

Decreased Production of Interferon-Gamma by Human Neonatal Cells

Intrinsic and Regulatory Deficiencies

Christopher B. Wilson, Judith Westall, Laura Johnston, David B. Lewis, Steven K. Dower, and Alan R. Alpert
Division of Infectious Disease, Children's Orthopedic Hospital and Medical Center, and Department of Pediatrics, University of Washington and Immunex Corporation, Seattle, Washington 98105

Abstract

Human neonatal lymphocytes produced little macrophage activation factor in response to mitogens. This correlated with decreased production of interferon-gamma (IFN γ): adult lymphokines contained 894.2 \pm 177.1 U/ml, whereas neonatal cord and peripheral lymphokines contained 66.9 \pm 17.0 and 116.7 \pm 29.6 U/ml by bioassay. Results by radioimmunoassay (RIA) for IFN γ were similar. In contrast, the interleukin 2 content of cord lymphokines was greater ($P < 0.01$) and that of neonatal peripheral blood lymphokines similar to that of adults. Interleukin 1 production and interleukin 2 receptor expression and affinity were similar for adult and neonatal cells. Interleukins 1 and 2 in amounts comparable to those in adult lymphokines did not increase production of macrophage activation factor or IFN γ by neonatal cells. Neonatal cells did not contain intracellular IFN or degrade exogenous IFN. Excess suppressor activity was not found in neonatal cultures. Addition of IFN α , 10,000–50,000 U/ml of interleukin 2 or phorbol myristate acetate (PMA) to cord mononuclear cells or of adult monocytes or PMA to cord T cells increased IFN γ production compared to cells stimulated with concanavalin A (ConA) alone. Nevertheless, under optimal conditions (T cells + PMA + Con A), adult cells produced much more IFN γ (1,360 \pm 261 U/ml by RIA) than cord cells (122 \pm 37 U/ml). Staphylococcal enterotoxin A (SEA) stimulated cord cell IFN γ production at low cell densities; nevertheless, adult cells produced more IFN in response to SEA (1,341 \pm 350 U/ml) than cord cells (350 \pm 33 U/ml). Decreased production of IFN γ by neonatal cells appears to be due both to differences in their intrinsic capacity to produce IFN γ and to differences in regulatory mechanisms.

Introduction

The human fetus and neonate are unusually susceptible to infection with intracellular pathogens resistance to which appears to be mediated at least in part by T lymphocyte-dependent macrophage activation (1). We previously reported that adult but not neonatal blood mononuclear cells (MC)¹ produced a factor(s)

that activated adult and neonatal monocyte-derived and tissue macrophages (M ϕ) to kill or inhibit the replication of the intracellular pathogen, *Toxoplasma gondii* (2). We and others have found that interferon-gamma (IFN γ) was the critical M ϕ activation factor (MAF) in adult MC culture supernatants, and that purified or recombinant IFN γ but not IFN α or β were equally effective (2–4). The importance of IFN γ in control of *Toxoplasma* infection is supported by studies in mice. Murine resistance to this infection correlates directly with the amount of IFN γ produced by lymphocytes in response to *Toxoplasma* (5) and passively administered murine IFN γ is protective (6). IFN γ may also be important in mediating resistance to viral infection and tumors (7). Consistent with the decreased ability of neonatal MC supernatants to activate M ϕ , Bryson et al. (8) and we (2) found that neonatal MC produces less IFN γ than adult MC. We have now performed further studies to determine the basis for deficient IFN γ production in the neonate. We report that IFN γ production by blood MC of neonates is markedly decreased in spite of apparently normal production of interleukin 1 (IL-1), interleukin 2 (IL-2), and IL-2 receptors. Our data suggest that decreased production of IFN γ by neonatal T cells is due both to intrinsic differences in capacity to produce IFN γ and to differences in regulatory cell interactions.

