Interferon-Gamma Expression in Young Foals When Treated With an Immunostimulant or Plasma

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Production of interferon-gamma is fundamental in the maturation of the neonatal immune response. Although stimulation by external antigens is thought to stimulate interferon-gamma production, neither an immunostimulant nor plasma seemed to provide the necessary signals in our foals. Authors’ address: Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY 40546; e-mail: Tracy.Sturgill-Wright@uky.edu. © 2006 AAEP.

1. Introduction

Respiratory disease in foals remains a persistent management problem for the equine industry. Young foals in particular exhibit a unique susceptibility to respiratory infections not normally encountered in the adult.1–3 For example, bronchopneumonia caused by Rhodococcus equi strikes foals <6 mo of age.4 This susceptibility is thought to be the result of the relative immaturity of the foal’s immune system. We have recently shown that neonatal foals exhibit a reduced capacity to produce interferon (IFN)-gamma compared with older foals and adults.5 The mechanism responsible for this deficiency is not known. It is reasonable to propose that increased immune competency in the foal should lead to increased resistance to infectious disease.

Hyperimmune plasma (HIP) has been used on endemic farms to prevent disease in animals with high exposure to R. equi. Experimentally, the results of giving HIP have been contradictory. Although initial reports indicated that HIP could protect foals from R. equi infection,6,7 conflicting results have been obtained more recently.8,9 Furthermore, the protective component of the sera is unknown, because non-immune sera confers protection in some cases.9 Indeed, the overall role of humoral immune responses in protection against R. equi pneumonia remain unclear.10–13 Other effects of plasma on the immune system are also possible.14,15

Immunostimulants are widely used in equine medicine for the treatment of respiratory disease.16 Previous studies of EqStim® in healthy foals17 indicate that treatment causes a decrease in lymphocyte numbers in the lung and an increase in total white cell count in the peripheral blood. CD4+ T lymphocyte counts increased in the peripheral blood while decreasing in the bronchoalveolar lavage (BAL). This study did not determine the affect of EqStim on IFN-gamma production either in the blood or the lung, although a study performed in adult horses showed gene expression of IFN-gamma after treatment with EqStim.18
The purpose of this study was to determine whether giving foals an immunostimulant or equine plasma would enhance their ability to produce IFN-gamma.

2. Materials and Methods

Foals originated from a breeding herd that has been maintained on Veterinary Science Farm property for several years. Eighteen pony foals were randomly divided into three groups with one group receiving an immunostimulant, one group receiving plasma, and the control group receiving no treatment. All foals had rectal temperature, heart rate, and respiratory rate recorded on the days of sample collection. Treatments were administered between 72 and 96 h after birth and repeated between days 28 and 30. Hi-Gamm equi plasma (250 ml, IV) was administered one time during each time point, as recommended by the manufacturer. The immunostimulant EqStim (1 ml, q 48 h, IV) was administered for a total of three treatments, as recommended by the manufacturer.

Peripheral blood was collected from each foal at birth and on days 14, 28, and 56 by jugular venipuncture. Heparinized blood was overlaid on Ficoll-Paque Plus for isolation of peripheral blood mononuclear cells (PBMCs). PBMCs were washed three times in sterile PBS (pH 7.2) and enumerated using a ViCell-XR instrument. The cells were resuspended at 3 x 10^6/ml in medium. They were then supplemented with 2.5% fetal equine serum (FES), 2 mM glutamine, 100 U/ml penicillin/streptomycin, and 55 μM 2-mercaptoethanol and cultured in vitro.

BAL samples were collected from each foal at ~45 and 90 days of age. BAL tubes were prepared by inserting polyethylene tubing into outer tubing. A 5.5-in, 16-g IV catheter was inserted into and glued to the inner tubing. The assembled tubing was placed in a sterilization pouch and gas sterilized. Foals were administered a sedative cocktail containing xylazine (0.15 mg/kg, IV), acepromazine (0.01 mg/kg, IV), and butorphanol tartrate (0.01 mg/kg, IV). Sterile BAL tubes were lubricated at the distal end with sterile lubricating jelly and passed into the lung until gently seated in a bronchiole. Luer-lock 60-ml syringes were filled with sterile saline (0.9% NaCl) and attached to the catheter on the proximal end of the inner BAL tube. The saline was slowly instilled into the lung and immediately withdrawn. This was repeated until at least 150 ml of BAL fluid (BALF) had been collected, which was typically 50–70% of the volume of saline instilled. The BAL fluid was centrifuged at 100 x g for 10 min, the pellet was resuspended in 10 ml PBS, and the cells were enumerated. BAL cells were resuspended in medium at 3 x 10^6/ml and cultured in vitro.

