[CANCER RESEARCH 51, 5304–5307, October 1, 1991]

Antiproliferative Activity of a Pregnancy Recognition Hormone, Ovine Trophoblast Protein-1[1]

Carol H. Pontzer, Fuller W. Bazer, and Howard M. Johnson

Departments of Microbiology and Cell Science [C. H. P., H. M. J.] and Animal Science [F. W. B.], University of Florida, Gainesville, Florida 32611

ABSTRACT

Ovine trophoblast protein-1 (oTP-1) is the α-interferon (IFNα) variant, secreted by conceptuses and referred to as type I trophoblast interferon, that is responsible for maternal recognition of pregnancy in sheep. We have previously shown that oTP-1 is as potent an antiviral agent as any known IFN. IFNs also possess anticellular activity and are, in fact, used in cancer therapy and have been found to be effective in the treatment of cancers such as myelogenous and hairy cell leukemias. A significant problem with the currently used IFNs is the undesirable side effect of toxicity at high concentrations. In this study, we examined the anticellular activity and toxicity of oTP-1. It inhibited proliferation but did not exhibit toxicity at high concentrations, unlike known IFNαs. In an anticellular assay using colony formation of both the human amnionic line, WISH, and the bovine epithelial line, MDBK, oTP-1 inhibited both colony size and number. oTP-1 was as effective as human and bovine IFNαs on human and bovine cells, respectively; thus, it displays potent cross-species activity. Its activity was dose dependent, and inhibition of proliferation could be observed at concentrations as low as 1 unit/ml. Concentrations as high as 50,000 units/ml stopped proliferation, while viability was not impaired. Cell cycle analysis revealed an increased proportion of cells in S phase and a corresponding decreased proportion of cells in G_2/M after 48 h of oTP-1 treatment. Therefore, oTP-1 appears to inhibit progress of cells through S phase. oTP-1 antiproliferative effects can be observed as early as 12 h after after the initiation of culture and are maintained through 6 days. Thus, oTP-1 exhibits potent anticellular activity without toxicity across species and may have therapeutic potential as an antitumor agent without the toxic effects generally associated with IFNs.

INTRODUCTION

Conceptuses of sheep secrete a protein called oTP-1[2] since it is the first major protein secreted by the membranes (trophoblast) of the conceptus. oTP-1 was initially identified as the major conceptus secretory protein responsible for inhibition of pulsatile secretion of prostaglandin $F_{2\alpha}$ by the uterus which allows for morphological and functional maintenance of the corpus luteum (1). Recently, the complementary DNA for oTP-1 was expressed, and surprisingly, it exhibited 45–55% homology with various IFNαs from human, mouse, rat, and pig and 70.3% homology with bovine $IFN\alpha_{II}$ (2). Thus, oTP-1 appeared to be a novel IFNα, currently called type I trophoblast interferon. This was confirmed by receptor competition and the demonstration by us that purified oTP-1 was as potent as any known IFNα with specific activity of $2–3 \times 10^8$ antiviral units/mg protein (3, 4). The prodigious secretion of oTP-1 by the conceptus is unique for any known "natural" IFN in that a single Day 16 sheep conceptus secretes 200 μg (40,000,000 units) or more of IFN during a 30-h period. Furthermore, a synthetic gene for oTP-1 has been produced and the protein expressed (5). There is evidence that humans also produce a pregnancy-related IFN, and thus, the human placenta may be a practical source of a human counterpart of oTP-1 (6). This would suggest that natural IFNs could be produced under conditions in which they could be used to treat diseases such as cancer. Furthermore, oTP-1-type IFNs lack the toxicity that is normally associated with high doses of IFNs. While the antiviral and antileuteolytic properties of this unusual IFN have been examined, its potential antiproliferative capacity has not been exploited. In this study, we assessed the antiproliferative potency, cross-species activity, and lack of toxicity of oTP-1.

