BIOLOGY OF REPRODUCTION **53,** 1500–1507 (1995)

Secretion of Biologically Active Interferon Tau by In Vitro-Derived Bovine Trophoblastic Tissue[1]

Miodrag Stojkovic,[3] Eckhard Wolf,[3] Mathias Büttner,[4] Ulrike Berg,[3]
Gilles Charpigny,[5] Andrea Schmitt,[3] and Gottfried Brem[2,6]

Lehrstuhl für Molekulare Tierzucht und Haustiergenetik[3] and Institut für Medizinische Mikrobiologie[4]
Ludwig-Maximilians-Universität, Munich, Germany
Station de Physiologie Animale,[5] INRA, Jouy-en-Josas, France
Institut für Tierzucht und Genetik,[6] Veterinärmedizinische Universität, Vienna, Austria

ABSTRACT

Secretion of interferon tau (IFNτ) by trophoblastic tissue has been shown to be the first embryonic signal for pregnancy recognition. Therefore we tried to derive biologically active trophoblastic tissue by in vitro techniques. Since conventional in vitro conditions for bovine embryo development were not sufficient for long-term culture, we tested more complex culture conditions, including Ménézo B2 or Buffalo rat liver (BRL) cell-conditioned medium, for their ability to support proliferation and IFNτ secretion by in vitro-derived trophoblastic tissue. IFNτ activity was determined by using a biological assay based on the inhibition of the cytopathic effect of vesicular stomatitis virus on Madin-Darby bovine kidney cells. When cultures of individual hatched blastocysts were started in 60-μl drops of BRL cell-conditioned medium, mean IFNτ secretion (antiviral units/ml/48 h) corresponded to 1200 on Day 11 and to 5000 on Day 13 ($p < 0.01$). To characterize trophoblast cell-specific secretions, the inner cell mass was removed from all embryos by microsurgery on Day 13. IFNτ secretion by trophoblastic tissue increased to mean levels of $> 10^5$ antiviral units/ml/48 h on Day 23, stayed high for about 1 wk, and then slowly declined to levels below 10^3 antiviral U/ml/48 h. The specificity of the cytoprotective effect of IFNτ was tested by Western blot analysis and by immunoneutralization with use of a polyclonal antiserum specific to IFNτ. Our results demonstrate that viable trophoblastic tissue can be maintained entirely in vitro and secretes high amounts of IFNτ.

INTRODUCTION

Early embryonic mortality, a major economic problem in cattle production [1], is most probably due to pathophysiological alterations in the biochemical network of embryo-maternal communication [2]. The first embryonic signal for pregnancy recognition in domestic ruminants is interferon tau (IFNτ) [3], which has high amino acid sequence homology with α-interferon [4–7] and was originally named trophoblastin [8], trophoblast protein-1 [9], or type I trophoblast interferon [10–13]. IFNτ is a major secretory protein produced by the trophectoderm cells of bovine conceptuses at about Day 15 and plays a very important role in establishment of pregnancy by its antiluteolytic effect (for review, see [14]). In addition, IFNτ has potent antiviral [15–18] and antiproliferative activity [19, 20].

A number of studies have demonstrated that trophoblastic tissue has an antiluteolytic effect. Transfer of ex vivo-derived trophoblastic vesicles to cyclic heifers resulted in a prolongation of the estrous cycle [21]. Cotransfer of in vivo-derived trophoblastic vesicles with frozen-thawed blastocysts [22] or embryos consisting of reduced cell numbers [23] increased pregnancy rate. In addition, a positive effect on embryo development of trophoblastic secretions was seen in coculture experiments of preimplantation-stage bovine embryos with trophoblastic vesicles [24, 25]. In view of these potential applications of trophoblastic cells or secretions, their production exclusively by in vitro techniques would be useful for basic studies on trophoblast cell physiology and for practical purposes as well. Since attempts using conventional systems for bovine embryo development have been unsuccessful at maintaining high levels of IFNτ secretion [26], we used more complex culture conditions—Ménézo B2 (B2) [27] and Buffalo rat liver (BRL) cell-conditioned medium [28]. B2 has been used successfully for short-term culture of ex vivo-derived trophoblastic vesicles [21]. BRL cell-conditioned medium is known to promote proliferation and to inhibit differentiation of mouse embryonic stem cells [28].

In this study, we investigated effects of stage of embryo development, types of culture conditions, presence or absence of feeder cells, and duration of culture on development and IFNτ secretion by bovine embryos produced in vitro or trophoblastic tissues that were maintained in vitro. Our experimental hypothesis was that prolonged viability of trophoblastic tissue and secretion of IFNτ may not—as previously hypothesized [26]—require a uterine environment but may be maintained by the complex culture systems described above.

Accepted August 2, 1995.
Received January 4, 1995.
[1]This study was supported by EC grant BIO2-CT92-0067 and by a grant from the Bayerische Forschungsstiftung.
[2]Correspondence: Prof. Dr. Dr. habil. Gottfried Brem, Institut für Tierzucht und Genetik, Veterinärmedizinische Universität Wien, Linke Bahngasse 11, A-1030 Vienna, Austria. FAX: 43-1-7149104.

