Human Recombinant Interferon \( \gamma \) 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-\( \gamma \) (IFN-\( \gamma \)) is a lymphokine that has been shown to activate phagocytic cells, and IFN-\( \gamma \) messenger RNA production by neonatal mononuclear leukocytes has been reported to be depressed. In the present studies, we found that recombinant human IFN-\( \gamma \) 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-\( \gamma \) production resulting in deficiency of this critical phagocyte-activating lymphokine.

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-PM 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 x 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 wavelengths before FMLP (10^-7 M) (Sigma Chemical Co., St. Louis, MO) 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) 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 ~50 ± 7 nM. There was no difference in baseline values between PMNs from adults and neonates. In the experiments with IFN-\( \gamma \), the cells were preincubated with the indicated concentration of rhIFN-\( \gamma \) (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-µm pore size micropore filters (Millipore Corp., Bedford, MA). 50 µl containing 10⁶ 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 10x ocular, 45x objective, and a 5 x 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 x 10⁸ 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 ~2 x 10⁶ 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 rIFN-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 incubation 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.

**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 (p) | Adult PMNs/ 10 hpf (p) |
|-----------|--------------------------|------------------------|
| 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 ± SE.

* Concentration 0.01 U/ml of cytokine.

**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.
The enhancing effect of IFN-γ on neonatal PMN chemotaxis was not limited to studies in which FMLP was used as the chemotactant. 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 ± 5% of baseline) was significantly less than with PMNs from nine adults (200 ± 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 calcium 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,
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 infections 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.

We wish to thank Jeannette Rejali for secretarial assistance and Don Morse for art work.

This work was supported by U.S. Public Health Service Grants AI-19094 and AI-13150 and a grant from Genentech.

Address correspondence to Harry R. Hill, Division of Clinical Immunology and Allergy, 58114, University of Utah School of Medicine, Salt Lake City, UT 84132.

Received for publication 13 August 1990 and in revised form 10 December 1990.

References

1. Sacchi, F., and H.R. Hill. 1984. Defective membrane potential changes in neutrophils from human neonates. J. Exp. Med. 160:1247.
2. Mattoth, Y. 1952. Phagocytic and ameboid activities of the leukocytes in the newborn infants. Pediatrics. 9:748.
3. Miller, M.E. 1971. Chemotactic function in the human neonate: Humoral and cellular aspects. Pediatr. Res. 5:487.
4. Klein, R.B., T.J. Fischer, S.E. Gard, M. Biberstein, K.C. Rich, and E.R. Stiehm. 1977. Decreased mononuclear and polymorphonuclear chemotaxis in human newborns, infants and young children. Pediatrics. 60:467.
5. Hill, H.R., N.H. Augustine, J.A. Newton, A.O. Shigeoka, E. Morris, and F. Sacchi. 1987. Correction of a developmental defect in neutrophil activation and movement. Amer. J. Pathol. 128:307.
6. Bruce, M.C., J.E. Baley, K.A. Medvick, and M. Berger. 1987. Impaired surface membrane expression of C3b but not C3b receptors on neonatal neutrophils. Pediatr. Res. 21:306.
7. Sacchi, F., N.H. Augustine, M.M. Coello, E.Z. Morris, and H.R. Hill. 1987. Abnormality in actin polymerization associated with defective chemotaxis in neutrophils from neonates. Int. Arch. Allergy Appl. Immunol. 84:32.
8. Hilmo, A., and T.H. Howard. 1987. F-actin content of neonate and adult neutrophils. Blood. 69:945.
9. Bryson, Y.J., H.S. Winter, S.E. Gard, T.J. Fischer, and E.R. Stiehm. 1980. Deficiency of immune interferon production by leukocytes of normal newborns. Cell. Immunol. 55:191.
10. Lewis, D.B., M. Weaver, K. Prickett, and C.B. Wilson. 1989. Restricted production of IL-4 compared to IFN-gamma by human T cells during postnatal development. J. Cell. Biochem. 13(A):233.
11. Seki, H., K. Taga, A. Matsuda, N. Uwadana, M. Hasui, T. Miyawaki, and N. Taniiguchi. 1986. Phenotypic and functional characteristics of active suppressor cells against IFN-gamma production in PHA-stimulated cord blood lymphocytes. J. Immunol. 137:3158.
12. Lewis, D.B., A. Larsen, and C.B. Wilson. 1986. Reduced interferon-γ mRNA levels in human neonates. J. Exp. Med. 163:1018.
13. Ezekowitz, R.A.B., M.C. Dinauer, H.S. Jaffe, S.H. Orkin, and P.E. Newburger. 1988. Partial correction of the phagocyte defect in patients with X-linked chronic granulomatous disease by subcutaneous interferon gamma. N. Engl. J. Med. 319:146.
14. Ezekowitz, R.A.B., and the International Collaborative Study Group to Assess the Efficacy of rIFN-gamma in CGD. 1990. Clinical efficacy of recombinant human interferon-gamma (rIFN-gamma) in chronic granulomatous disease (CGD). Clin. Res. 38:465a.
15. Grynkiewicz, G., M. Poenie, and R.Y. Tsien. 1985. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J. Biol. Chem. 260:3440.
16. Kemmerich, B., G.J. Small, and J.E. Pennington. 1986. Relation of cytosolic calcium to the microbicidal activation of blood monocytes by recombinant gamma interferon. J. Infect. Dis. 154:770.
17. Wilson, C.B., J. Westall, L. Johnston, D.B. Lewis, S.K. Dower, and A.R. Alpert. 1986. Decreased production of interferon-gamma by human neonatal cells. Intrinsic and regulatory deficiencies. J. Clin. Invest. 77:860.
18. Hill, H.R. 1987. Biochemical, structural, and functional abnormalities of polymorphonuclear leukocytes in the neonate. Pediatr. Res. 22:275.
19. Sacchi, F., G. Rondini, G. Mingrat, M. Stronati, G.P. Gancia, G.L. Marseglia, and A.G. Siccardi. 1982. Different maturation of neutrophil chemotaxis in term and preterm newborn infants. J. Pediatr. 101:173.