes. This observation also suggests that it is not a trophoblast-lymphocyte cross-reactive antigen such as has been described for the human (Faulk & Hsi, 1983). A role for the SN1/38 antigen in attachment is possible in view of the fact that it appears before implantation and is maintained throughout a substantial period of pregnancy. It is also present on 'trophospheres' after 10 days in culture, suggesting that its synthesis is controlled by the blastocyst itself and is not regulated by a maternal factor. This would indicate that it is not a modulating antigen which induces tolerance by responding to maternal immunoglobulins directed towards it.

The epitope is species-specific (Table 1), although it is impossible with one monoclonal antibody to know whether the antigen itself is species-specific. Mouse and human trophoblast have been shown, by use of polyclonal antisera, to possess species-specific antigens (Searle, Billington, Whyte & Loke, 1981).

It is acknowledged that the trophoblast expresses antigenic products demonstrable in xenogeneic systems and which are tissue-specific (Searle & Jenkinson, 1978; Whyte & Loke, 1979). In man one of these antigens is probably the transferrin receptor (Booth & Booth, 1982). Another is the placental form of the alkaline phosphatase enzyme. Antibodies to placental alkaline phosphatase are a major component of antisera raised against human trophoblast plasma membranes (Kantor, Galbraith, Emerson & Galbraith, 1981). In the case of SN1/38, however, the epitope does not appear to be on pig placental alkaline phosphatase. Antiserum to human placental alkaline phosphatase inhibits the mixed lymphocyte culture (MLC) reaction (Kantor, Galbraith, Emerson & Galbraith, 1983) in a way similar to that induced by antiserum to HLA-D, although with different dose-response and time-course characteristics (Kantor *et al.*, 1983). Antisera to normal human serum and to transferrin had no effect on the MLC (Kantor *et al.*, 1983). McIntyre & Faulk (1979a, b) showed that trophoblast membranes, and antisera raised to them, blocked the allogeneically stimulated MLC reaction, but had no effect on mitogen-treated lymphocytes. These are in-vitro experiments, however, and there is no evidence that these observations have any analogous in-vivo correlate. These are also non-specific MLC experiments. In spite of these reservations, however, it is possible that substances on the trophoblast membrane could have an important role in the materno-fetal immunological interaction. The discovery of a trophoblast-specific antigen in the pig may further advance our understanding of pregnancy in this animal.

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