MATERIALS AND METHODS

Reagents. Conceptuses were collected from Day 16 (Day 0, first day of estrus) pregnant sheep, cultured *in vitro* in an HMEM, and oTP-1 was purified from conceptus culture medium as described previously (4). oTP-1 was homogeneous as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein determinations were performed using the bicinchoninic assay (Pierce Chemical Co., Rockford, IL). Recombinant bovine IFNα (rBoIFNα) was kindly supplied by Genentech, Inc. (South San Francisco, CA) and Ciba-Geigy (Basel, Switzerland). A reference preparation of recombinant human IFNα (rHuIFNα) was supplied by the National Institutes of Health, and commercial rHuIFNα was purchased from Lee Biomolecular (San Diego, CA). All tissue culture media, sera, and IFNs used in this study were negative for endotoxin, as determined by assay with *Limulus* amebocyte lysate (Associates of Cape Cod, Woods Hole, MA) at a sensitivity of 0.07 ng/ml.

Antiviral Assay. Activity is expressed in terms of antiviral units/ml as assessed in a standard cytopathic effect assay (7). Briefly, dilutions of oTP-1 or the various IFNs were incubated with MDBK cells for 16–18 h at 37°C. Following incubation, inhibition of viral replication was determined in a cytopathic effect assay using vesicular stomatitis virus as challenge. One antiviral unit caused a 50% reduction in destruction of the monolayer.

Colony Inhibition Assay. Anticellular activity was examined using a modification of a colony inhibition assay (8). Human amnion (WISH) or MDBK cells were plated at low cell densities to form colonies originating from single cells. Cells were cultured at 200 or 400 cells/well in 24-well plates in HMEM supplemented with 2% FBS and essential and nonessential amino acids. Various dilutions of interferons were added to triplicate wells, and the plates were incubated for 8 days to allow colony formation. Colonies were visualized after staining with crystal violet and were then counted.

Cell Cycle Analysis. MDBK cells were synchronized in G_0/G_1 phase by culturing confluent monolayers with HMEM containing 0.5% "spent" media for an additional 7 days. WISH cells were used without being synchronized. For examination of oTP-1 activity, cells were replated at 2.5×10^5 cells/well in HMEM with 10% FBS in 6-well plates. Various dilutions of IFNs alone or in combination with peptides were added to achieve a final volume of 1 ml. Plates were incubated at 37°C in 5% CO_2 for 12, 15, 18, 24, or 48 h. Following incubation, cells were collected by trypsinization and washed. Cell counts were performed with a hemocytometer, and cell viability was assessed by trypan blue dye exclusion. For propidium iodide staining, the cell pellet was blotted to dryness and 250 μl nuclear staining solution (5 mg propidium iodide, 0.03 ml Nonidet P-40, and 0.1 g sodium citrate in 100 ml distilled H_2O) was added to each tube and incubated at room temper-

Received 2/6/91; accepted 7/24/91.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This study was supported by NIH Grants HD 26006 and CA 38587 to H. M. J. This paper is published as University of Florida Agricultural Experiment Station journal series 1201790.

[2] The abbreviations used are: oTP-1, ovine trophoblast protein-1 or type I trophoblast interferon; IFNα, α-interferon; rBoIFNα, recombinant bovine α-interferon; rHuIFNα, recombinant human α-interferon; HMEM, modified minimal essential medium; MDBK, Madin-Darby bovine kidney; FBS, fetal bovine serum; LSD, least significant differences.

ature. After 10 min, 250 μl RNase (500 units/ml in 1.12% sodium citrate) was added per tube and incubated an additional 20 min. Nuclei were filtered through 44-μm mesh and analyzed on a FACStar (Becton Dickinson, Mountain View, CA) using the DNA2.0 software.

RESULTS

oTP-1 inhibits colony formation by the human cell line WISH cultured at low cell densities. At doses of 100 and 1000 antiviral units/ml, oTP-1 decreased the number (Table 1), as well as size, of colonies (Fig. 1). The anticellular effect of oTP-1 was comparable to that of equivalent doses of rHuIFNα on the human cell line. Therefore, oTP-1 is a potent anticellular agent with activity across species. Colony formation by the bovine cell line MDBK could also be inhibited by oTP-1 (Table 2). Direct colony counts showed that the reduction in number of visible colonies was significant ($P < 0.01$) at 1000 units/ml of both oTP-1 and rBoIFNα. This confirms the anticellular activity of oTP-1 across species.

