

Brief Definitive Report**Human Recombinant Interferon γ Enhances Neonatal Polymorphonuclear Leukocyte Activation and Movement, and Increases Free Intracellular Calcium**

By Harry R. Hill,* Nancy H. Augustine,* and Howard S. Jaffe†

From the *Division of Clinical Immunology and Allergy, Departments of Pathology and Pediatrics, University of Utah School of Medicine, Salt Lake City, Utah 84132, and †Genentech, South San Francisco, California 94080

Summary

In previous studies, we have reported that after chemotactic factor stimulation, PMNs from neonates fail to undergo certain critical activation steps. Furthermore, the concentration of free intracellular calcium reached is significantly below that of PMNs from adults. Interferon- γ (IFN- γ) is a lymphokine that has been shown to activate phagocytic cells, and IFN- γ messenger RNA production by neonatal mononuclear leukocytes has been reported to be depressed. In the present studies, we found that recombinant human IFN- γ markedly enhanced the chemotactic responses of PMNs from neonates to levels that were not different from that of PMNs from adults. Furthermore, preincubation of the neonatal cells with this recombinant human lymphokine also corrected the abnormality in intracellular calcium metabolism. These results suggest that this developmental defect in phagocytic cell movement may be the result of an intrinsic defect in IFN- γ production resulting in deficiency of this critical phagocyte-activating lymphokine.

Although a number of abnormalities in the neonate's host defense system have been described, one of the most consistent defects is in the ability of the PMNs from neonates to move in a unidirectional fashion toward a chemotactic stimulus (1-5). In previous studies, we reported in this journal (1) that PMNs from neonates fail to increase their intracellular concentration of free calcium to levels approximating that of PMNs from adults after chemotactic factor stimulation. This deficiency in signal transduction may contribute to the defect in cell movement as well as the failure of these cells to upregulate surface expression of the adhesive glycoprotein CD 11b/18 complex and polymerize globular into filaments actin (6, 7, 8).

Interferon γ is a glycoprotein varying in molecular mass between 20 and 25 kD, depending on the degree of glycosylation, encoded for by a single gene on chromosome 12 in the human. It is produced by CD4⁺ and CD8⁺ T cells as well as natural killer cells. Production of IFN- γ by neonatal mononuclear cells has been reported to be decreased by several investigators (9-12). Recently, Ezekowitz et al. (13, 14) have reported that IFN- γ treatment of patients with chronic granulomatous disease of childhood (CGD) enhances phagocyte respiratory burst activity and significantly decreases these patient's relative risk for acquiring serious infection. For these reasons, we have examined the effect of recombinant human IFN- γ (rhIFN- γ) on the responses of PMNs from human neonates.

Materials and Methods

Polymorphonuclear Leukocyte Preparations. Whole blood was obtained from peripheral veins of healthy adults and term neonates as well as from umbilical veins in acid citrate dextrose (Becton Dickinson & Co., Mountain View, NJ). The blood was allowed to settle in 1% dextran (Pharmacia Fine Chemicals, Piscataway, NJ), and the PMNs were separated on Ficoll-Hypaque (Pharmacia Fine Chemicals) gradients. Contaminating erythrocytes were hypotonically lysed, and the PMN suspensions (98% pure) were washed in PBS (pH 7.4). The cells were resuspended in a modified HBSS as previously described (1).

Changes in Intracellular Free Calcium. Changes in intracellular free calcium were monitored spectrofluorometrically using the calcium sensitive probe Indo-1. Indo-1AM (Molecular Probes, Eugene, OR) in a 5- μ M concentration was incubated with the PMNs with rotation for 10 min at 37°C. The cells were then washed twice in modified HBSS and resuspended in the same medium at a concentration of 5×10^6 /ml. Spectrofluorescence was monitored at 10-s intervals for 60 s in a spectrofluorometer (SLM-Aminco, Urbana, IL) with the excitation wave length set at 355 nm and emission wave lengths of 405 and 485 nm. Baseline fluorescence was established at both emission wave lengths before FMLP (10^{-7} M) (Sigma Chemical Co., St. Louis, MO) was added to the suspensions. Changes in intracellular calcium were determined from the ratio of emissions at 405 and 485 nm (15). The baseline intracellular free calcium concentration in PMNs is $\sim 50 \pm 7$ nM. There was no difference in baseline values between PMNs from adults and neonates. In the experiments with IFN- γ , the cells were preincubated with the indicated concentration of rhIFN- γ (Genentech,