Methods

Reagents. Reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Hank's balanced salt solution (HBSS), calf serum, L-glutamine, penicillin, and streptomycin were obtained from Gibco (Grand Island, NY). When analyzed by *Limulus* amoebocyte lysate assay (Pyrotell Associates of Cape Cod, Inc., Woods Hole, MA), the RPMI 1640 contained <0.06 ng/ml of reference *Escherichia coli* endotoxin. Fetal calf serum (FCS) was obtained from Sterile Systems, Inc. (Logan UT); FCS contained <0.003–0.05 ng/ml endotoxin and lacked detectable (<1:4) *Toxoplasma* antibody in the Sabin-Feldman dye test. Concanavalin A (ConA), Ficoll-Hypaque (FH), and Percoll were obtained from Pharmacia Fine Chemicals (Piscataway, NJ) [³H]thymidine, Omnifluor, and Aquasol 2 were obtained from New England Nuclear (Boston, MA). Phytohemagglutinin-P (PHA) was obtained from Difco Laboratories (Detroit, MI). Staphylococcal enterotoxin A (SEA) was obtained from Dr. Reginald Bennett, Food and Drug Administration (Washington, DC). Glutaraldehyde was obtained from Ted Pella, Inc. (Irvine, CA). Paraformaldehyde was obtained from J. T. Baker Co., (Phillipsburg, NJ). Diff Quik was obtained from Dade Diagnostics (Aguado, PR). Lymphokine was obtained from One Lambda, Inc. (Los Angeles, CA).

Partially purified human IFN γ (Gg 23-901-530) was obtained from the National Institutes of Health; this preparation was used as a standard to determine the IFN γ content of other materials. Recombinant IFN γ and monoclonal antibody 12.2067 (IgG1), which neutralizes recombinant and native human IFN γ , were provided by Genentech, Inc. (S. San Francisco, CA). Purified human IL-2, which lacked detectable IFN, was obtained from Electro Nucleonics, Inc. (Silver Spring, MD); this material contained 6.1 \times 10⁴ laboratory units (U)/ml of IL-2. Recombinant human IL-2 (rIL-2) was provided by Immunex Corp. (Seattle, WA); this material contained 1.2 \times 10⁶ U/ μ g and lacked detectable IFN. Reference purified human IL-2, which contained 1.31 \times 10⁷ U/mg, was obtained from the National Institutes of Health. Human IL-1, which was purified from

Address reprint requests to Dr. Wilson, Division of Infectious Diseases, Children's Orthopedic Hospital and Medical Center, P. O. Box C5371, Seattle, WA 98105.

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1. **Abbreviations used in this paper:** ConA, concanavalin A; FCS, fetal calf serum; FH, Ficoll Hypaque; IFN, interferon; IL-1, IL-2, interleukins 1 and 2; LK, lymphokines; LPS, lipopolysaccharide; MAF, macrophage activation factor; MC, mononuclear cells; M ϕ , macrophage; PGE₂, prostaglandin E₂; PHA, phytohemagglutinin-P; PMA, phorbol myristate acetate; RIA, radioimmunoassay; rIL-2, recombinant interleukin 2.

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supernatants of endotoxin-stimulated monocytes by chromatography on DEAE, was provided by Immunex Corp. (9).

Monoclonal antibodies used to detect T lymphocytes were CD2 [T_{gp}50] 9.6, obtained from Dr. J. Hansen, Fred Hutchinson Cancer Research Center, Seattle, WA; CD3 [T_{gp}19–20] 64.1, obtained from Genetic Systems Corp. (Seattle, WA); CD8 [T_{gp}32–33] OKT8, obtained from Ortho Diagnostics (Raritan, NJ). 2A3 (IgG1), which recognizes the IL-2 receptor on activated T cells (10), was obtained from Immunex. M21 (IgG1), myeloma protein from the MOPC-21 cell line, was obtained from Litton Bionetics (Kensington, MD) and was used as a control antibody. Fluorescein isothiocyanate labeled (Fab₂) goat anti-mouse IgG was obtained from Boehringer Ingelheim (La Jolla, CA).