Dose-response studies of inhibition of proliferation by oTP-1 indicated that oTP-1 is efficacious over a broad range of concentrations (Fig. 2). At 3, 4, and 5 days of oTP-1 treatment, doses as low at 1 antiviral unit/ml produced significant reduction in proliferation of MDBK cells. Increasing concentrations further reduced proliferation. This potency was comparable to that previously observed for the IFNαs in other systems such as on the sensitive Daudi cell line (9).

Cell cycle analysis was used to examine the control point within the cell cycle for oTP-1 activity. Human cells were exposed to oTP-1 or rHuIFNα for 48 h, and the relative changes in the percentage of cells in each phase of the cycle was determined. Table 3 shows the increased percentage of cells in S phase and corresponding decreased percentage of cells in G_2/M produced by oTP-1 treatment. oTP-1 was as effective in retarding progression through the cell cycle as is rHuIFNα on the human line.

The cell cycle of the bovine cells was also perturbed by oTP-1 treatment. Inhibition of the transition from G_0/G_1 into S can be seen in time-course studies (Fig. 3). Synchronized MDBK cells were replated in fresh media with or without oTP-1. Twelve h after initiation of culture the majority of both control and oTP-1-treated cells remained in the first peak corresponding to G_0/G_1. By 15 h of treatment, control cells began to progress into S phase (intermediate area), while few S phase

Table 1 *Anticellular activity of oTP-1 on a human cell line*

Results are expressed as the number of colonies ± SD from three replicate experiments. WISH cells were plated at either 200 cells/well (Experiments 1 and 2) or 400 cells/well (Experiment 3) in HMEM with 2% FBS with 10, 100, or 1000 antiviral units/ml of IFNs and allowed to incubate undisturbed for 8 days. Colonies were visualized after staining with crystal violet.

Treatment	Experiment No. 1	2	3
Media	24 ± 3	32 ± 4	82 ± 5
oTP-1 (antiviral units/ml)			
10	26 ± 6	28 ± 7	74 ± 2[a]
100	23 ± 3	23 ± 1[a]	66 ± 5[a]
1000	4 ± 2[a]	22 ± 4[a]	58 ± 2[a]
rHuIFNα (antiviral units/ml)			
10	20 ± 2	25 ± 2	71 ± 2[a]
100	24 ± 3	22 ± 1[a]	64 ± 7[a]
1000	23 ± 4	21 ± 2[a]	60 ± 9[a]

[a] $P < 0.05$ as determined by analysis of variance followed by LSD.

Table 2 *Anticellular activity of oTP-1 on a bovine cell line*

Results are expressed as the number of colonies ± SD from three replicate experiments. MDBK cells were plated at 200 cells/well in HMEM with 2% FBS with 10, 100, or 1000 antiviral units/ml of IFNs and allowed to incubate undisturbed for 8 days. Colonies were visualized after staining with crystal violet.

Treatment	Experiment No. 1	2	3
Media	24 ± 6	27 ± 4	25 ± 4
oTP-1 (antiviral units/ml)			
10	22 ± 4	25 ± 3	20 ± 3
100	16 ± 7	14 ± 1[a]	15 ± 1[a]
1000	11 ± 2[a]	4 ± 2[a]	9 ± 1[a]
rBoIFNα (antiviral units/ml)			
10	17 ± 3	26 ± 5	25 ± 4
100	14 ± 1[a]	25 ± 2	18 ± 3
1000	16 ± 1[a]	21 ± 1	16 ± 1[a]

[a] $P < 0.01$ as determined by analysis of variance followed by LSD.