MATERIALS AND METHODS

In Vitro Production (IVP) of Day 8 Bovine Blastocysts

IVP of bovine embryos was essentially carried out as previously described [29]. Briefly, ovaries from nonpregnant heifers and cows were obtained from the local slaughterhouse. Oocytes were aspirated from follicles with a diameter between 2 and 6 mm. Oocytes with a complete dense cumulus and a dark, evenly granulated cytoplasm were selected for in vitro maturation (IVM). The IVM medium was Hepes/carbonate-buffered (4.43 mM Hepes/33.9 mM sodium bicarbonate) tissue culture medium 199 (TCM 199) supplemented with 2 mM sodium pyruvate, 2.92 mM calcium lactate, 10 μg/ml FSH (FSH-p, Schering Pharmaceuticals, Kenilworth, NJ), 60 μg/ml gentamicin (Sigma, St. Louis, MO), and 10% (v/v) heat-inactivated serum from cows at estrous (ECS). Oocytes were matured for approximately 24 h at 39°C in an atmosphere of 5% CO_2 in air and maximum humidity. For in vitro fertilization, matured oocytes (30/400 μl) were maintained in a Tyrode's-Albumin-Lactate-Pyruvate (TALP) medium containing 6 mg/ml BSA, 10 μg/ml heparin, and frozen/thawed semen (10^6 spermatozoa/ml) that had been subjected to a swim-up procedure described by Parrish et al. [30]. Oocytes were maintained in this medium for 20 h under the conditions that had been used for IVM. Subsequently, oocytes were cultured together with attached granulosa cells from the cumulus oophorus in TCM 199 supplemented with 10% ECS at 39°C in a humidified atmosphere of 5% CO_2:5% O_2:90% N_2.

On Day 8 (fertilization = Day 0), embryos were evaluated morphologically for developmental stage and quality [31]. Only excellent and good blastocysts or hatched blastocysts were used for the culture experiments described below. For statistical analysis, blastocysts and expanded blastocysts as well as hatching and hatched blastocysts were pooled. The two groups are referred to as blastocysts and hatched blastocysts.

Culture Systems for Day 8 to Day 13 Embryos and > Day 13 Trophoblastic Tissue

On Day 8, blastocysts were transferred individually to 60-μl microdrops of various media under equilibrated paraffin oil (DAB 7160; Merck, Darmstadt, Germany). Embryos were distributed among treatment groups according to a completely randomized design created by the PLAN procedure (SAS Institute Inc., Cary, NC). The culture conditions were as follows: 1a) TCM 199 supplemented with 10% heat-inactivated ECS; 1b) Ménézo B2 (ref. 01001; BioMérieux SA, Marcy-l'Étoile, France) supplemented with 15% heat-inactivated fetal bovine serum (FBS; Biochrom, Berlin, Germany); 1c) Dulbecco's modified Eagle's medium supplemented with 60% BRL cell-conditioned Glasgow BHK 21 medium, 10^{-4} M 2-mercaptoethanol (Merck) and 15% FBS (GLCM); 1d) GLCM in the presence of mitotically inactivated (γ-irradiation; 10 min; 5000 rad) primary mouse embryonic fibroblast feeder cells (GLCM/F).

Culture was carried out at 39°C with 5% CO_2 (a and b) or at 37°C with 10% CO_2 (c and d) in humidified air.

Different serum supplementations (ECS vs. FBS) were used, since TCM 199 is supplemented with ECS in our routine bovine embryo production system, while all other media have been used only with FBS [21, 32]. Cultures in GLCM were maintained at 37°C and 10% CO_2 since these conditions have been found suitable for mouse embryonic stem cells.

On Day 11 and on Day 13, 30-μl samples of each drop were taken for quantification of IFNτ and replaced by the same amount of fresh medium. Samples were stored individually at −20°C until further analysis. From Day 13, after removal of the inner cell mass by microsurgery, 30-μl medium samples were taken from each drop every other day and replaced with fresh medium.

Estimation of Trophoblastic Cell Viability and Proliferation

To estimate the amount of trophoblastic cells, we calculated the area of trophoblastic cells after measuring the mean radius (r) of embryos, spherical trophoblastic vesicles, or attached trophoblastic tissue. Trophoblastic area was calculated as $4r^2\pi$ (spherical) or as $r^2\pi$ (attached). These calculations give only an approximate estimation of trophoblastic cell growth, since embryos and trophoblastic vesicles may collapse and re-expand. However, these estimations can—in contrast to more accurate methods—be done repeatedly for prolonged periods without detrimental effects to the trophoblastic cells. Individual embryos or trophoblastic structures were evaluated morphologically and measured simultaneously with changes of medium. Embryos and trophoblastic structures that appeared dark and shrunken and did not show cell proliferation were judged to be degenerated.

Biological Assay for IFNτ Bioactivity

IFNτ secretion was quantified by means of a bioassay based on the inhibition of the cytopathic effect of vesicular stomatitis virus (Indiana strain) on Madin-Darby bovine kidney (MDBK; ATCC CRL-6071) cells [33]. The NIH recombinant human IFNαII reference preparation (No. Gxa01–901–535, NIH-Research Reference Reagent Note No. 31, 1984) was included in each assay. The antiviral activity was shown to be IFN, as the effects of supernatant and appropriate control IFN preparations were blocked by specific anti-IFN sera.

Immunoneutralization of IFNτ

The following antisera with specific reactivity against different IFN preparations were used to demonstrate blocking of antiviral activity: 1) anti-recombinant bovine trophoblast

TABLE 1. Development of Day 8 in-vitro produced bovine embryos in different culture systems.

Day	Embryo condition	TCM 199 n	TCM 199 (%)	B2 n	B2 (%)	GLCM n	GLCM (%)	GLCM/F n	GLCM/F (%)
11	Spherical	—	—	42	(82)[a]	42	(84)[a]	30	(60)[b]
	Attached	—	—	—	—	2	(4)[a]	11	(22)[b]
	Degenerated	30	(100)[a]	9	(18)[b]	6	(12)[b]	9	(18)[b]
13	Spherical	—	—	28	(55)[a]	37	(74)[b]	21	(42)[a]
	Attached	—	—	1	(2)[a]	3	(6)[a]	20	(40)[b]
	Degenerated	—	—	22	(43)[a]	10	(20)[b]	9	(18)[b]

Proportions with different superscripts are significantly different between culture conditions (chi-square analysis: $p < 0.05$).

IFN (rbIFNτ; kindly provided by Dr. R.M. Roberts, University of Missouri, Columbia, MO), 2) anti-human IFNα (no. 853 593; Boehringer-Mannheim, Indianapolis, IN).

