Defective Production of Interleukin-6 by Monocytes: A Possible Mechanism Underlying Several Host Defense Deficiencies of Neonates[1]

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ABSTRACT. Several deficiencies in antibacterial defense have been described in neonates. Among those best characterized are delayed maturation of B cells into antibody producing cells, deficient T-cell maturation, and delayed cycling of hematopoietic progenitor cells after an infectious challenge. No unifying theory has been forwarded, however, to explain the concomitance of these three developmental deficiencies. IL-6, a cytokine produced primarily by monocytes and macrophages in response to stimulation by IL-1, is involved in the regulation of these three processes. Thus, we postulated that defective production of IL-6 could be a mechanism underlying these immune deficiencies of neonates. Indeed, we observed that at peak production, cells of five term neonates produced only one half as much IL-6 (14 120 ± 2590 pg IL-6/10^6 monocytes) as those of five adults (28 940 ± 1680 pg, $p < 0.001$). Peak production was lower still by monocytes of six preterm neonates (7190 ± 1400 pg, $p < 0.001$ *versus* term). Production of IL-6 protein was inhibited by actinomycin D and the IL-6 mRNA content of monocytes from neonates, as assessed by competitive polymerase chain reaction, was less than that of adult monocytes. We speculate that defective IL-6 transcription might underlie some of the defects in immune regulation observed in neonates. (*Pediatr Res* 31: 18–21, 1992)

Abbreviations

PCR, polymerase chain reaction
LPS, lipopolysaccharide

Infectious diseases are among the major causes of morbidity and mortality in neonates (1). Human and animal studies indicate that, compared with adults, neonates have an increased susceptibility to acquiring certain infections (2–4) and when infected have an increased likelihood of developing a severe resultant illness (4–6). This increased susceptibility is particularly marked among neonates delivered prematurely.

The reduced host defense status of neonates appears to be the result of multiple factors, including hyporesponsiveness of B

cells, T cells, and granulocytes, and reduced quantities of granulocytes and their progenitors (7, 8). Specific defects include delayed maturation of B cells into antibody-producing cells (9–12), deficient stimulation of T-cell maturation (13–15), and delayed induction of hematopoietic progenitor cell cycling (16). The clinical deficiencies resulting from these immature cellular processes include blunted synthesis of specific IgG (12, 17, 18) and delayed up-regulation of neutrophil production (19, 20).

Although these developmental deficiencies are well described individually, no unifying theory has been forwarded to explain their concomitance. Indeed, if a common underlying defect were identified, such recognition might form the basis of new strategies for the prevention or treatment of neonatal infections.

Striking similarities are observed between the actions of the cytokine IL-6 and the host defense deficiencies of neonates listed above. Specifically, IL-6, which is produced primarily by monocytes and macrophages in response to inflammatory mediators such as IL-1(21, 22), induces antigen-specific antibody production by B cells (23, 24), stimulates T-cell growth and differentiation (25, 26), and induces hematopoietic progenitor cells from the quiescent state into active cell cycle (27, 28). Thus, we postulated that a defective capacity to generate IL-6 might underlie several of the host defense defects observed in neonates. The following experiments were designed to assess the kinetics of IL-6 production by monocytes obtained from adults and from term and preterm neonates.

MATERIALS AND METHODS

Subjects. Blood was obtained from five healthy adults by venipuncture and from five term neonates and six preterm neonates (23–34 wk gestation) by needle puncture of an umbilical vessel immediately after delivery. Exclusion criteria for infants included infection, fetal distress, and presence of congenital anomalies. The studies were performed in accordance with protocols approved by the University of Utah Institutional Review Board, and informed consent was obtained from the participants.

Preparation of cells. Blood mononuclear cells were obtained by density centrifugation with Ficoll-Hypaque (sp gr < 1.077). Monocyte-enriched populations were obtained by incubating mononuclear cells with murine antibodies directed against human T lymphocytes (anti-human leu-1 and anti-human leu-5b; Becton Dickinson, San Jose, CA), B lymphocytes (anti-human leu-12), progenitor cells (anti-human progenitor cell antigen-1), and nucleated red blood cells (anti-glycophorin antibodies kindly supplied by Dr. Richard Langlois, Lawrence Livermore Laboratories, Livermore, CA). The antibody-bound cells were partitioned from the remaining mononuclear cells using magnetic beads coated with goat anti-mouse antibodies (Dynal Inc., Great Neck, NY). The plated cells from adults consisted of 91 ± 3%