Cell preparations. Blood MC were routinely obtained by FH density gradient separation of adult or neonatal peripheral blood or neonatal umbilical cord blood as previously described (11). In certain experiments, cells from the FH interface were treated with mononuclear cell Lymphokwik as described by the manufacturer to deplete preparations of contaminating granulocytes and platelets. When MC were to be used for IL-1 production, cells from the FH interface were washed once in HBSS at 200 *g* for 10 min, centrifuged through a cushion of calf serum at 200 *g* for 10 min to decrease platelet contamination, and washed twice in HBSS before use. MC were resuspended in RPMI 1640 containing 2 mM l-glutamine, 25 mM HEPES buffer, 50 U/ml penicillin G and 50 μg/ml streptomycin containing 5% human type AB serum (RPMI + 5% AS) for MC cultures or 20% autologous or type AB serum (RPMI + 20% AS) for preparation of monocyte-Mφ monolayers. The percentage of cells that were monocytes or granulocytes was determined as previously described (12, 13). MC suspensions from adult, neonatal cord, and neonatal peripheral blood contained 25.8±1.6%, 19.1±1.7%, and 11.7±1.2% monocytes and 1.0±0.2%, 8.7±0.9%, and 14.5±2.4% granulocytes, respectively. MC suspensions of FH cells isolated after treatment with MC Lymphokwik contained <2% granulocytes and less than one platelet per 20 cells.

T lymphocytes were prepared from MC by nylon column adherence (14) followed by incubation of nonadherent cells with T cell Lymphokwik for 45 min at 37°C. T cells were then separated from nonviable cells by centrifugation as specified by the manufacturer, washed twice in HBSS and resuspended in RPMI + 5% AS. Such cell preparations routinely contained >90% T cells (CD2 or CD3 positive) and ≤1% monocytes.

Monocyte monolayers were prepared in Linbro trays (Linbro Scientific, Hamden CT) as previously described (12). For IL-1 production experiments, the monocyte monolayers were used immediately. For *Toxoplasma* experiments wells contained 15-mm round glass coverslips and the adherent cells were cultured in vitro for 7–9 d before they were infected, by which time the monocytes had assumed the characteristics of Mφ (15). Monocyte suspensions were prepared by recovering adherent cells from 100-mm Petri dishes (Corning Glass Works, Corning, NY) after incubation in phosphate-buffered saline, pH 7.4 (PBS), containing 2 mM EDTA at 4°C for 15 min and gently resuspending them with a rubber policeman; these suspensions were routinely >85% monocytes.

Preparation of lymphokines (LK). LK were the supernatants of cells (5×10^6 /ml) cultured at 37°C in the presence of optimal concentrations of ConA (20–50 μg/ml), PHA (10–40 μg/ml), or SEA (0.1–0.2 μg/ml). Optimal concentration ranges were determined in preliminary experiments. Where indicated, phorbol myristate acetate (PMA) was added in a concentration determined to be optimal for IFNγ production (50 ng/ml). Culture supernatants were collected 24 h later unless otherwise indicated. As indicated in the results, certain cultures were supplemented with IL-1 (100 U/ml) or IL-2 in the indicated concentrations at the time cultures were initiated. Lymphocyte transformation in response to the same stimuli was assessed as previously described (11).

IL-1 production. Lipopolysaccharide (LPS) from *E. coli* 026:B26 was added to monolayers of freshly isolated blood monocytes at a final concentration of 10 μg/ml. After incubation for 24 h culture supernatants were harvested and stored at –70°C until assayed. To determine numbers of adherent cells, monolayers were lysed by the addition of a 1% cetyltrimethylammonium bromide–naphthol blue black solution as described by Nakagawara and Nathan (16), and nuclei were counted in a hemacytometer.

In preliminary experiments addition of indomethacin or dialysis of supernatants before they were assayed were found not to affect the results.