Both antisera as well as normal rabbit serum were used in dilutions from 1:80 to 1:5120. In IFN neutralization experiments, 400 antiviral U/50 μl of trophoblast tissue supernatants were tested in checkerboard titrations after incubation (1 h at 37°C) with the different sera.

Western Blot Analysis of Medium Samples for IFNτ

Samples of medium conditioned by Day 23–25 IVP trophoblastic tissue were diluted 1:2 with sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% [w/v] SDS, 10% [w/v] sucrose, and 5% [v/v] 2-mercaptoethanol); boiled (5 min); and electrophoresed on a 5% stacking/15% separating SDS-polyacrylamide gel. Separated proteins were transferred to polylidene difluoride (PVDF) membrane (Bio-Rad, Munich, Germany). Immunodetection of IFNτ was performed according to Amersham ECL (Amersham Buchler, Braunschweig, Germany) Western blot detection protocols. Briefly, the membrane was blocked in a solution of 5% BSA in PBS containing 0.1% Tween 20 (PBS-T) for 2 h, rinsed several times with PBS-T, and incubated in a 1:1000 dilution of rabbit anti-rbIFNτ serum in PBS-T at 37°C for 12 h. Bound IFNτ-specific antibodies were detected by means of peroxidase-conjugated goat-anti-rabbit antibodies (1:1000 in PBS-T; A9169, Sigma, Deisenhofen, Germany) and Amersham ECL detection reagents. As a control, a second blot was produced and treated identically except for the IFNτ-specific antiserum, which was replaced by the same dilution of a normal rabbit serum.

Statistical Analysis

Developmental rates of embryos cultured in different conditions were evaluated by chi-square analysis. Trophoblastic area and IFNτ production by Day 11 and Day 13 embryos were analyzed by means of a linear model, taking effects of stage of embryo development on Day 8, culture system, time in culture (= age) and all possible interactions between these factors into account. Data were transformed to natural logarithms before analysis to approximate normal distribution of values. The GLM procedure (SAS Institute Inc.) was used to calculate least squares means (LSMs), which were compared by Student's *t*-tests. The values presented are antilogarithms of LSMs of logarithmically transformed data. A univariate repeated-measures analysis of variance was used for the evaluation of growth and IFNτ secretion by > Day 13 trophoblastic tissue to take the correlations between measurements into account. The model fitted included culture condition as well as individual trophoblastic sample within culture condition as fixed effects, and time in culture as covariate as well as the interaction between time and culture condition. Normal distribution of residual errors was tested by the Shapiro-Wilk test. Homogeneity of variances of residual errors was tested by an *F* test.

RESULTS

Development of IVP Day 8 Bovine Embryos in Different Culture Systems

The numbers of blastocysts and hatched blastocysts randomly distributed among treatment groups were 10 and 20 (TCM 199), 13 and 38 (B2), 36 and 14 (GLCM), and 31 and 19 (GLCM/F), respectively. As outlined in Table 1, TCM 199 was not sufficient for culturing IVP blastocysts. On Day 11, all embryos were degenerated in this culture system. With B2 or GLCM, more than 80% of the embryos were alive on Day 11. The presence of feeder cells resulted in a significantly elevated proportion of attached embryos ($p < 0.05$). On Day 13, GLCM proved to be superior to B2 ($p < 0.05$), in which more than 40% of the embryos were degenerated. The survival rates of embryos in GLCM and GLCM/F were 80% and 82%, respectively. The effect of feeder cells on the attachment of embryos was more pronounced on Day 13 than on Day 11.

Since the proportions of blastocysts and hatched blastocysts were not homogeneous among treatment groups (chi-square analysis: $p < 0.05$), we used analysis of variance to estimate fixed effects of culture condition, stage of the embryo on Day 8 (blastocyst vs. hatched blastocyst), time in culture (= age), and all possible interactions between these factors on trophoblastic growth and IFNτ secretion. Logarithmic transformation reduced skewness of data from 1.89 to −0.06 (trophoblastic area) and from 5.67 to 0.27 (IFNτ secretion). The corresponding levels of kurtosis were 3.86 and −0.17, and 36.65 and −0.10. The results of analysis of variance are shown in Table 2. Error variances of transformed data did not significantly deviate from normal distribution and were homogeneous among experimental groups.

Although there was no significant difference in trophoblastic area or IFNτ production by embryos cultured in B2,

TABLE 2. Factors affecting trophoblastic growth and IFNτ secretion by Day 11 and Day 13 IVP bovine embryos.[a]

Parameter	Effect[#]	DF	Mean square	F
ln trophoblastic area	Medium	2	2.36	4.18*
	Embryo developmental stage	1	12.21	21.59***
	Age	1	7.71	13.63***
	Medium × age	2	2.02	3.57*
	Embryo developmental stage × age	1	0.76	1.35 n.s.
ln IFNτ secretion	Medium	2	8.92	6.07**
	Embryo developmental stage	1	11.47	7.80**
	Age	1	97.22	66.08***
	Medium × age	2	1.05	0.71 n.s.
	Embryo developmental stage × age	1	0.01	0.01 n.s.

[a]DF = degrees of freedom; F = F value; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; n.s. = not significant.
[#]Other interactions between the main effects were not significant and were therefore omitted from the final model.

GLCM, or GLCM/F on Day 11, GLCM was superior to B2 on Day 13 with respect to both parameters ($p < 0.01$). The presence or absence of feeder cells did not play a significant role for trophoblastic growth and IFNτ production (Fig. 1A). Embryos that had already hatched on Day 8 displayed greater trophoblastic growth (Day 11: $p < 0.05$; Day 13: $p < 0.001$) and IFNτ secretion (Day 11: $p < 0.05$; Day 13: $p = 0.07$) than did nonhatched blastocysts (Fig. 1B). There was a marked increase of trophoblastic area ($p < 0.01$ except for B2) and IFNτ secretion ($p < 0.01$) from Day 11 to Day 13. IFNτ secretion was significantly ($p < 0.01$) correlated with trophoblastic area under all culture conditions evaluated (Table 3).