Aliquots of 3 x 10^6 PBMC or BAL cells from each foal were placed in 24-well plates in either 1 ml of medium or in 1 ml of medium supplemented with phorbol 12-myristate 13-acetate (PMA; 25 ng/ml) and ionomycin (1 μM). Brefeldin A (10 μg/ml) was added to both medium- and PMA-stimulated cultures to ensure that the intracellular accumulation of proteins synthesized during the incubation. Plates were incubated for a total of 4 h at 37°C with 5% CO2 in air. The cells were transferred to duplicate wells of a 96-well V-bottom microtiter plate.

The plates were centrifuged at 500 x g for 5 min, and each cell pellet was fixed in 100 μl 2% paraformaldehyde and stored overnight at 4°C. Fixed cells were washed once in PBS-saponin (PBS-S; PBS supplemented with 1% FBS, 0.1% saponin, and 0.1% sodium azide) and then incubated on ice for 30 min with 100 μl CC302 fluorescein isothiocyanate (FITC) conjugated mouse-anti-bovine IFN-gamma or an isotype-control antibody (FITC-conjugated mouse IgG1) at a concentration of 1 μg/ml in PBS-S. Unbound antibody was removed by washing the cells twice with PBS-S, and each cell aliquot was resuspended in fluorescent activated cell sorter (FACS) buffer for flow cytometric analysis.

Mononuclear cells were identified and gated based on forward and side-scatter parameters. At least 50,000 gated events were acquired using a FACSCalibur flow cytometer. The data were analyzed using Cell Quest software. Markers were set on isotype-control antibody-labeled PMA-stimulated samples from each foal such that 1% cells were positive. These markers were then used to determine the percentage of IFN-gamma–producing mononuclear cells in the corresponding PMA-stimulated cell cultures labeled with anti–IFN-gamma–specific antibody (CC302). Results are expressed as the percentage of mononuclear cells that produced IFN-gamma in response to PMA-stimulation after subtraction of the isotype-control detection (1% by definition).

Statistical analysis was performed using SigmaStat. Data was considered significant when p < 0.05.

3. Results

Neither the immunostimulant nor plasma treatment had a statistically significant effect on the acquisition of IFN-gamma production by PBMCs (Fig. 1). Although there was a significant (p < 0.025) increase in IFN-gamma production over time in the BAL cells, the immunostimulant treatment failed to augment this response. Plasma treatment, however, caused a significant (p < 0.017) suppression of IFN-gamma production at both time points (Fig. 2).

4. Discussion

Experiments in adult animals indicate a Th1-immune response featuring IFN-gamma and interleukin (IL)-2 production that results in the clearance of R. equi. Additionally, a Th2 response with IL-4, IL-5, and IL-10 being produced results in disease. Diminished immune capacity in foals could account for their susceptibility to rhodococcal pneu-
In general, the ability of the neonatal immune system to confer protection against viral, bacterial, and fungal infection is inadequate compared with adults. An age-related susceptibility to intracellular pathogens is seen in mice, and this has been attributed to a defect in neonatal T cells. Unlike naive adult T cells, murine neonatal T cells make little IL-2 or IFN-gamma but produce high levels of IL-4 in response to primary stimulation in vitro. As such, neonatal T cells seem to be heavily biased to Th2 responses both in vitro and in vivo. Because protection against intracellular pathogens is dependent on the induction of Th1 cytokines, such biasing to a Th2 response could have dire consequences after exposure to intracellular pathogens. The reasons for this bias are unclear but could be the result of exposure to the maternal environment that favors Th2 induction, possibly as a means of preventing fetal allograft rejection. These maternal influences may persist in the neonate and lead to the predilection for Th2-immune responses. Indeed, it was recently shown that it is the neonatal lung environment in the mouse that favors the induction of Th2 responses, because neonatal T cells transplanted into adult lungs develop normal Th1 responses to intracellular pathogens. Although less is known about neonatal immune maturation in other species, including the horse, Th2 skewing seems to be a universal phenomenon unique to the neonatal period. Our recent results indicate that young foals are likewise biased against a Th1 immune response as evidenced by reduced levels of IFN-gamma in both the periphery and lungs during the first 3 mo of life. With time, IFN-gamma production in the foal increases, and this seems to coincide with increased resistance to Rhodococcal infections. What is unclear is the process whereby IFN-gamma production is increased.