IL-1 assay. The IL-1 content of culture supernatants and reagents was determined by the capacity to stimulate the proliferation of mouse thymocytes in the presence of a suboptimal concentration (1 μg/ml) of PHA, as described by Mizel (17). Thymocytes were obtained from 2–6-wk-old C3H/HeN mice, which were obtained from the Seattle Veterans Administration Hospital, or C3H/HeJ mice, which were obtained from Jackson Laboratories (Bar Harbor, ME). A standard preparation of purified IL-1 was included in each assay. 1 U/ml of IL-1 is that which stimulated 50% of maximal proliferation obtained using partially purified IL-1. Results were comparable with thymocytes from both strains of mice and were pooled.

IL-2 assay. The IL-2 content of culture supernatants and reagents was determined by the capacity to maintain proliferation of murine CTLL-2 cells, as previously described (18). One laboratory unit per milliliter of IL-2 is that which will maintain 2×10^3 CTLL-2 cells at 50% of maximal proliferation over this time period. 27.8±11.6 laboratory units are equal in activity to 1 U of the proposed NIH reference IL-2. A standard preparation of crude rat IL-2 or of purified human IL-2 was used in each assay to determine maximum proliferation (18).

Interferon assays. The interferon content of LK was determined by inhibition of vesicular stomatitis versus plaque formation as described (19), except that human WISH cells were used rather than human fibroblasts. A standard preparation of IFNγ was used in each assay and activity is reported in units per milliliter of the NIH reference IFNγ standard. The sensitivity of the assay was ~5 U/ml of IFNγ and the lowest dilution assayed was 1:5. For the purposes of data analysis, samples lacking detectable activity were considered to contain 20 U/ml.

We also assayed IFNγ by a specific radioimmunoassay (RIA) that detects biologically active but not heat- or acid-inactivated IFNγ (20). Under our conditions, this assay detected ≥0.5 U/ml of reference IFNγ. When adult supernatants ($n = 18$) were assayed in both assays, values in the RIA correlated ($r = 0.62$, $P < 0.01$) with values in the bioassay but were somewhat lower (453.4±131.8 vs. 761.2±163.7, $P < 0.05$).

Toxoplasma assay. Mφ were processed and survival and replication of *Toxoplasma* assessed microscopically (2, 15).

Assay for IL-2 receptors. MC were cultured as described above for the preparation of LK. After incubation for 24 h (or at other intervals as indicated), the cells were harvested by centrifugation at 200 *g* for 10 min, washed twice with cold (4°C) PBS, and resuspended in cold PBS containing 1% bovine serum albumin (BSA) and 0.1% Na azide (PBS + BSA + azide). Monoclonal antibodies were added in a predetermined optimal concentration for 30 min at 4°C, cells were washed twice, and fluorescein isothiocyanate labeled (Fab₂) goat anti-mouse IgG was added. After incubation for 30 min at 4°C, cells were washed twice. For double-labeling experiments, cells were then incubated with phycoerythrin-conjugated 2A3 and washed. Cells were then fixed in 2% paraformaldehyde in PBS pH 7.0 for 10 min, collected by centrifugation, resuspended in PBS + BSA + azide and analyzed in an EPICS-C fluorescence-activated cell sorter (Coulter Instruments, Hialeah, FL).

IL-2 was radiolabeled with ¹²⁵I by using the Enzymobead radioiodination reagent (Bio-rad Laboratories, Richmond, CA) as described by the manufacturer and had a specific activity of 1.5×10^{15} cpm/mmol. MC were enriched for T cells by a single nylon column adherence, stimulated with ConA + PMA for 48 h and then washed free of IL-2 by the method of Robb et al. (21). Specific and nonspecific binding of ¹²⁵I-IL-2 was determined and data was analyzed as described (22).

Statistics. The significance of the difference between means was evaluated by two-tailed Student's *t* test or by paired *t* test as appropriate. Results are expressed as mean±standard error.