South San Francisco, CA) for 15 min. As additional controls, we incubated PMNs from neonates and adults with other cytokines: IFN- α (Roche Pharmaceuticals, Nutley, NJ); IFN- β (Triton Biosciences, Emeryville, CA); granulocyte/macrophage (GM)-CSF (ICN Biochemicals, Cleveland, OH); G-CSF (Amgen Biologicals, Thousand Oaks, CA); TNF- α (Cetus Corp., Emeryville, CA); IL-1 (Genzyme, Boston, MA); and IL-8 (Amgen Biologicals, Thousand Oaks, CA) in a similar manner and examined their chemotactic responsiveness. All experiments were performed three times with duplicate or usually triplicate determinations, and means and standard errors were calculated. The figures show the means and standard errors (bars) of the mean.

Chemotaxis Assay. Chemotaxis was assessed in multiwell microchemotaxis chambers (Neuroprobe Inc., Cabin John, MD) with 5- μ m pore size micropore filters (Millipore Corp., Bedford, MA). 50 μ l containing 10^5 PMNs were added to the top or cell side of the chamber, and 10^{-8} M FMLP or recombinant human C5a (100 ng/ml; Sigma Chemical Co., St. Louis, MO) was added to the bottom.

The chambers were incubated for 2 h at 37°C in a humidified chamber with 5% added CO₂. Filters were removed, dried in alcohol, and stained with hematoxylin. The PMNs that had migrated completely through the filter in 10 random fields were counted employing a 10 \times ocular, 45 \times objective, and a 5 \times 5-mm photographic reticule. Three and five replicates were run for each variable, and the mean was determined. The person reading the slides was blinded to the experimental protocol in all instances.

Interferon- γ . rIFN- γ (Genentech) cloned in *Escherichia coli* was supplied as an endotoxin free lyophil containing 2×10^7 U/mg protein. The lyophilized material was diluted in modified HBSS, aliquoted, and stored at 4°C until use.

Interferon- γ mAb. Murine mAb to human IFN- γ was purchased from Chemicon International (Los Angeles, CA). The antibody is an IgG2a monomer that neutralizes $\sim 2 \times 10^4$ U/ml.

Statistical Analyses. Statistical analyses were carried out using the unpaired student's *t* test.

Results

The mean number of PMNs from 10 neonates migrating completely through the filter in 10 random fields was 38 ± 5 compared with 73 ± 6 in 11 adults (Fig. 1; $p < 0.02$). We next examined the chemotactic responsiveness of PMNs from neonates after preincubation for 15 min with 0.01 U/ml of rhIFN- γ . As shown (Fig. 1), the chemotactic response of the PMNs from neonates increased significantly to a level (63 ± 9 PMNs/10 high power fields; 166%) that was not statistically different from that of untreated PMNs from adults. Adult PMNs incubated with IFN- γ also had enhanced responses (99 ± 13) but the increase was not statistically significant.

As added controls, we examined the effect of incubating PMNs from three neonates and three adults with similar concentrations of seven additional cytokines. The data in Table 1 are derived from three separate experiments using different donors. In these studies, only IFN- γ (156%; $p = 0.002$) and G-CSF (124%; $p = 0.04$) enhanced the chemotaxis of PMNs from neonates. In contrast, IL-1 (78%; $p = 0.05$) and IL-8 (70%; $p = 0.04$) mildly suppressed movement of these cells. Adult PMNs also had enhanced responsiveness after incuba-

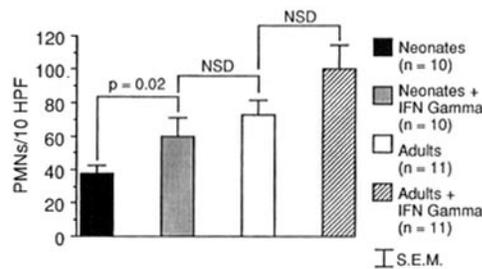


Figure 1. Chemotactic responses of the PMNs of 10 neonates and 11 adults to FMLP (10^{-8} M). Recombinant human IFN- γ (0.01 U/ml) was preincubated with the cells for 15 min. The differences between the untreated PMNs from neonates and adults was significant with a *p* value of < 0.02 employing the unpaired T test.

tion with IFN- γ but again the difference was not statistically significant (116%; $p = 0.21$).

The increase in chemotactic responsiveness after incubation with IFN- γ by the PMNs from neonates was dose dependent with enhancement occurring at concentrations from 0.001 to 1 U/ml (Fig. 2). Concentrations > 1 U produced (10–10,000 U) a plateau in the response.

