Differences in Lymphocyte-Regulatory Activity Among Variants of Ovine IFN-τ

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ABSTRACT

Interferon-τ (IFN-τ) is secreted from trophectoderm of the ruminant preimplantation conceptus and functions during pregnancy to prevent luteolysis. In addition, IFN-τ can inhibit proliferation of peripheral blood lymphocytes (PBL) and other cells. Several distinct ovine IFN-τ (OvIFN-τ) gene variants exist; three of these (IFN-τ4, IFN-τ6d, IFN-τ11) differ in their ability to prevent luteolysis (IFN-τ4 > IFN-τ6d > IFN-τ11), inhibit growth of Daudi cells (IFN-τ4 > IFN-τ6d > IFN-τ11), and induce an antiviral state (IFN-τ4 > IFN-τ6d = IFN-τ11). The present objective was to compare variants for differences in ability to inhibit proliferation of phytohemagglutinin-stimulated PBL. At equal concentrations, IFN-τ4 was more inhibitory than IFN-τ6d, IFN-τ11, or an IFN-ω control. Similar differences in potency were seen when IFN-τ variants were tested at equal antiviral concentrations. Thus, the sheep trophectoderm secretes variants of IFN-τ that differ in ability to regulate lymphocyte function. The nature of the effect of the trophectoderm on endometrial lymphocytes may depend on the relative amount of each variant produced.

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equal in relative abundance.\(^{(13)}\) OvIFN-\(\tau 4\) is the most potent antiviral IFN-\(\tau\)\(^{(12,13,33)}\) and is more potent than IFN-\(\tau 6d\) and IFN-\(\tau 11\) in inhibiting growth of human Daudi cells.\(^{(12)}\) Moreover, the IFN-\(\tau\) variants have different potencies in eliciting antitumorolytic actions, with IFN-\(\tau 4\) being more effective at extending the life span of the corpus luteum than IFN-\(\tau 6d\), IFN-\(\tau 2c\), and IFN-\(\tau 11\)\(^{(12,13)}\).

The objective of this study was to compare IFN-\(\tau\) variants for their potency in ability to inhibit mitogen-induced proliferation of sheep PBL. Demonstration that IFN-\(\tau\) variants differ in biologic activity would imply that the immunoregulatory actions of the conceptus during the perimplantation period would depend on the mix of IFN-\(\tau\) variants secreted by the trophoblast.

For the experiment, adult cycling ewes of predominantly Rambouillet genotype were used as blood donors. Tissue culture medium-199 (TCM-199), Eagle’s minimal essential medium (MEM), glutamine, penicillin-streptomycin, phytohemagglutinin-L (PHA), red cell lysis buffer, \(\beta\)-mercaptoethanol, and Triton-X 100 were purchased from Sigma Chemical (St. Louis, MO). Fico-Lite-1077 was from Atlanta Biologicals (Norcross, GA). Horse serum was from HyClone (Logan, UT). Fetal bovine serum (FBS) was from Intergen (Purchase, NY). \(^{[3]}\)H\thymidine (specific activity 670 mCi/mmol) was from Amersham Life Sciences (Piscataway, NJ). Cell culture flasks were from Sarstedt (Newton, NC), and 96-well culture plates were from Becton Dickinson (River Falls, NJ). Production and purification of recombinant IFN-\(\tau 4\), IFN-\(\tau 6d\), and IFN-\(\tau 11\) were performed as previously described.\(^{(12)}\) The OvIFN-\(\omega\) (see ref. 2 for sequence) was generously provided by R.M. Roberts, University of Missouri. The gene was cloned into the pET15b bacterial expression plasmid (Novagen, Madison, WI) and used to transform BL21(DE3) pLysS Escherichia coli (Promega, Madison, WI).

Cells were fermented in Luria broth containing ampicillin (50 \(\mu g/ml\)) and chloramphenicol (17 \(\mu g/ml\)) at 37°C. Recombinant protein expression was induced at the proper cell density (OD\(_{600}\) = 1.0) with 0.5 mM isopropyl-\(\beta\)-thiogalactoside (IPTG) (AlexisCorp., San Diego, CA). After 6 h, cells were collected and lysed, and insoluble proteins were isolated and refolded as described previously.\(^{(29)}\) Approximately 90% of the resulting protein preparation was estimated to represent OvIFN-\(\omega\), as determined visually after SDS-PAGE and Coomassie staining.

Antiviral activity of each recombinant protein was determined as the ability of IFN to inhibit vesicular stomatitis virus (VSV)-induced cytolysis of Madin-Daily bovine kidney (MDBK) cells.\(^{(10)}\) One antiviral unit of recombinant OvIFN (rOvIFN) was defined as the concentration of IFN that prevented cytolysis by 50%. Using this measure, antiviral activity was 178.21 pM for IFN-\(\tau 4\), 609.5 pM for IFN-\(\tau 6d\), 330.2 pM for IFN-\(\tau 11\), and 1592 pM for IFN-\(\omega\).