Results

Production of MAF and IFNγ by blood MC. As previously reported (2), both LK from ConA-stimulated adult MC and re-

Table I. Production of MAF and IFN by ConA-stimulated Blood MC

Culture	Mφ activation*		n	Interferon	n
	Mφ infected	Toxoplasma/vacuole			
	%			U/ml‡	
ConA-stimulated LK§					
Adult	35.7±2.4 ^{¶¶}	60.2±6.8 [¶]	6	894.2±177.1 ^{¶¶}	23
Neonatal cord	114.3±14.2	91.0±7.5	4	66.9±17.0	24
Neonatal peripheral	87.3±8.3	102.0±7.6	3	116.7±29.6	10

* Results are expressed as percent of the concomitant control values 20 h after infection, which were 38.0±8.6% infected Mφ and 5.2±0.2 *Toxoplasma* per vacuole. Values at 1 h after infection were 51.3±9.0% infected Mφ, <1.2 *Toxoplasma* per vacuole and 2.0±0.2 *Toxoplasma* per infected Mφ. The Mφ were derived by culturing adult monocytes in vitro for 6–8 d. ‡ Bioassay (see Methods for details). § Supernatants of ConA-stimulated MC cultures harvested at 24 h. ¶ P < 0.05 vs. controls without LK and neonatal cord and peripheral LK. ¶¶ P < 0.001 vs. neonatal cord and peripheral LK.

combinant IFN γ enhanced the anti-*Toxoplasma* activity of adult monocyte-derived Mφ; this was indicated by a decrease in the percentage of infected Mφ and a decrease in the mean number of *Toxoplasma* per vacuole 20 h after infection (Table I). In contrast, LK from ConA-stimulated neonatal cord or peripheral blood (Table I) or from unstimulated adult MC did not enhance Mφ anti-*Toxoplasma* activity.

The ability of LK to enhance *Toxoplasma* activity (MAF activity) correlated with IFN content. Adult LK contained 894.2±177.1 U/ml IFN by bioassay (Table I). In contrast, neonatal cord and peripheral blood MC LK contained 66.9±17.0 and 116.7±29.6 U/ml, respectively. 1 of 23 adult, 15 of 24 neonatal cord, and 3 of 10 neonatal peripheral LK contained \leq 20 U/ml. Comparable results were obtained when PHA was used as the stimulus instead of ConA.

Relationship of IFN γ production to IL-1 and IL-2 production and IL-2 receptor expression. In contrast to the decreased production of IFN γ and MAF by neonate MC, replication of these cells in response to ConA, as indicated by [³H]thymidine incorporation, was similar to that by adult MC (Table II). IL-2 production by neonatal cord blood MC was significantly greater and that by neonatal peripheral blood MC was similar to that by adult MC. Similarly, production of IL-1, which is thought to be necessary both for induction of (23) and response to (24) IL-2 by T cells, by LPS-stimulated adult and neonatal cord and peripheral blood adherent monocytes (Table III) and MC in suspension (not shown) was similar. Unstimulated cells did not produce detectable IL-1. Further, although addition of purified IL-1 to ConA-stimulated neonatal peripheral blood MC cul-

tures slightly but not significantly enhanced IL-2 production (1126±769 U/ml, n = 6) compared to controls (531±162), MAF activity was not enhanced. IL-1 supplementation did not increase production of IL-2 or MAF by adult or cord blood MC. Similarly, addition of 2,000 U/ml of rIL-2 (Table IV) or purified IL-2 (not shown) to ConA-stimulated adult or neonatal MC cultures did not increase IFN or MAF production.

As shown in a representative two-color fluorescence experiment (Fig. 1), nearly all (>90%) of ConA-stimulated adult and neonatal peripheral blood T cells bound anti-IL-2 receptor antibody. Results with neonatal cord blood MC were similar. 2A3 binding to unstimulated MC was similar to that of control M21 myeloma protein. The percentage of MC that were T cells, as determined by staining with a monoclonal antibody to the E-rosette receptor (CD2), were also similar in six experiments: adult = 62.3±9.4%, cord = 71.1±13.0%, and neonatal peripheral = 50.3±8.6%, respectively.