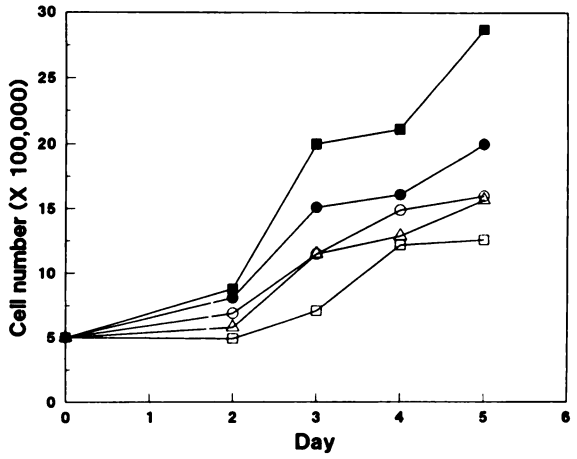

Fig. 2. Low concentrations of oTP-1 inhibit proliferation. Synchronized MDBK cells were plated at 5×10^5 cells/well in 6-well plates with or without various doses of oTP-1. At 2, 3, 4, and 5 days, triplicate wells were harvested by trypsinization and scraping and counted. The results of one of two replicate experiments are presented, and the data are expressed as mean cell numbers. Concentrations of oTP-1 used were 0 (■), 1 (●), 10 (○), 100 (△) and 1000 (□) antiviral units/ml. The mean coefficient of variation was <10%. Significant differences were determined by analysis of variance followed by LSD.

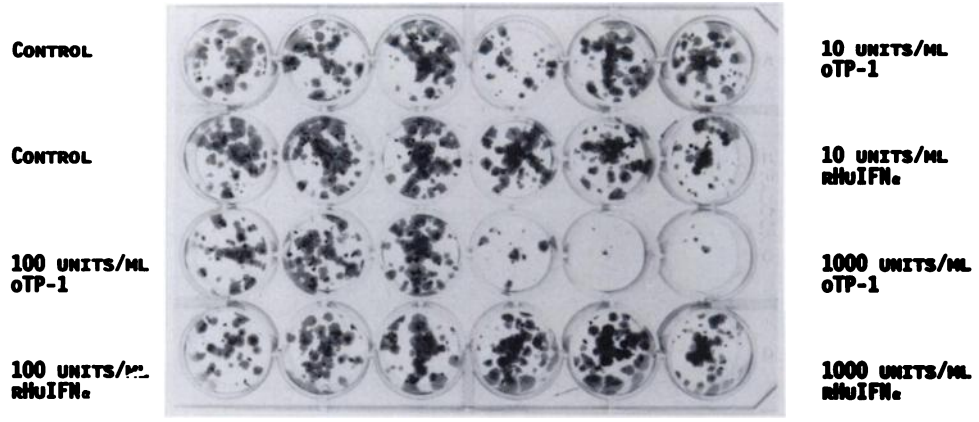

Fig. 1. oTP-1 exerts an antiproliferative effect on human cells. WISH cells were plated at 200 cells/well in HMEM/2% FBS in media alone or in the presence of various doses (antiviral units/ml) of oTP-1 or rHuIFNα. Cells were incubated 8 days at 37°C and colonies visualized with crystal violet. Samples were assessed in triplicate, and representative results of one of three replicates are presented. Data are numerically represented in Table 1.

Table 3 *Cell cycle analysis of oTP-1- and rHuIFNα-treated human WISH cells*

WISH cells (1×10^6) were incubated for 48 h with or without 1000 units/ml of either IFN in 2% FBS. Propidium iodide staining and flow cytometric analysis were used for determination of cell cycle. Results from three replicate experiments are expressed as percentage of cells in each phase of the cell cycle.