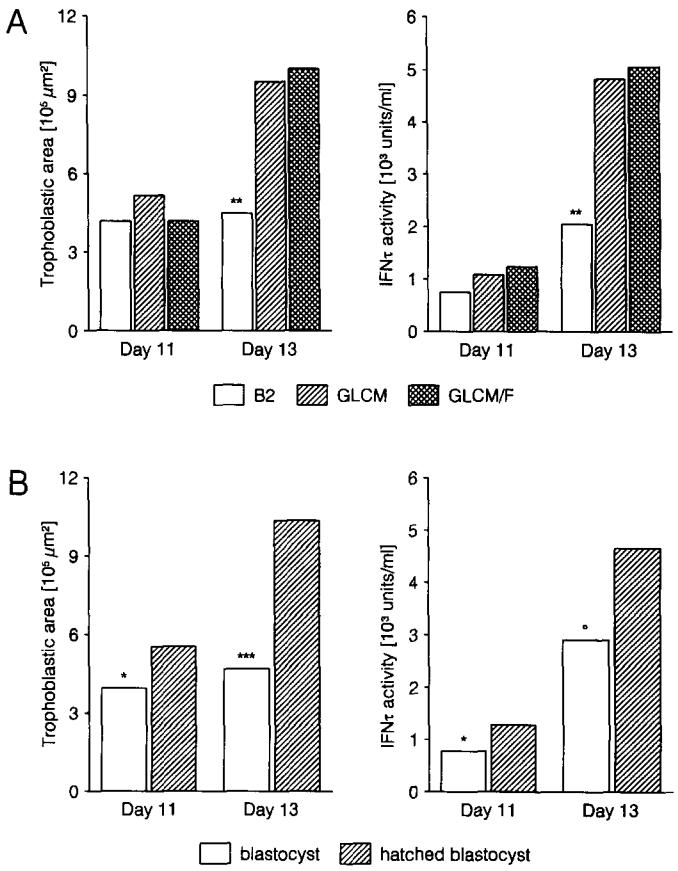

FIG. 1. Effects of culture system (**A**) and developmental stage of embryos on Day 8 (**B**) on trophoblastic area and IFNτ secretion by Day 11 and Day 13 IVP bovine blastocysts. Figure shows antilogarithms of LSMs of logarithmically transformed data. Antilogarithms of standard errors of LSMs ranged from 1.11 to 1.83. LSMs were compared by Student's *t*-tests. Significant differences are marked: $°p = 0.07$; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Growth and IFNτ Secretion by IVP Trophoblastic Tissue

On Day 13, the inner cell mass was microsurgically removed from all embryos, and culture of trophoblastic tissue from individual embryos was continued in the same culture system. Antilogarithms of means of logarithmically transformed data obtained for trophoblastic growth and IFNτ secretion are shown in Figure 2. There was an increase in trophoblastic area and IFNτ secretion from Day 15 to Day 23 in all culture systems. Media conditioned by feeder cells alone did not possess any antiviral activity (data not shown). Regression analysis showed that trophoblastic growth was significantly influenced by the effects of individual trophoblastic tissue within culture condition ($p < 0.001$), time in culture ($p < 0.001$), and the interaction time in culture × culture condition ($p < 0.01$), whereas the effect of culture condition reached the borderline of statistical significance ($p = 0.07$). IFNτ secretion was affected by individual trophoblastic tissue within culture condition ($p < 0.001$) and time in culture ($p < 0.001$). The statistical model used explained 84.4% and 84.9% of the total variation of data obtained for trophoblastic growth and IFNτ production, respectively. Distribution of residual errors did not significantly deviate from normal distribution. Error variances were homogeneous among experimental groups. Regression coefficients for time in culture on logarithmically transformed data obtained for trophoblastic growth in GLCM (0.26) and GLCM/F (0.30) were significantly ($p <$

TABLE 3. Linear correlations between logarithmically transformed values of trophoblastic area and IFNτ production by Day 11 to Day 13 in-vitro produced bovine embryos.

Medium	n	Pearson correlation coefficient	*p*
B2	71	0.315	0.0075
GLCM	82	0.340	0.0018
GLCM/F	82	0.451	0.0001
Overall	235	0.391	0.0001

The CORR procedure (SAS Institute Inc., Cary, NC) was used for linear correlation analysis of data obtained for trophoblastic growth and IFNτ secretion by Day 11 and Day 13 IVP bovine embryos maintained under different culture conditions; Pearson correlation coefficients were greatest after logarithmic transformation of data.