As determined by binding of ¹²⁵I-rIL-2, adult and neonatal T cells (n = 2) had similar numbers of low-affinity (K_d ~ 2 × 10⁻⁷ M) IL-2 receptors (1.5–5.3 × 10⁴/T cell and 3.6–4.6 × 10⁴/T cell, respectively) and high-affinity (K_d ~ 5 × 10⁻¹⁰ M) IL-2 receptors (1,053–1,566 per T cell and 923–1,818 per T cell, respectively).

Mechanisms for decreased IFN γ production in spite of IL-2 production by neonatal MC. To determine whether there might be a trivial explanation for decreased IFN γ production by neonatal MC, we performed a series of experiments: (a) Because, as reported by others (25), FH-separated neonatal cord and peripheral blood MC but not adult MC were contaminated by

Table II. Lymphocyte Transformation and IL-2 Production by MC

	n	Lymphocyte transformation		IL-2	
		Unstimulated	ConA	Unstimulated	ConA
		cpm	cpm	U/ml	U/ml
Adult	38	978±177*	67,692±4,052	<20	848.3±129.4
Neonatal cord	32	4,055±645	59,209±5,534	<20	1462.8±197.3‡
Neonatal peripheral	21	1,178±197	54,869±8,110	<20	531.2±161.9

* Mean±SE.

‡ P < 0.01 compared to adult, P < 0.005 compared to neonatal peripheral.

Table III. IL-1 Production by LPS-stimulated Monocytes

	n	IL-1 U/ml
Adult	9	495±213*
Neonatal cord	8	880±332
Neonatal peripheral	3	1,632±639

* Mean±SE.

~10% granulocytes and each had some platelet contamination (adult more than neonatal), we depleted MC of granulocytes and platelets with monoclonal antibodies and complement; this did not affect results in that IFN γ production by neonatal cells (cord and peripheral) was 105±34% of controls ($n = 8$). (b) To determine whether neonatal MC might degrade IFN γ , we added 1,000 U/ml of rIFN γ to cultures at the time they were initiated; 2,562±926 U ($n = 2$) were recovered from adult MC cultures and 1,160±320 U ($n = 3$) were recovered from neonatal MC cultures. (c) To determine whether neonatal MC produced but did not secrete IFN, we washed MC and then assayed MC (10^7 cells) sonicates for IFN activity; adult MC contained 125±55 U whereas neonatal cells did not contain detectable IFN ($n = 2$). (d) When IFN γ was assayed by a sensitive RIA, results were similar to the bioassay although mean values for each were somewhat lower than by bioassay (adult = 284.1±53.8, cord = 12.0±2.4, neonatal peripheral = 10.0±1.4 U/ml). (e) We also assayed 48-h supernatants of ConA-stimulated MC cultures by the RIA; adult MC supernatants contained 336.3±163.6 U/ml, whereas cord MC supernatants contained 36.3±14.9 U/ml.

We performed additional studies to determine whether decreased IFN γ production by neonatal MC was intrinsic to the producing cells, due to abnormal regulatory interactions, or both. For these studies we used the IFN γ RIA because of its greater sensitivity and its specificity. Because IFN γ production by neonatal cord and peripheral blood MC was similar, we performed these studies with cord MC because larger numbers of cells were available.

Cord MC did not have excess suppressive activity, in that IFN γ production by adult MC cocultured with an equal number of cord MC was similar to adult MC alone (Table V). Addition of the cyclooxygenase inhibitor indomethacin (0.5 or 5 μ g/ml) at the initiation of the cultures (Table V) or depletion of T8 cells with antibody and complement (not shown) did not significantly