Treatment	Phase	Experiment No. 1	2	3
Media	G_0/G_1	64	66	66
	S	16	21	18
	G_2/M	20	13	16
oTP-1	G_0/G_1	64	69	63
	S	24	22	23
	G_2/M	12	9	14
rHuIFNα	G_0/G_1	62	66	52
	S	23	24	36
	G_2/M	15	10	12

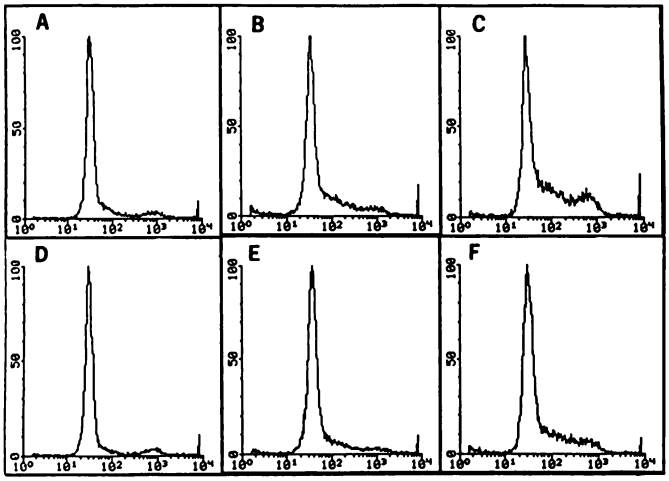

Fig. 3. oTP-1 inhibits progression into S phase. MDBK cells were grown to confluence and synchronized in G_0/G_1 by continued culture for 7 days in the presence of 0.5% spent media. Renewed proliferation was stimulated by replating the cells at 2.5×10^6 cells/ml in HMEM/10% FBS. Progress through the cell cycle was examined using propidium iodide, and representative results of one of four replicate experiments are presented as one-parameter fluorescence histograms. *Horizontal axis*, relative fluorescence intensity; *vertical axis*, number of cells. *A*, *B*, and *C*, media controls; *D*, *E*, and *F*, oTP-1 (1000 antiviral units/ml) treated cells, at 12, 15, and 18 h after the initiation of culture, respectively.

cells were present in the oTP-1-exposed wells. At 18 h, a large proportion of control cells were in S phase, and some of these had progressed to G_2/M (the final peak). oTP-1-treated cells still failed to enter in large numbers into S. Thus, the progression of cells from resting (G_0/G_1) into S phase appears to be inhibited by oTP-1.

The time course of oTP-1 antiproliferative activity was extended to 6 days of treatment (Table 4). During that time, the control cells had passed through more than three generations, while oTP-1-treated cells had undergone only slightly more than one doubling time. Control cells progressed more quickly into S and G_2/M phase, and even at 48 h no oTP-1-treated cells were in G_2/M. By Day 6, the monolayers in the control cultures were confluent, and the cells were accumulating in G_0/G_1. Therefore, oTP-1 exerts a long-term effect on cell growth. It is not cytotoxic because basel cell numbers remained constant rather than decreased. The reduced growth is a function of slower progression through the cell cycle resulting in fewer mitoses.

Confirmation of cytostatic rather than cytotoxic effects of oTP-1 was obtained by treatment of synchronized MDBK cells with a very high dose (50,000 antiviral units/ml) of either oTP-1 or rBoIFNα for 48 h, after which the IFNs were removed and the cells examined for evidence of toxicity at 48 and 96 h of culture. As seen in Table 5, both oTP-1 and rBoIFNα completely stopped MDBK proliferation at 50,000 antiviral units/ml. Furthermore, rBoIFNα-treated cells exhibited significant cell death that was not present in oTP-1-treated cells. This is consistent with previous reports of the unusual lack of toxicity of high doses of oTP-1 using human and feline peripheral blood lymphocytes (10). When the IFNs were removed, both oTP-1- and rBoIFNα-treated cells resumed cycling. This indicates that oTP-1 is a very effective cytostatic agent, capable of being used at high doses, and its effects can be reversed when removed.

DISCUSSION

Examination of the ability of oTP-1 to inhibit cell proliferation reveals that oTP-1 exhibits potent anticellular activity across species. It appears to decrease the rate of progression through the cell cycle, specifically the transition from G_0/G_1 into, as well as progression through, S phase. Although contro-

Table 4 *Time course of oTP-1 antiproliferative activity*

MDBK cells were plated at 1×10^6 cells/well in 6-well plates and incubated with 1000 antiviral units/ml of oTP-1 for the indicated times. Cells were trypsinized, counted, and stained with propidium iodide for cell analysis.