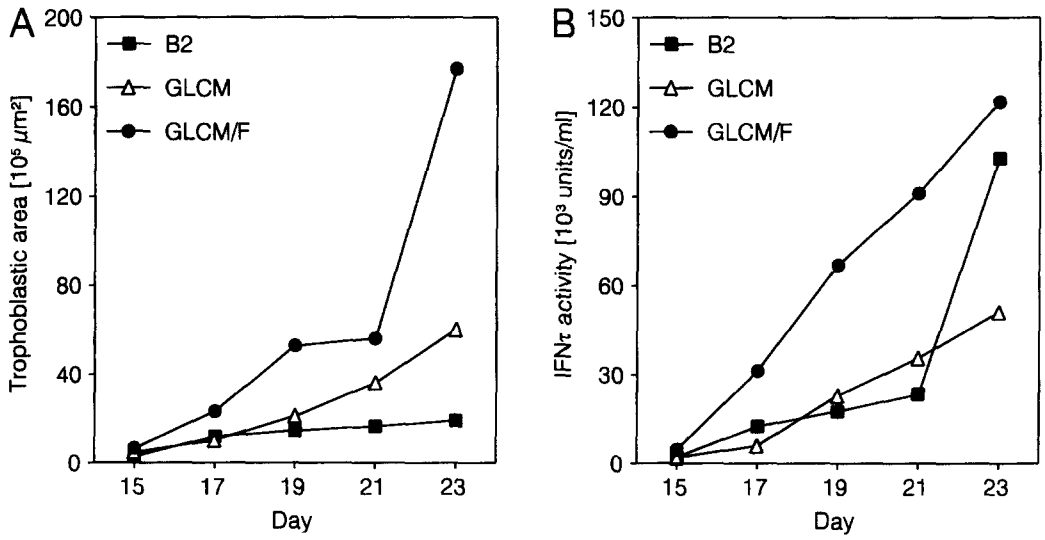

FIG. 2. Effects of different culture systems and time in culture on trophoblastic area (A) and IFNτ secretion (B) by IVP trophoblastic tissue. Figure shows antilogarithms of means of logarithmically transformed data. For trophoblastic area, antilogarithms of standard deviations ranged between 2.7 and 5.8 (B2), between 2.8 and 3.9 (GLCM), and between 1.7 and 4.2 (GLCM/F). For IFNτ secretion, antilogarithms of standard deviations ranged from 2.7 to 5.5 (B2), from 3.1 to 6.3 (GLCM), and from 1.9 to 5.4 (GLCM/F).

0.01) greater than in B2 (0.11). The corresponding regression coefficients for IFNτ secretion were not significantly different between culture systems (B2: 0.32; GLCM: 0.34; GLCM/F: 0.32).

Growth behavior of trophoblastic tissue was different in various culture conditions. In B2, trophoblastic tissue rarely (13%) attached to the culture dish but formed trophoblastic vesicles. Sometimes one large trophoblastic vesicle was obtained from one embryo, but also aggregates of up to 16 small vesicles were seen. In GLCM/F, almost all trophoblastic tissue attached to the feeder cells, forming large sheets of trophoblastic cells (diameter up to 7.5 mm). In GLCM, trophoblastic tissue from about 50% of the embryos attached within the experimental period, whereas the remaining trophoblastic pieces displayed spherical growth.

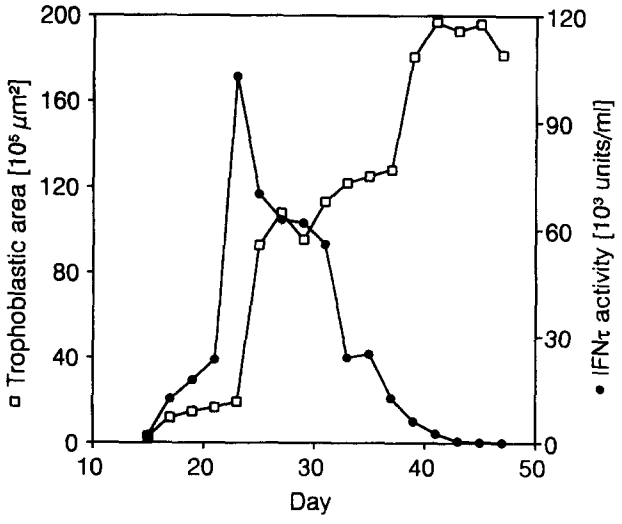

FIG. 3. Growth and IFNτ secretion by IVP trophoblastic vesicles during long-term culture in B2. Figure shows antilogarithms of means of logarithmically transformed data. Antilogarithms of standard deviations ranged from 1.5 to 6.2 (trophoblastic area), and from 1.7 to 6.1 (IFNτ secretion).

On Day 23, trophoblastic tissue produced in GLCM or GLCM/F was frozen, but trophoblastic tissue maintained in B2 was further examined until Day 47. A few trophoblastic vesicles continued to develop until Day 39 and then started to degenerate. IFNτ secretion by trophoblastic tissue in B2 reached a maximum on Day 23 and then slowly declined to reach levels below 10^3 antiviral U/ml/48 h on Day 43 (Fig. 3). Representative IVP trophoblastic vesicles are shown in Figure 4.

Western Blot Analysis of IVP Trophoblastic Secretions

Western blot analysis of medium samples conditioned by Day 23–25 IVP trophoblastic tissue revealed bIFNτ-specific proteins between 21.5 and 23 kDa (Fig. 5). The intensity of bands was consistent with the biological activity. Fresh GLCM and GLCM conditioned by feeder cells did not show a reaction with bIFNτ-specific antibodies. There was no bIFNτ-specific signal in B2 supplemented with 10% normal rabbit serum. The IFNτ-specific bands were absent in a corresponding experiment in which the anti-IFNτ serum was replaced by normal rabbit serum (data not shown).

Immunoneutralization of IFNτ Produced by IVP Trophoblastic Tissue

The cytoprotective effect of IVP trophoblastic secretion (400 antiviral U/50 μl) was completely neutralized by a 1:80

dilution of an antiserum to rbIFNτ but not by an antiserum to human IFNα or by normal rabbit serum (data not shown).

DISCUSSION

The aim of our study was to establish culture conditions in which IFNτ-secreting trophoblastic tissue can be generated and maintained in vitro. When IVP blastocysts were cultured in TCM 199 supplemented with 10% FBS, they secreted only small amounts of IFNτ and degenerated [26]. This is consistent with our findings that TCM 199 is not sufficient to maintain later-stage embryos in culture. When blastocysts were transiently transferred to the uteri of synchronized cows, recovered 4 days later, and then cultured, they survived in vitro and started to produce high amounts of IFNτ [26]. The authors concluded that blastocyst viability and sustained IFNτ secretion may require some exposure to a uterine environment. Our study was based on the assumption that later-stage blastocysts may require more complex culture conditions than TCM 199. Therefore we chose Ménézo B2, which has already been used for the culture of ex vivo-derived bovine trophoblastic tissue [21], and BRL cell-conditioned medium, which was used to maintain mouse embryonic stem cells in culture [28]. We have shown that BRL cell-conditioned medium markedly stimulates the proliferation of mouse embryonic stem cells but inhibits their differentiation less efficiently than the purified cytokines leukemia inhibitory factor (LIF), ciliary neurotrophic factor, and oncostatin M [32, 34]. BRL cells are known to produce a plethora of growth factors including LIF [28], insulin-like growth factor-II [35], and transforming growth factor β [36]. BRL cell-conditioned medium has successfully been used for in vitro production of bovine embryos [37]. Addition of purified LIF to the medium has been shown to increase hatching rate and trophectoderm growth of mouse preimplantation embryos in culture. Similar results were obtained with ovine embryos (for review, see [38]).