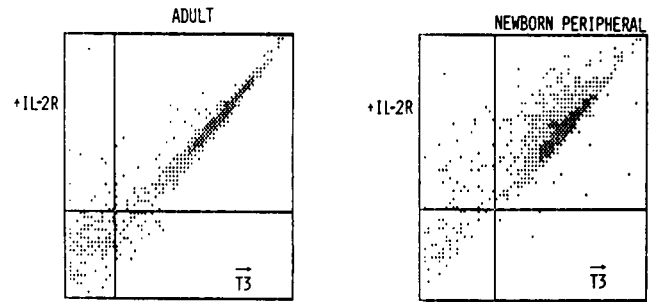


Figure 1. IL-2 receptors on T cells. Two-color fluorescence histogram of 2A3 (anti-IL-2 receptor) antibody on the ordinate (IL-2) and CD3, (T cell) antibody on the abscissa (T3). This is a representative experiment with adult MC (left) and neonatal peripheral MC (right). >90% of adult and neonatal T cells bound 2A3 antibody.

increase IFN γ production by cord or by adult MC. The suppressive effect of prostaglandin E $_2$ (PGE $_2$) on lymphocyte proliferation in adults (26) and on IFN γ production in neonates (27, 28) is reported to be abrogated when MC are cultured for 24 h before stimulation with ConA. However, IFN γ production by cord blood MC that were cultured for 24 h and then stimulated was not different from controls either in 24-h supernatants of standard tube cultures (6.2±3.0 U/ml) or in 72-h supernatants of microwell cultures (9.8±5.7 U/ml) performed as described by Wakasugi et al. (27, 28).

As noted above, IFN recovery from cultures to which 1,000 U/ml of IFN γ were added was >100%. Similarly, IFN γ production by adult and to a greater extent cord MC was increased in cultures to which rIFN α A (1,000 U/ml) was added at the outset (Table V); addition of rIFN α A just before supernatants were harvested was without effect (not shown).

We also examined the effects of PMA on IFN γ production. PMA can totally replace the monocyte-M ϕ requirement for mitogen-stimulated IFN γ production and proliferation (29, 30). In the presence of PMA, ConA-stimulated cord MC and adult MC produced about six- to eightfold more IFN γ than controls (Table IV). When purified T cells were stimulated with ConA, they produced little IFN γ (adult = 9±6, cord = 2±1 U/ml). With addition of PMA, ConA-stimulated cord T cells produced ~40-fold more IFN γ than paired MC controls. Nevertheless, under these conditions, cord blood MC produced much less IFN γ (<1–329 U/ml) than adult T cells (573–2,359 U/ml). The increase in IFN γ production paralleled a comparable increase in IL-2 production. IL-2 production by ConA-stimulated MC

Table IV. Effects of IL-2 on IFN γ and MAF Production*

	IFN γ			MAF M ϕ infected <i>Toxoplasma</i> /vacuole (% control at 20 h)				
	n	ConA	ConA + IL-2	n	ConA	ConA + IL-2‡	ConA	ConA + IL-2
		U/ml	U/ml		%	%	%	%
Adult	7	841.2±319.3§	686.5±293.1	5	35.7±2.4	44.0±6.2	60.2±6.8	60.2±8.2
Neonatal cord	5	108.1±36.3	75.6±33.2	4	114.3±14.2	160.8±29.4	91.0±7.5	99.8±9.1
Neonatal peripheral	5	111.7±41.5	93.0±23.2	3	87.3±8.3	74.0±6.6	102.0±7.6	95.3±8.4

* Assays were performed and results are expressed as in Table I. ‡ Mean±SE. § 2,000 U/ml of recombinant IL-2 were added at the time cultures were initiated. Supernatants were harvested 24 h later.

Table V. Effects of Different Culture Conditions on IFN γ Production in Response to ConA

	Adult cells	Cord cells
	U/ml-% control (n)	U/ml-% control (n)
MC	284 \pm 54—100% (26)*	13 \pm 3 (19)
Coculture‡	572 \pm 135—94% (9)	
+ indomethacin	127 \pm 41—103% (5)	16.6 \pm 9.2—97% (8)
+ IFN α	170 \pm 66—201% (4)	63 \pm 30—780% (10)
+ PMA	837 \pm 202—678% (8)	49 \pm 16—791% (13)
+ IL-2 (10,000 U/ml)	136 \pm 68—122% (3)	53 \pm 22—1723% (3)
+ IL-2 (50,000 U/ml)	277—247% (1)	94 \pm 17—4329% (3)
T cells	Adult T cells	Cord T cells
+ PMA	1,360 \pm 261—1587% (8)	122 \pm 37—4291% (6)
+ adult monocytes§	85 \pm 31 (5)	61 \pm 19 (5)
+ cord monocytes	53 \pm 14 (5)	15 \pm 10 (4)