monocytes (mean ± SD), and those from term and preterm neonates contained 94 ± 3% and 88 ± 4% monocytes, respectively, as determined by staining with α-naphthyl esterase (Sigma Diagnostics, St. Louis, MO). Cells (0.5 × 10⁶ cells/well) were plated under serum-free conditions using minimal essential medium with alpha modification (Hyclone Laboratories, Logan, UT) plus 1% nutridoma (Boehringer Mannheim, Indianapolis, IN) and incubated with various concentrations of IL-1-α (R&D Systems, Minneapolis, MN; 0–20 ng/mL) in 5% CO_2 at 37°C. In other studies, monocytes were incubated with IL-1 (10 ng/mL) alone or in combination with actinomycin D (1 μg/mL from Sigma Chemical Co., St. Louis, MO). Purified recombinant human IL-1-α had a sp act of ≥5 × 10⁸ units/mg and a purity of ≥95% by SDS-PAGE. Endotoxin (LPS) concentrations in the reagents were less than 0.05 ng/mL. Supernatants were assayed for IL-6 using ELISA (R&D Systems).

RNA isolation. Monocytes (10⁶/mL) were obtained from the blood of five adults and five term neonates. After 4 and 12 h of incubation with 5 ng IL-1/mL, RNA was extracted using a guanidinium thiocyanate extraction buffer, isolated by centrifugation through a cesium chloride gradient, and purified by phenol chloroform extraction (29).

Reverse transcriptase reaction. First strand DNA, required for the subsequent competitive PCR, was prepared from RNA using a modification of the procedure described by Sambrook *et al.* (29). Briefly, 0.1 μg of total cellular RNA was incubated with 200 U of Moloney Murine Leukemia Virus Reverse Transcriptase (Gibco BRL, Gaithersburg, MD) for 1 h at 37°C. Reaction components included 50 mM KCl; 10 mM Tris-Cl, pH 8.3; 1.5 mM MgCl₂; 0.01% gelatin; 1 mM DTT; 100 pmol of random hexamers (Boehringer Mannheim, Indianapolis, IN); 500 μM deoxynucleotide triphosphates (Perkin Elmer Cetus, Norwalk, CT), and 40 U of RNAasin placental ribonuclease inhibitor (Promega, Madison, WI) in a final reaction volume of 20 μL. The reverse transcriptase was heat-inactivated at 95°C for 5 min.

Competitive template preparation. A 314-bp fragment of the human IL-6 gene between positions 1223 and 1536 (Kishimoto T *et al.*, Genebank Accession Y00081) was amplified, using PCR, from high molecular weight DNA isolated from L-2379 K 1462, a lymphoblastoid cell line. This fragment is in the 5' coding region of the IL-6 gene and includes the 18 bp in exon 1, the entire 153 bp of intervening sequence 1, and the first 142 bp of exon 2. The primer pair used was upstream primer 5'-ATG AAC TCC TTC ACA-3' and downstream primer 5'-CAA TTC GTT CTG AAG AGG-3'. These same primers amplify the 161-bp fragment from the reverse transcribed IL-6 mRNA.

Competitive PCR. The competitive PCR techniques used in this study are modifications of methods previously described (30–32). Briefly, 1 pg of competitive template DNA in 10 μL water was added to each 0.65-mL microcentrifuge tube. Samples were overlayed with mineral oil, heat-denatured for 10 min at 94°C, and quenched in ice. A master mix was prepared and added on ice such that the final concentration of reagents for each sample was 2.5 U of Amplitaq DNA polymerase (Perkin Elmer Cetus), 200 μM deoxynucleotide triphosphates (Perkin Elmer Cetus), 1.25% formamide (33), 50 mM KCl, 10 mM Tris-Cl (pH 8.3 at 22°C), 1.5 mM MgCl₂, 0.01% gelatin, 100 pmol of upstream and downstream primers, and water to a volume of 35 μL. A 5-μL aliquot of each 20-μL reverse transcriptase reaction was added to the tubes, bringing the final volume of each tube to 50 μL. The samples were kept on ice until the block of the Perkins Elmer Cetus thermocycler was at 94°C, whereupon the samples were immediately placed into the block for 2 min. Samples were amplified for 30 cycles of 1 min at 94°C followed by 1 min annealing at 49°C followed by 2 min of extension at 72°C. Upon completing the final cycle, samples were incubated for 5 min at 72°C.

Analysis and densitometry. Electrophoresis was performed on a 30-μL aliquot of each sample through a 2.5% NuSieve/1% Seakem (FMC, Rockland, ME) agarose gel in 1 × Tris acetate

running buffer for 3 h at 2 V direct current/cm constant current. Gel and running buffer each contained 0.5 μg ethidium bromide/mL. The gels were illuminated with UV 280-nm light and photographed with type 55 positive/negative polaroid film through a no. 15 wratten and 2E UV filter. The negative was transmissively scanned, and integrated intensities of the bands were determined with the Bio Image Visage 60 system (Millipore Corp., Milford, MA) which uses a high-speed two-dimensional array camera providing 512 × 512 pixel resolution.