Neonatal PMNs as well as PMNs from adults were incubated with IFN- γ (0.01 U/ml) for 1, 2, 5, 15, 30, and 60 min, washed, and examined for chemotactic responsiveness to FMLP. In these experiments, no effect was observed after 1 min, but enhancement was prominent (219%) after only 2 min of exposure of the neonatal PMNs to the IFN- γ . In these experiments, the maximum response (465% of baseline FMLP-stimulated chemotaxis) occurred after 15 min of incubation with IFN- γ .

We also attempted to determine how long the IFN- γ enhancement of neonatal PMN chemotaxis persisted. Cells were exposed to the IFN- γ for 15 min, washed, and held at room temperature for 60 and 120 min. In two experiments with eight replicates per assay, enhancement persisted at 60 (205%) but not 120 min (102%).

Table 1. Effect of Additional Cytokines on the Chemotactic Responses of PMNs from Neonates ($n = 3$) and Adults ($n = 3$) to FMLP (10^{-8} M)

Cytokine*	Neonatal PMNs/ 10 hpf (<i>p</i>)	Adult PMNs/ 10 hpf (<i>p</i>)
Medium 199	25.1 ± 1.4	53.1 ± 5.4
IFN- γ	39.1 ± 3.7 (0.002)	61.5 ± 3.9 (0.21)
IFN- α	28.6 ± 1.6 (0.12)	56.4 ± 7.0 (0.71)
IFN- β	28.9 ± 2.2 (0.15)	52.8 ± 5.8 (0.97)
GM-CSF	21.8 ± 2.6 (0.23)	49.6 ± 5.0 (0.65)
G-CSF	31.3 ± 2.7 (0.04)	49.8 ± 3.0 (0.61)
TNF- α	21.5 ± 2.2 (0.16)	43.9 ± 4.7 (0.22)
IL-1	19.5 ± 2.4 (0.05)	49.7 ± 6.7 (0.23)
IL-8	17.4 ± 4.2 (0.04)	43.2 ± 4.6 (0.30)

Data represent means \pm SE.

* Concentration 0.01 U/ml of cytokine.

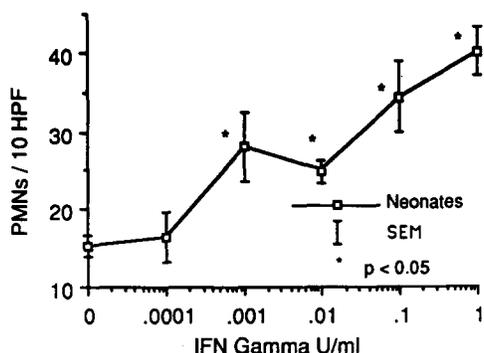


Figure 2. Effect of different concentrations of rhIFN- γ on neonatal PMN chemotactic responses to FMLP (10^{-8} M). IFN- γ was preincubated with the cells for 15 min at 37°C. Statistical differences between baseline with media alone and the different concentrations of IFN- γ were calculated using the unpaired T test.

The enhancing effect of IFN- γ on neonatal PMN chemotaxis was not limited to studies in which FMLP was used as the chemoattractant. Enhancement was also observed when recombinant human C5a was used as the chemoattractant. In additional experiments, PMNs from eight neonates were examined. In the absence of IFN- γ , C5a (100 ng/ml) attracted an average of 16.8 ± 1.1 PMNs per 10 hpf. After incubation with IFN- γ (0.01 U/ml), the response increased to 25.2 ± 1.8 (150%) ($p = 0.001$).

We next examined the effects of IFN- γ on chemotactic factor (FMLP)-induced changes in free intracellular calcium using the fluorescent calcium probe Indo-1AM. The rise in intracellular calcium measured with Indo-1 after chemotactic factor exposure of the PMNs from eight neonates ($180 \pm 5\%$ of baseline) was significantly less than with PMNs from nine adults ($200 \pm 4\%$ of baseline; $p < 0.05$). After incubation of the cells from neonates with 0.01 U of IFN- γ , the calcium response of the PMNs from neonates rose dramatically to a level equivalent to that of the adult cells exposed, or not exposed, to the lymphokine (Fig. 3). Of the other cytokines tested, only G-CSF, which like IFN- γ also enhanced neonatal PMN chemotaxis, increased the calcium response (135%; data not shown).