PBL were obtained by fractionation of heparinized whole blood from adult cyclic ewes using density gradient centrifugation on Fico-Lite-1077. Cells were washed twice in 2 ml TCM-199 and resuspended at \(1 \times 10^6\) cells/ml in modified TCM-199 (TCM-199 containing 5% [v/v] horse serum, 200 U/ml penicillin, 0.2 mg/ml streptomycin, 2 mM extra glutamine, and \(10^{-5}\) M \(\beta\)-mercaptoethanol). Briefly, \(1 \times 10^6\) PBL in 100 \(\mu l\) total volume were placed in individual wells of 96-well culture plates with various concentrations of IFN-\(\tau\) variants and 2 \(\mu g\) PHA (dissolved in DPBS; volume 10 \(\mu l\)) in a final volume of 170 \(\mu l\) modified TCM-199. For control wells, an equivalent volume of DPBS was added instead of IFN. After cells were incubated at 37°C and 5% CO\(_2\) for 2 h, \(^{[3]}\)H\thymidine (0.1 \(\mu Ci/well\)) was added in 30 \(\mu l\) modified TCM-199. Cells were incubated overnight at 37°C and 5% CO\(_2\) and then harvested onto glass-fiber filters using a cell harvester (Brandel, Gaithersburg, MD) and consecutive washes with 0.9% (w/v) NaCl and distilled water. The amount of \(^{[3]}\)H\thymidine on the filters was measured using a liquid scintillation counter (LKB Wallac Model 1219, Turku, Finland).

In the first experiment, IFN-\(\tau\) variants were tested at final concentrations (i.e., after addition of \(^{[3]}\)H\thymidine) of 1, 10, and 10 ng/ml. The experiment was replicated three times with PBL from different ewes for each replicate. The second experiment was conducted similarly except that concentrations of 20, 60, and 100 ng/ml IFN-\(\tau\) were tested. For the last experiment, concentrations of IFN-\(\tau 6d\), IFN-\(\tau 11\), and IFN-\(\omega\) were tested at antiviral units equivalent to antiviral activity of 1, 10, and 20 ng/ml of IFN-\(\tau 4\). Thus, IFN-\(\tau 6d\) (3.42 times less potent than IFN-\(\tau 4\)) was tested at 3.42, 34.2, and 68.4 ng/ml, IFN-\(\tau 11\) (1.86 times less potent than IFN-\(\tau 4\)) was tested at 1.86, 18.6, and 37.2 ng/ml, and IFN-\(\omega\) (8.94 times less potent than IFN-\(\tau 4\)) was tested at 8.94, 89.4, and 178.8 ng/ml. The experiment was replicated three times with PBL from a different ewe for each replicate.

Data were analyzed by least squares analysis of variance using the general linear models procedure of the Statistical Analysis System.\(^{(34)}\) Ewe was considered a random effect, and other main effects were considered as fixed. Differences in proliferation between control lymphocytes and individual concentrations of each IFN were determined by the pdiff mean separation test of SAS. To determine differences in potency of individual IFN, the dataset was restricted to exclude data for control lymphocytes, and orthogonal contrasts were used to determine differences between IFN. The contrasts were IFN-\(\tau 4\) vs. others, IFN-\(\tau 6d + IFN-\tau 11\) vs. IFN-\(\omega\), and IFN-\(\tau 6d\) vs. IFN-\(\tau 11\).

Results of the first two experiments are shown in Figure 1. When IFN-\(\tau\) variants were tested at 1, 10, and 20 ng/ml, all concentrations of each IFN-\(\tau\) variant inhibited \(^{[3]}\)H\thymidine uptake by PHA-stimulated PBL (\(p < 0.05\)). However, IFN-\(\tau 4\) was more inhibitory than either IFN-\(\tau 6d\), IFN-\(\tau 11\), or an IFN-\(\omega\) control (treatment \times concentration; \(p < 0.03\) (Fig. 1, left). A similar result was obtained when IFN-\(\tau\) variants were tested at concentrations of 20, 60, and 100 ng/ml (Fig. 1, right). All concentrations of each IFN inhibited \(^{[3]}\)H\thymidine uptake (\(p < 0.025\), IFN-\(\tau 4\) was more inhibitory than the other IFN (\(p < 0.001\), and IFN-\(\omega\) was the least inhibitory (IFN-\(\omega\) vs. IFN-\(\tau 6d + IFN-\tau 11\); \(p < 0.001\)). The inhibition of proliferation was dose dependent. However, inhibition plateaued at 60 ng/ml, and the addition of 100 ng/ml did not cause further inhibition. Moreover, at the highest concentrations tested, inhibition was only \(\sim 50\%\) for IFN-\(\tau 4\), 36% for IFN-\(\tau 6d\), 31.5% for IFN-\(\tau 11\), and 15% for IFN-\(\omega\).