Current opinion is that encounters with microbial antigens that stimulate cytokine production may be required for the maturation of the immune systems of neonates. This likely involves the recognition of conserved pathogen-associated molecular patterns (PAMP) through Toll-like receptors (TLRs) present on macrophages and other antigen-presenting cells. This stimulation presumably leads to the age-dependent increase in IFN-gamma production. Other forms of stimulation of the neonatal immune system may provide these signals. Macrophage activation by immune stimulants may provide one such signal. Therefore, we were interested in the role that immune modulators may play in this process. Immunostimulants are widely used in equine medicine for the treatment of respiratory disease. EqStim contains inactivated Propionibacterium acnes, a known immunostimulant that contains a number of different PAMPs and thus, stimulates multiple TLRs. Previous work has shown that Propionibacterium acnes stimulates IFN-gamma production in horses, and it was detected 7 days after initiation of therapy. Although we did see evidence of some enhancement of this response in the peripheral blood at 14 days, this was neither statistically significant nor prolonged. Cytokine responses in the periphery may not be a good measure of the protective immune response to R. equi, but the challenge is located
primarily in the lung. Thus, we also measured the effect of treatment on BAL cells. There was no effect of immunostimulant treatment on IFN-gamma production by BAL at either time sampled. It is possible that greater enhancement may have occurred at an earlier time post-treatment. Alternatively, this failure to enhance IFN-gamma production in the foals may be caused by altered TLR signaling in the neonate.35

Hyperimmune plasma is frequently used in hopes of preventing rhodococcal pneumonia; however, the contribution of Rhodococcus-specific antibodies in protection is unclear. Indeed, non-immune plasma may also confer protection in some cases.9 The possibility exists that some other component or activity of the plasma may be responsible for this effect. To test the hypothesis, we used non-immune plasma to treat the foals and assessed its effect on IFN-gamma production. There was no enhancement of IFN-gamma production by PBMC. Surprisingly, plasma treatment significantly (p < 0.015) reduced production of IFN-gamma in the BAL at all sampling times. The significance of this depression of IFN-gamma production in the lungs is not known because there was no effect seen in the periphery. This decreased IFN-gamma production was not the result of alterations in cellular composition because there were similar numbers of macrophages and lymphocytes in the BAL samples from each of the treatment groups (data not shown). These results differ from an earlier report regarding Propionibacterium acnes effects on foals17 in which the proportion of macrophages in the BAL increased at 14 days post-treatment; however, this may be the result of differences in sampling schedules between the two studies. Alternatively, the difference could be attributed to the difference in age of the foals used for the two studies, because the Flaminio et al.17 study was performed on 6–8-mo-old foals. The effect on IFN-gamma production either in the blood or the lung was not determined in their study, but a study performed in adult horses showed gene expression of IFN-gamma after treatment with EqStim.18

The mechanism responsible for this decreased IFN-gamma production in foals is not known. Likewise, little is known about the process whereby IFN-gamma production increases as foals age. Although stimulation by external antigens is thought to stimulate IFN-gamma production, neither the immunostimulant nor plasma seemed to provide the necessary signals in our foals.

The authors thank the Grayson Jockey Club Foundation, Inc. and Fort Dodge Animal Health for financial support. The EqStim was a kind gift from Neogen Corporation. The Hi-Gamm equi was kindly gifted by Lake Immunogenics, Inc. kindly gifted by Lake Immunogenics, Inc.

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