Statistical analysis. The *t* test was used for assessing differences in IL-6 concentrations and IL-6 mRNA content. The *t* test with Bonferroni correction for multiple observations was used to assess differences in IL-6 concentrations in supernatants of monocytes from different groups.

RESULTS

Production of IL-6 by monocytes incubated for 24 h with various concentrations of IL-6 is shown in Fig. 1. In the absence of IL-6, supernatants of monocytes from adults contained more IL-6 (6148 ± 990 pg/10⁶ monocytes, mean ± SEM) than did supernatants of monocytes from term (2343 ± 958 pg/10⁶, $p <$ 0.05) and preterm monocytes (441 ± 198 pg/10⁶, $p < 0.001$ *versus* adult). Peak IL-6 concentrations appeared in all groups when stimulated by IL-1 at ≥5 ng/mL. Combining plateau points, IL-6 concentration from the adult monocytes was 28 937 ± 1683 pg/10⁶ monocytes (mean ± SEM). This was significantly greater than plateau concentrations from term (14 117 ± 2592 pg/10⁶, $p < 0.005$) and preterm cells (7186 ± 1407 pg/10⁶, $p < 0.005$).

The production of IL-6 by monocytes at various times after stimulation with IL-1 is shown in Figure 2. IL-6 was undetectable in supernatants of all groups at 2 h after the addition of IL-1. At 6 h IL-6 was present in the supernatants in all groups; however,
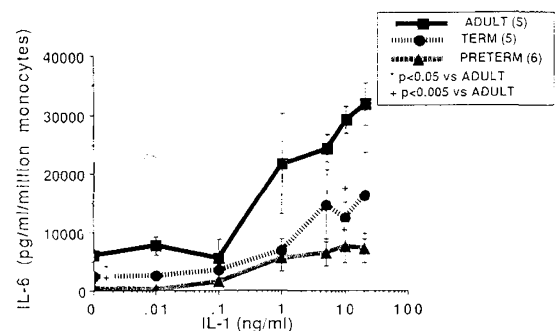

Fig. 1. Accumulation of IL-6 in supernatants of monocytes from adults and from term and preterm infants induced by various concentrations of IL-1-α keeping time constant.
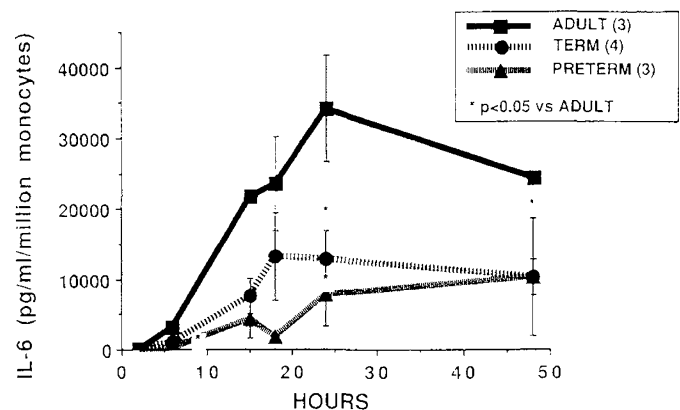

Fig. 2. Accumulation of IL-6 in supernatants from adults and from term and preterm neonates stimulated by IL-1 for varying lengths of time.

levels were significantly lower in cells from preterm infants than in cells from adults ($p < 0.05$). Peak IL-6 production was noted in supernatants of cells from all groups by 24 h, at which time concentrations were significantly greater in adult cells than in cells of neonates ($p < 0.05$). IL-6 production was abolished by actinomycin D.

As assessed by competitive PCR, monocytes from adults contained more IL-6 mRNA than did monocytes from neonates (Fig. 3), ($p < 0.005$). After 4 h of incubation, the RNA/DNA proportions were $100 \pm 0\%$ for adult and $82 \pm 10\%$ for neonatal monocytes. At 12 h, IL-6 RNA/DNA proportions were $99 \pm 1\%$ and $62 \pm 14\%$ for adults and neonates, respectively ($p < 0.05$).

DISCUSSION

Newborn infants, particularly those delivered prematurely, have deficiencies in antibacterial defense (5-10). Among the best-characterized deficiencies are delayed maturation of B cells into antibody producing cells (9-11, 18), deficient T-cell maturation (12-14, 18), and delayed cycling of hematopoietic progenitors after an infectious challenge (16). No unifying theory has been forwarded, however, to explain the concomitance of these deficiencies. IL-6 is a cytokine produced primarily by monocytes and macrophages in response to stimulation by IL-1 (22). The recognized actions of IL-6 (23-28) led us to postulate that its defective production by monocytes might underly developmental immune deficiencies in neonates.