Differences in activity of IFN-\(\tau\) variants were also apparent when concentrations of each IFN were adjusted for their antiviral activity (Fig. 2). Again, all concentrations of each IFN inhibited \(^{[3]}\)H\thymidine uptake (\(p < 0.001\)). However, even though concentrations of IFN were adjusted for differences in antiviral potency, IFN-\(\tau 4\) was more inhibitory than other IFN (\(p < 0.01\), and IFN-\(\omega\) was the least inhibitory (\(p < 0.02\)). It has been reported previously that OvIFN-\(\tau\) variants showed...
differences in antiviral and antiluteolytic activity\(^{(11-13,33,35)}\) with IFN-\(\tau 4\) being the most active variant. Parallel to these reports, present results indicate that IFN-\(\tau \) variants IFN-\(\tau 4\), IFN-\(\tau 6d\), IFN-\(\tau 11\), and IFN-\(\omega\) vary in effectiveness in inhibiting PHA-induced proliferation of lymphocytes, with IFN-\(\tau 4\) being the most potent inhibitor among the variants tested. Fillion et al.\(^{(31)}\) previously showed that five variants of IFN-\(\tau \) purified from conditioned medium of cultured sheep conceptuses were immunosuppressive toward PHA-induced lymphocyte proliferation. However, differences among these variants in inhibiting proliferation were not evaluated.

Differences in potency of lymphocyte inhibition between IFN-\(\tau \) variants were not simply a reflection of differences in receptor affinity. This is because IFN-\(\tau 4\) inhibited a greater proportion of lymphocyte proliferation when variants were tested at saturating concentrations of IFN-\(\tau \) or when variants were tested at the same antiviral concentration. These observations suggest that some populations of lymphocytes are capable of responding to IFN-\(\tau 4\) but not to IFN-\(\tau 6d\), IFN-\(\tau 11\), and IFN-\(\omega\). Failure of some lymphocyte populations to be inhibited by IFN-\(\tau \) is probably not because of lack of receptor expression, given the near ubiquitous distribution of the type I IFN receptor.\(^{(36)}\) Inhibition of proliferation by IFN-\(\alpha\) requires components of the T cell receptor (TCR) complex.\(^{(37)}\) Perhaps the assembly of the complex among IFN-\(\tau\), type I receptor, and other proteins required for signal transduction is favored for some IFN-\(\tau\) variants in a cell type-specific manner. There is evidence that the specific signal transduction pathway varies between IFN-\(\alpha\) and IFN-\(\beta\)\(^{(38)}\) and between HuIFN-\(\alpha 4\alpha\) and HuIFN-\(\alpha 2\alpha\)\(^{(39)}\) In the uterus, too, there is evidence that the nature of the response to type I IFN depends on IFN type. Although IFN-\(\tau\) and IFN-\(\alpha\) have the same receptor in the endometrium, only IFN-\(\tau\) induces granulocyte chemotactic protein-2 gene expression.\(^{(18)}\)

**FIG. 1.** The effect of OvIFN variants on PHA-induced proliferation of PBL at equal concentrations. Lymphocytes were incubated for 72 h with IFN-\(\tau\) variants and IFN-\(\omega\), pulsed with \(^{3}\)H thymidine overnight, and harvested. Results are the least squares means ± SEM of results from three ewes.

**FIG. 2.** The effect of OvIFN variants on PHA-induced proliferation of PBL at adjusted equal antiviral concentrations. Lymphocytes were incubated for 72 h with IFN-\(\tau\) variants and IFN-\(\omega\), pulsed with \(^{3}\)H thymidine overnight, and harvested. Results are the least squares means ± SEM of results from three ewes.
The well-studied observation that IFN-τ, like other type I IFN, can inhibit lymphocyte proliferation has been interpreted to indicate that one function of IFN-τ is to block maternal lymphocyte responses directed against the conceptus. Consistent with this finding is the observation that numbers of intraepithelial lymphocytes in the luminal epithelium decline from day 20 until day 27 of pregnancy, following the period of maximal IFN-τ secretion. However, this interpretation might be oversimplified, as type I IFN have been shown to augment skin allograft rejection and activate NK cells actions that could compromise immunotolerance toward the conceptus. The nature of the effect of IFN-τ probably depends on the particular lymphocytes in the uterine epithelium. For example, IFN-τ stimulates WC1<sup>+</sup>CD8<sup>-</sup>γδ T cells and suppresses WC1<sup>+</sup>CD8<sup>-</sup>γδ T cells.

In conclusion, sheep trophectoderm secretes variants of IFN-τ that differ in their ability to regulate maternal lymphocyte function. Thus, the nature of the effect of the trophectoderm on endometrial lymphocytes may depend on the type and the relative amounts of each IFN-τ variant produced during pregnancy.

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