Time (h)	Control Cell count	Control Cell cycle phase (%) G_0/G_1	S	G_2/M	oTP-1 Cell count	oTP-1 Cell cycle phase (%) G_0/G_1	S	G_2/M
18	1.0×10^6	77	22	0	0.8×10^6	91	9	0
24	1.7×10^6	56	40	4	1.6×10^6	79	22	0
42	1.5×10^6	33	55	12	1.5×10^6	44	56	0
48	2.3×10^6	35	51	14	1.8×10^6	31	69	0
72	3.9×10^6	52	20	28	1.5×10^6	32	50	18
144	6.3×10^6	91	1	7	2.8×10^6	84	3	12

Table 5 *Lack of toxicity of oTP-1*

Synchronized MDBK cells were plated at either 3×10^5 (Experiment 1) or 1.5×10^5 (Experiment 2) cells/well in 6-well plates and incubated for 48 h with or without 50,000 antiviral units/ml of oTP-1 or rBoIFNα. At 48 h, triplicate wells were harvested for viability determinations or cell cycle analysis was performed on single wells in which 10,000 cells were examined. The remaining wells were washed 3 times with media containing no IFNs and incubated without IFNs until 96 h. Cell cycle results are expressed as percentage of cells in each phase of the cell cycle.

Time (h)	Treatment	Experiment 1: cell No. (×10⁵) Live	Dead	Viability (%)	Experiment 2: cell No. (×10⁵) Live	Dead	Viability (%)	Experiment 3: cell cycle G_0/G_1	S	G_2/M
48	Media	5.0 ± 0.6	0.5 ± 0.2	91	3.2 ± 0.6	0.3 ± 0.1	91	23	61	16
	oTP-1	3.3 ± 0.5	0.4 ± 0.1	89	1.7 ± 0.3	0.1 ± 0	94	97	3	0
	rBoIFNα	3.1 ± 0.5	0.9 ± 0.1[a]	78	1.6 ± 0.2	0.6 ± 0[a]	73	100	0	0
96	Media	18.3 ± 4.2	0.3 ± 0.1	98	7.7 ± 0.2	1.2 ± 0.5	87	73	25	2
	oTP-1	14.9 ± 1.6	0.3 ± 0.2	98	[b]	[b]	[b]	26	51	23
	rBoIFNα	19.0 ± 3.2	0.2 ± 0.1	99	5.5 ± 0.5	1.3 ± 0.3	80	37	41	22

[a] $P < 0.05$ as determined by analysis of variance followed by LSD.
[b] Not done.

versy persists regarding the point of IFN interaction with the cell cycle (reviewed in Ref. 11), inhibition of transition out of G_0/G_1 into S phase and prolongation of S by oTP-1 is consistent with studies of IFNα effects on human melanoma cells (12). In addition, oTP-1 exhibits identical antiproliferative potency to other known IFNs. Remarkably, it lacks the toxic effects on cells observed when cells are exposed to high doses of other natural and recombinant IFNs.

In tumor immunotherapy, the acute antitumor effect appears to result from direct cytostatic activity of IFNαs rather than from indirect immunomodulation (13). Clinical trials of lymphoma treatment indicate that increasing doses of IFNs are associated with increasing effectiveness. High doses of recombinant IFNs, however, produced intolerable fever and chills, anorexia, weight loss, and fatigue (13). Therefore, the value of an antitumor agent such as oTP-1, which has potent cytostatic activity in the absence of toxicity, is apparent.

The antiproliferative activity, as well as antiviral, immunosuppressive, and antileuteolytic effects, of oTP-1 may have potentially important physiological roles in pregnancy. The antiproliferative effect may function in the pregnant uterus to slow growth of the endometrium during the period when the conceptus is elongating to establish contact with a high percentage of the uterine luminal surface epithelium. The antiviral activity of oTP-1 could potentially serve to protect the peri-implantation conceptus from viral infection (4). Finally, immunosuppressive activity, detected by the inhibitory effect of oTP-1 on blastogenesis of sheep lymphocytes in response to mitogens (14), may facilitate survival of the conceptus allograft.