High production of IFNτ by IVP trophoblastic tissue in both B2 and BRL cell-conditioned media suggests that a stimulation of trophoblastic cells by a uterine environment is not absolutely necessary.

In addition to the culture condition, the developmental stage of the embryo and the presence or absence of feeder cells significantly influenced trophoblastic growth and IFNτ secretion.

When embryo cultures were started with hatched Day 8 blastocysts, trophoblastic growth and IFNτ secretion was significantly greater than when nonhatched Day 8 blastocysts were used as starting material. This is consistent with findings by Hernandez-Ledezma et al. [39] that IFNτ secretion is positively correlated with embryo quality or developmental stage.

Use of feeder cells together with BRL cell-conditioned medium significantly increased the proportion of attached

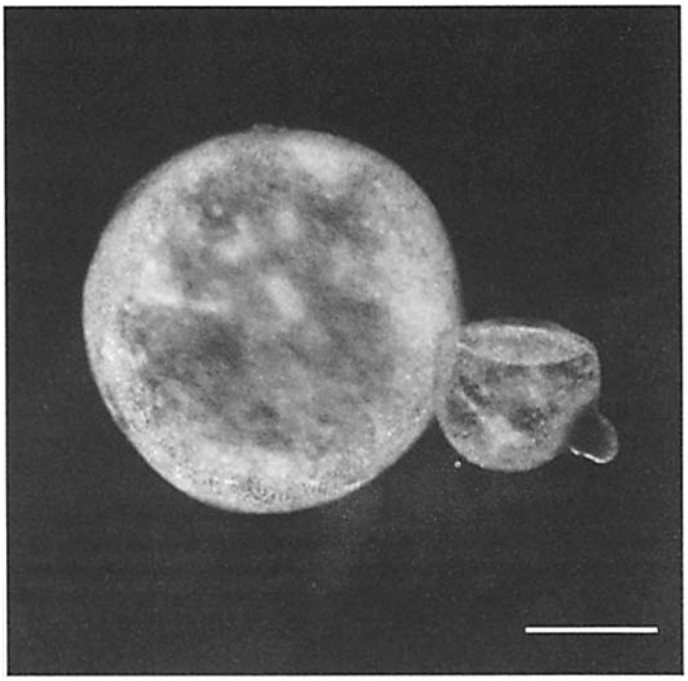

FIG. 4. Representative Day 23 in vitro-derived trophoblastic vesicles maintained in B2. Bar = 500 μm.

embryos on Day 11 and Day 13 but did not affect embryonic IFNτ secretion. After removal of the inner cell mass on Day 13, trophoblastic growth and IFNτ production were greatest in the presence of feeder cells. Feeder cells may act unspecifically by providing a mechanically suitable substrate for other cells or by detoxification of medium. On the other hand, they may secrete specific growth factors that stimulate cell proliferation and protein synthesis [40].

Since cryopreservation would greatly facilitate the prac-

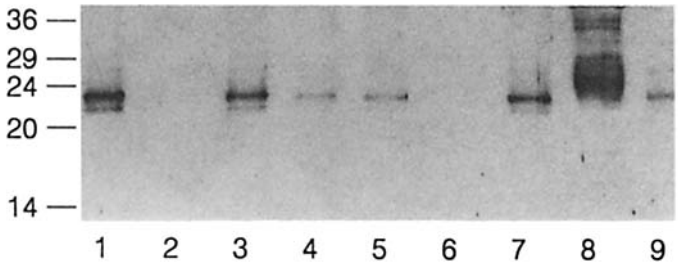

FIG. 5. Western blot analysis of IVP trophoblastic secretions using polyclonal rabbit antiserum against rbIFNτ. Lanes 1, 3–5, 7, and 9: medium samples conditioned by Day 23–25 IVP trophoblastic tissue. Antiviral activity (U/ml) was $> 2 \times 10^5$ (lanes 1, 3, and 7), 1×10^5 (lanes 5 and 9), and 0.5×10^5 (lane 4). Controls were GLCM (lane 2), GLCM/F (lane 6), and B2 supplemented with 10% normal rabbit serum (lane 8). Signal in lane 8 was also present on corresponding blot that had been incubated with normal rabbit serum instead of the anti-rbIFNτ serum (data not shown) and is most likely due to binding of secondary antibody (goat anti-rabbit) to rabbit immunoglobulin G light chains. Relative mobility of molecular size markers is indicated in kDa at left of blot.

tical use of IVP trophoblastic tissue for cotransfer with embryos, we stopped culture experiments in GLCM on Day 23 and froze the trophoblastic tissue. Our preliminary results indicate that IVP trophoblastic tissue may survive cryopreservation and secrete high levels of IFNτ after thawing; however, the viability is markedly influenced by the amount of frozen tissue [41]. Therefore, only large pieces (> 500 μm in diameter) of trophoblastic tissue should be used for cryopreservation.

Long-term cultures in B2 indicated that IVP trophoblastic vesicles may survive until Day 47 in culture. IFNτ secretion reached a maximum on Day 23 and then slowly declined to reach levels below 10^3 antiviral U/ml/48 h on Day 43. In vivo, IFNτ synthesis is maximum between Days 16 and 19 but can be detected from Day 12 and until at least Day 38 [42, 43] of pregnancy. The phenomenon of a later increase of IFNτ secretion to maximum levels in vitro needs to be clarified. Since we removed the inner cell mass on Day 13, this increase could be due to lack of factors produced by the inner cell mass that might down-regulate IFNτ secretion. However, the level and timing of IFNτ secretion was very similar in intact nuclear transfer embryos that were cultured for up to 24 days [44]. Thus differences between the timing of trophoblastic secretions in vivo and in vitro are more likely to be due to uterine factors and/or factors provided by the culture medium.