Additional experiments were carried out with the PMNs from three neonates to determine the specificity of the IFN- γ response. One U of anti-IFN- γ mAb was incubated with 1 U of the rhIFN- γ for 30 min before addition to neonatal PMNs. The antibody inhibited rhIFN- γ induced enhancement in chemotaxis (Baseline 21.2 ± 2.1 PMNs/10 hpf vs. 37.9 ± 5.5 PMNs/10 hpf with IFN- γ vs. 27.1 ± 2.7 with IFN- γ plus IFN- γ mAb).

Discussion

Chemotaxis of the PMNs of neonates is significantly depressed compared with PMNs from adults, and the intracellular free calcium response is also blunted. IFN- γ appears to correct not only the movement defect but also improves the calcium response. Kemmerich et al. (16) reported that IFN- γ enhanced Con A-induced rises in free intracellular cal-

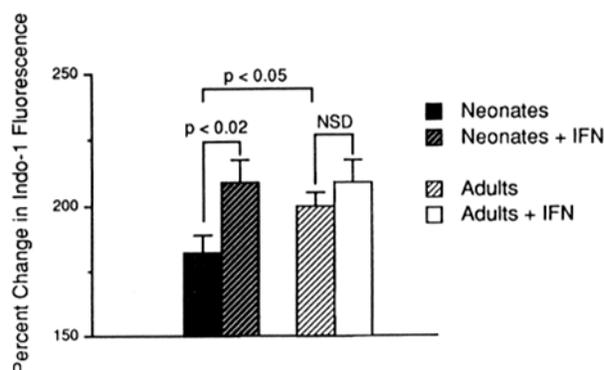


Figure 3. Effect of rhIFN on FMLP (10^{-8} M) induced changes in intracellular free calcium as measured by Indo-1 fluorescence. IFN- γ was preincubated with the cells for 15 min. The baseline intracellular free calcium concentration is ~ 50 nM (± 7 nM). There was no difference in baseline values between PMNs from adults and neonates. As shown, stimulated calcium concentrations increased from 180 to 210% of baseline in neonatal and adult PMNs, respectively (90 and 105 nM, respectively).

cium in peripheral blood monocytes from adults. Studies employing TMB-8, which prevents the release of intracellular calcium, suggested that the IFN- γ exerted its effect by increasing the release of these stores rather than increasing the influx of calcium. In our previous studies (1), we showed that the rise in intracellular calcium associated with chemotactic factor stimulation comes mostly from intracellular stores since the rise occurred in both adult and neonatal PMNs in the absence of extracellular calcium. This ability to enhance the release of intracellular calcium may partially explain the mechanism of IFN- γ correction of the cell movement defect in the PMNs of neonates.

Several investigators (9-12) have demonstrated that neonatal lymphocytes are strikingly deficient in their ability to make IFN- γ in response to a variety of stimuli. This is in contrast to their ability to make other lymphokines such as IL-2 (17). The defect in IFN- γ production by neonatal lymphocytes may be a critical one in host resistance not only to viral infections like herpesvirus and cytomegalovirus but also to pyogenic bacterial infections in which phagocyte activation, movement, and bactericidal activity are critical.

Ezekowitz et al. (14) in a recent double-blind placebo-controlled trial have shown a dramatic effect of rhIFN- γ , administered subcutaneously in a dose of 50 mcg/m² three times per wk, to significantly decrease the relative risk of serious infection in patients with CGD of childhood. Surprisingly, in contrast to pilot study data (13), the CGD trial failed to establish a consistent IFN- γ effect on oxidative metabolism of PMNs from patients as measured by superoxide production. Clearly, other mechanisms must be involved.

Human neonates have an increased susceptibility to serious bacterial infection, including ones that are common in patients with classic PMN disorders such as CGD (cutaneous infections with bacteria and yeast, pneumonia, and tissue infections). Furthermore, they clearly have defects in neutrophil movement and may have deficient bactericidal activity under conditions of stress (18). Studies by Sacchi et al. (7,

19) suggest that this defect in cell movement and activation persists for between 2 and 6 wk after birth, a period when the infant is at highest risk for infection. Only through efforts at enhancing the infant's own host defense system are we likely to continue to lower the morbidity and mortality due to in-

fections in this age group. The present studies suggest that rhIFN- γ at concentrations easily achieved in the clinical setting may correct the major deficit in neonatal PMN function *in vitro*. These data suggest the need for further investigations of the effects of IFN- γ on host defenses in the neonate.

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Address correspondence to Harry R. Hill, Division of Clinical Immunology and Allergy, 5B114, University of Utah School of Medicine, Salt Lake City, UT 84132.

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