The myriad of biological activities associated with oTP-1 suggest that oTP-1 may have different functional domains. Insight into the structural requirements of elicitation of distinct activities will allow for manipulation of both the antiproliferative and reproductive effects of this molecule. Since oTP-1 is potent, nontoxic at high concentrations, and easily and inexpensively produced and purified in large quantities (5), its human counterpart which is currently being pursued could be a potential candidate for an efficacious antineoplastic biological response modifier.

REFERENCES

1. Godkin, J. D., Bazer, F. W., Moffatt, J., Sessions, F., and Roberts, R. M. Purification and properties of a major, low molecular weight protein released by the trophoblast of sheep blastocyst at day 13–21. J. Reprod. Fertil., *65:* 141–150, 1982.
2. Imakawa, K., Anthony, R. V., Kazemi, M., Marotti, K. R., Polites, H. G., and Roberts, R. M. Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophectoderm. Nature (Lond.), *330:* 377–379, 1987.
3. Stewart, H. J., McCann, S. H. E., Barker, P. J., Lee, K. E., Lamming, G. E., and Flint, A. P. F. Interferon sequence homology and receptor binding activity of ovine trophoblast antileuteolytic protein. J. Endocrinol., *115:* R13–R15, 1987.
4. Pontzer, C. H., Torres, B. T., Vallet, J. L., Bazer, F. W., and Johnson, H. M. Antiviral activity of the pregnancy recognition hormone ovine trophoblast protein-1. Biochem. Biophys. Res. Commun., *152:* 801–807, 1988.
5. Ott, T. L., Van Heeke, G., Johnson, H. M., and Bazer, F. W. Cloning and expression of *S. cerevisiae* of a synthetic gene for the pregnancy recognition hormone ovine trophoblast protein-1: purification and evidence for both antiviral and antileuteolytic activity. J. Interferon Res., in press, 1991.
6. Duc-Goiran, P., Robert-Galliot, B., Lopez, J., and Chany, C. Unusual apparently constitutive interferons and antagonists in human placental blood. Proc. Natl. Acad. Sci. USA, *82:* 5010–5014, 1985.
7. Familletti, P. C., Rubinstein, S., and Pestka, S. A convenient and rapid cytopathic effect inhibition assay for interferon. Methods Enzymol., *78:* 387–394, 1981.
8. Blalock, J. E., Georgiades, J. A., Langford, M. P., and Johnson, H. M. Purified human immune interferon has more potent anticellular activity than fibroblast or leukocyte interferon. Cell. Immunol., *49:* 390–394, 1980.
9. Gresser, I., and Tovey, M. G. Antitumor effects of interferon. Biochim. Biophys. Acta, *516:* 231–247, 1978.
10. Bazer, F. W., Johnson, H. M., and Yamamoto, J. K. Effect of ovine trophoblast protein-1 on replication of feline and human immunodeficiency viruses. (Abstract). Biol. Reprod., *40* (Suppl. 1)*:* 63, 1989.
11. Tamm, I., Jasny, B. R., and Pfeffer, L. M. Antiproliferative action of interferons. *In:* L. M. Pfeffer (ed.), Mechanisms of Interferon Actions, Vol. II, pp. 26–57. Boca Raton, FL: CRC Press, Inc., 1987.
12. Creasey, A. A., Bartholomew, J. C., and Merigan, T. C. Role of G_0–G_1 arrest in the inhibition of tumor cell growth by interferon. Proc. Natl. Acad. Sci. USA, *77:* 1471–1475, 1980.
13. Oldham, R. K. Biologicals for cancer treatment: interferons. Hosp. Pract., *20:* 71–91, 1985.
14. Newton, G. R., Vallet, J. L., Hansen, P. J., and Bazer, F. W. Inhibition of lymphocyte proliferation by ovine trophoblast protein-1 and a high molecular weight glycoprotein produced by peri-implantation sheep conceptus. Am J. Reprod. Immunol. Microbiol., *19:* 99–107, 1989.