Previous studies of IL-6 production by cells of neonates were performed using mixtures of various mononuclear cells or whole blood cell preparations. For instance, using "light-density" blood cells, composed predominantly of lymphocytes and monocytes, we previously observed less IL-6 accumulation in supernatants of cells of preterm neonates than in cells of adults (34). Using whole blood cell cultures, Yachie *et al.* (35) found no differences between term neonatal and adult cells in IL-6 accumulation after incubation with LPS derived from *Escherichia coli.* Interpretation of these studies is complicated by the fact that LPS from different bacterial species varies greatly in its capacity to induce

IL-6 (36) and also by the mixed cell populations used. Specifically, defective IL-6 production could be obscured under these conditions by the presence of various populations of cells, some of which produce and others that bind or degrade IL-6.

To reduce these variables, we devised a method for obtaining relatively pure populations of monocytes from blood using a technique of immunologically removing nonmonocytes from mononuclear cell preparations. The cells obtained using this method were 88-98% monocytes and, furthermore, the cells had not been subjected to selection procedures that result in activation, such as adherence to plastic or antibody attachment (37). Using this technique, we incubated monocytes from adults, term neonates, and preterm neonates with various concentrations of IL-1 for various periods of time and quantified IL-6 in the supernates.

In the IL-1 dose-response experiments as well as the kinetic studies, we observed substantially less IL-6 in supernatants from term and, particularly, preterm cells than from cells of adults. The IL-6 accumulation was inhibited by transcriptional inhibitors, consistent with the study of Navarro *et al.* (37) showing that IL-6 mRNA production by monocytes is regulated by transcription after stimulation. The hypothesis that the reduced IL-6 accumulation in monocytes of neonates was the result of decreased transcription is also supported by our observation of less IL-6 mRNA in stimulated neonatal than adult monocytes.

Whether IL-6 production in response to inflammatory mediators is reduced in infants *in vivo* remains to be determined. If such a defect is observed, however, it might underlie several of the immune deficiencies of neonates.

Fig. 3. IL-6 mRNA content, as determined by competitive PCR, in monocytes from five term neonates (*upper panel*) at 4 h (*row A*) and 12 h (*row B*) and from five adults (*lower panel*) at 4 h (*row A*) and 12 h (*row B*) after incubation with IL-1. All IL-6 mRNA were run in triplicate. The lanes are as follow: 123-kb ladder (*lanes 1A, 1B, 11A,* and *11B*), neonate 1 (*lanes 2A-4A*), neonate 2 (*lanes 5A-7A*), neonate 3 (*lanes 8A-10A* and *8B-10B*), neonate 4 (*lanes 2B-4B*), and neonate 5 (*lanes 5B-7B*). RNA samples from the adult subjects (*lower panel*) are as follow: 123-kb ladder (*lanes 1A, 1B, 11A,* and *11B*), adult 1 (*lanes 2A-4A* and *2B-4B*), adult 2 (*lanes 5A-7A*), adult 3 (*lanes 8A-10A*), adult 4 (*lanes 5B-7B*), and adult 5 (*lanes 8B-10B*).

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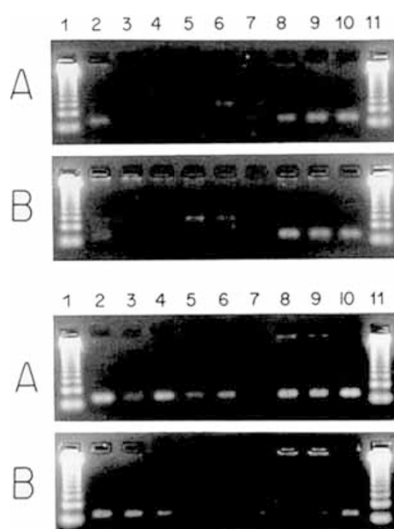18. Tosato G, Magrath IT, Koski IR, Dooley NJ, Blaese RM 1980 B cell differ-

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Erratum

In the article entitled "Requirements and Recommended Dietary Intakes of Protein during Infancy" by Samuel J. Fomon (Pediatr Res 30:391–395, 1991), an error was made in the last sentence discussing minimum protein content of infant formulas. It should read "For infants over 3 mo of age, a protein concentration of 1.8 g/100 kcal is considered adequate." The value given in the paper of 1.6 g/100 kcal was a typographical error. The author regrets this oversight.