The specificity of antiviral activity produced by IVP trophoblastic cells was shown by Western blot analysis using an antiserum raised against rbIFNτ and by immunoneutralization tests. Western blot analysis revealed bIFNτ immunoreactive proteins in the range of 21.5–23 kDa, which is consistent with findings by other investigators [18, 42, 45, 46]. Furthermore, the antiviral activity could be neutralized by an antiserum to rbIFNτ but not by an antiserum to human IFNα or normal rabbit serum. This is consistent with experiments involving immunoneutralization of rbIFNτ [47].

In summary, our results demonstrate that trophoblastic tissue secreting high amounts of biologically active IFNτ can be generated and maintained in vitro. Transfer and cotransfer experiments are in progress to investigate whether IVP trophoblastic tissue can inhibit luteolysis and support the in vivo development of frozen-thawed embryos or embryos with reduced cell numbers. Six recipients each received an IVP trophoblastic vesicle and an in vivo-flushed embryo. All recipients became pregnant. These preliminary results indicate that cotransfer of IVP trophoblastic tissue at least does not disturb the establishment of a pregnancy.

ACKNOWLEDGMENTS

We are grateful to Dr. R.M. Roberts for providing us with antiserum to rbIFNτ and to the Bayerische Klonierungsforschungs GmbH & Co KG for a continuous supply of embryos.

REFERENCES

1. Gerrits RJ, Blosser TH, Purchase HG, Terrell CE, Warwick EJ. Economics of improving reproductive efficiency in farm animals. In: Hawk HW (ed.), Beltsville Symposia in Agricultural Research. New York: John Wiley & Sons; 1979: 413–421.
2. Roberts RM, Schalue-Francis T, Francis H, Keisler D. Maternal recognition of pregnancy and embryonic loss. Theriogenology 1990; 33:175–183.
3. Roberts RM, Cross JC, Leaman DW. Interferons as hormones of pregnancy. Endocr Rev 1992; 13:432–452.
4. Stewart HJ, McCann SHE, Barker PJ, Lee KE, Lamming GE, Flint APF. Interferon sequence homology and receptor binding activity of ovine trophoblast antiluteolytic protein. J Endocrinol 1987; 115:R13-R15.
5. Imakawa K, Anthony RV, Kazemi M, Marotti KR, Polites GH, Roberts RM. Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophectoderm. Nature 1987; 330:377–379.
6. Charpigny G, Reinaud P, Huet J, Guillomot M, Charlier M, Pernollet J, Martal J. High homology between a trophoblastic protein (trophoblastin) isolated from ovine embryo and α-interferons. FEBS Lett 1988; 228:12–16.
7. Imakawa K, Hansen TR, Malathy P, Anthony RV, Polites GH, Marotti KR, Roberts RM. Molecular cloning and characterization of complementary deoxyribonucleic acids corresponding to bovine trophoblast protein-1: a comparison with ovine trophoblast protein-1 and bovine interferon-αII. Mol Endocrinol 1989; 3:127–139.
8. Martal J, Lacroix M-C, Loudes C, Saunier M, Wintenberger-Torrès S. Trophoblastin, an antiluteolytic protein present in early pregnancy in sheep. J Reprod Fertil 1979; 56:63–73.
9. Godkin JD, Bazer FW, Roberts RM. Ovine trophoblast protein 1, an early secreted blastocyst protein, binds specifically to uterine endometrium and affects protein synthesis. Endocrinology 1984; 114:120–130.
10. Capon DJ, Shepard MH, Goeddel DV. Two distinct families of human and bovine interferon-α genes are coordinately expressed and encode functional polypeptides. Mol Cell Biol 1985; 5:768–779.
11. Hauptmann R, Swetly P. A novel class of human type I interferons. Nucleic Acids Res 1985; 13:4739–4749.
12. Roberts RM, Cross JC, Leaman DW. Unique features of the trophoblast interferons. Pharmacol Ther 1991; 51:329–345.
13. Bazer FW. Mediators of maternal recognition of pregnancy in mammals. Proc Soc Exp Biol Med 1992; 199:373–384.
14. Bazer FW, Ott TL, Spencer TE. Pregnancy recognition in ruminants, pigs and horses: signals from the trophoblast. Theriogenology 1994; 41:79–94.
15. Betteridge KJ, Derbyshire JB, Rorie RW, Scodras JM, Johnson WH. Interferon-like activity released by bovine embryos and trophoblastic tissue in vitro. J Reprod Fertil 1988; (abstract series 1):21 (abstract 31).
16. Godkin JD, Lifsey BJ, Fujii DK, Baumbach GA. Bovine trophoblast protein-1: purification, antibody production, uterine cell interaction and antiviral activity. Biol Reprod 1988; 38(suppl 1):79 (abstract 91).
17. Pontzer CH, Torres BA, Vallet JL, Bazer FW, Johnson HM. Antiviral activity of the pregnancy recognition hormone ovine trophoblast protein-1. Biochem Biophys Res Commun 1988; 152:801–807.
18. Plante C, Hansen PJ, Thatcher WW, Johnson JW, Pollard JW, Mirando MA, Bazer FW. Purification of bovine trophoblast protein-1 complex and quantification of its microheterogeneous variants as affected by culture conditions. J Reprod Immunol 1990; 18:271–291.
19. Pontzer CH, Bazer FW, Johnson HM. Antiproliferative activity of a pregnancy recognition hormone, ovine trophoblast protein-1. Cancer Res 1991; 51:5304–5307.
20. Skopets B, Li J, Thatcher WW, Roberts RM, Hansen PJ. Inhibition of lymphocyte proliferation by bovine trophoblast protein-1 (type I trophoblast interferon) and bovine interferon αI1. Vet Immunol Immunopathol 1992; 34:81–96.
21. Heyman Y, Camous S, Fèvre J, Méziou W, Martal J. Maintenance of the corpus luteum after uterine transfer of trophoblastic vesicles to cyclic cows and ewes. J Reprod Fertil 1984; 70:533–540.
22. Heyman Y, Chesné P, Chupin D, Ménézo Y. Improvement of survival rate of frozen cattle blastocysts after transfer with trophoblastic vesicles. Theriogenology 1987; 27:477–484.
23. Loskutoff NM, Johnson WH, Betteridge KJ. The developmental competence of bovine embryos with reduced cell numbers. Theriogenology 1993; 39:95–107.
24. Heyman Y, Ménézo Y, Chesné P, Camous S, Garnier V. In vitro cleavage of bovine and ovine early embryos: improved development using coculture with trophoblastic vesicles. Theriogenology 1987; 27:59–68.
25. Pool SH, Rorie RW, Pendleton RJ, Menino AR, Godke RA. Culture of early-stage bovine embryos inside Day-13 and Day-14 precultured trophoblastic vesicles. Ann NY Acad Sci 1988; 541:407–418.
26. Hernandez-Ledezma J, Sikes JD, Murphy CN, Watson AJ, Schultz GA, Roberts RM. Ex-

pression of bovine trophoblast interferon in conceptuses derived by in vitro techniques. Biol Reprod 1992; 47:374–380.
27. Ménézo Y, Testart J, Perrone D. Serum is not necessary in human in vitro fertilization, early embryo culture and transfer. Fertil Steril 1984; 42:750–755.
28. Smith AG, Hooper ML. Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. Dev Biol 1987; 121:1–9.
29. Berg U, Brem G. In vitro production of bovine blastocysts by in vitro maturation and fertilization of oocytes and subsequent in vitro culture. Zuchthygiene (Berl) 1989; 24:134–139.
30. Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Critser ES, Eyestone WH, First NL. Bovine in vitro fertilization with frozen-thawed semen. Theriogenology 1986; 25:591–600.
31. Lindner GM, Wright RWJ. Bovine embryo morphology and evaluation. Theriogenology 1983; 20:407–416.
32. Wolf E, Kramer R, Polejaeva I, Thoenen H, Brem G. Efficient generation of chimeric mice using embryonic stem cells after long-term culture in the presence of ciliary neurotrophic factor. Transgenic Res 1994; 3:152–158.
33. Rubinstein S, Familletti PC, Pestka S. Convenient assay for interferons. J Virol 1981; 37:755–758.
34. Wolf E, Kramer R, Thoenen H, Brem G. Use of oncostatin M for mouse embryonic stem cell culture. Theriogenology 1994; 41:336 (abstract).
35. Marquardt H, Todaro GJ, Henderson LE, Oroszlan S. Purification and primary structure of a polypeptide with multiplication stimulating activity from rat liver cell cultures. J Biol Chem 1981; 256:6859–6865.
36. Massagué J, Kelly B, Mottola C. Stimulation by insulin-like growth factors is required for cellular transformation by type β transforming growth factor. J Biol Chem 1985; 260:4551–4554.
37. Vansteenbrugge A, Van Langendonckt A, Scutenaire C, Massip A, Dessy F. In vitro development of bovine embryos in buffalo rat liver- or bovine oviduct-conditioned medium. Theriogenology 1994; 42:931–940.
38. Fry RC. The effect of leukaemia inhibitory factor (LIF) on embryogenesis. Reprod Fertil Dev 1992; 4:449–458.
39. Hernandez-Ledezma J, Mathialagan N, Villanueva C, Sikes JD, Roberts RM. Expression of bovine trophoblast interferons by in vitro-derived blastocysts is correlated with their morphological quality and stage of development. Mol Reprod Dev 1993; 36:1–6.
40. Robertson EJ. Embryo-derived stem cell lines. In: Robertson EJ (ed.), Teratocarcinomas and Embryonic Stem Cells. A Practical Approach. Oxford, Washington DC: IRL Press; 1987: 71–112.
41. Stojkovic M, Willemsen D, Büttner M, Wolf E. Development and interferon production by frozen-thawed in vitro derived bovine trophoblastic vesicles. Theriogenology 1995; 43:326 (abstract).
42. Bartol FF, Roberts RM, Bazer FW, Lewis GS, Godkin JD, Thatcher WW. Characterization of proteins produced in vitro by periattachment bovine conceptuses. Biol Reprod 1985; 32:681–693.
43. Godkin JD, Lifsey BJ, Gillespie BE. Characterization of bovine conceptus proteins produced during the peri- and postattachment periods of early pregnancy. Biol Reprod 1988; 38:703–711.
44. Zakhartchenko V, Büttner M, Stojkovic M, Palma G, Wolf E, Brem G. Viability and interferon production by cloned bovine embryos during long-term culture in vitro. J Reprod Fertil 1995; (abstract series 15): 73 (abstract 215).
45. Helmer SD, Hansen PJ, Anthony RV, Thatcher WW, Bazer FW, Roberts RM. Identification of bovine trophoblast protein-1, a secretory protein immunologically related to ovine trophoblast protein-1. J Reprod Fertil 1987; 79:83–91.
46. Lifsey BJ, Baumbach GA, Godkin JD. Isolation, characterization and immunocytochemical localization of bovine trophoblast protein-1. Biol Reprod 1989; 40:343–352.
47. Klemann SW, Li J, Imakawa K, Cross JC, Francis H, Roberts RM. The production, purification, and bioactivity of recombinant bovine trophoblast protein-1 (bovine trophoblast interferon). Mol Endocrinol 1990; 4:1506–1514.