* Values are mean \pm SE (number of experiments) units per milliliter of IFN γ as determined by RIA followed by the % of the concomitant response by ConA-stimulated MC (% control).

‡ Equal numbers of adult and cord MC were cultured together.

§ Adult or cord monocytes were added to T cells to equal 10% of the T cell concentration.

alone, MC + PMA, and T cells + PMA was 796 \pm 189, 943 \pm 604, and 13,793 \pm 6,910 U/ml for adult cells and 1,575 \pm 219, 17,494 \pm 8,893, and 18,713 \pm 6,376 U/ml for cord cells, respectively. Addition of IL-2 to ConA-stimulated cord MC cultures in amounts similar to those detected in ConA + PMA-stimulated cultures produced similar results (Table V), suggesting that the effects of PMA on IFN γ production may have been mediated at least in part by increasing IL-2 production (31, 32). Although less than that by cord T cells + PMA, IFN γ production by purified cord T cells to which adult monocytes (10%) were added was greater than that by cord T cells + autologous cord monocytes or that by MC from which the T cells were derived (Table V). In contrast, IFN γ production by adult T cells to which autologous or cord monocytes were added were both less than that by the MC from which the T cells were derived, and markedly less than that by T cells supplemented with PMA.

SEA appears to be the most potent mitogen for stimulating IFN γ production by MC (33). T cells appear to be the principal source of SEA stimulated IFN γ (33, 34). In contrast to ConA and PMA, peak IFN γ production in response to SEA occurs later and appears to be partially dependent on cellular proliferation at least under certain culture conditions (34). Wakasugi et al. (27, 28) recently reported that SEA was the most effective stimulus for IFN γ by neonatal cord MC. We found that, under standard culture conditions (in tubes with 5 \times 10⁶ MC/ml), SEA-stimulated cord blood MC produced much less IFN γ than adult MC (Table VI). In contrast, 72-h supernatants of cord MC microwell cultures (1 \times 10⁶ MC/ml) contained significantly ($P < 0.005$) more IFN γ than other cord MC culture supernatants. Nevertheless, SEA-stimulated cord MC produced significantly ($P < 0.001$ – 0.02) less IFN γ than SEA-stimulated adult MC under all culture conditions. The 72-h supernatants of ConA-stimulated cord MC microwell cultures contained amounts of IFN γ similar to those of 24–48-h supernatants of standard tube cord MC cultures. The IL-2 content and lymphocyte transformation responses of SEA-stimulated cord MC cultures were similar to ConA-stimulated cultures (not shown).

Table VI. Production of IFN γ in Response to Staphylococcal Enterotoxin A*

	Culture conditions		
	Tube 24 h	Tube 72 h	Microwell 72 h
	U/ml (n)	U/ml (n)	U/ml (n)
Adult MC	126 \pm 31 (5)‡§	732 \pm 180 (3)§	1,341 \pm 350 (2)
Cord MC	18 \pm 7 (12)	52 \pm 26 (6)	350 \pm 33 (4)

* Cultures were stimulated with 0.1–0.2 μ g/ml SEA for the indicated times. 5 \times 10⁶ cells/ml were cultured in tubes and 1 \times 10⁶ cells/ml were cultured in microwells. Results are the maximum units per milliliter IFN γ by RIA at either concentration of SEA.

‡ Mean \pm SE (number of experiments).

§ $P < 0.01$ vs. comparable cord MC cultures.

^{||} $P < 0.02$ vs. comparable cord MC cultures.

Discussion

We previously reported that neither neonatal cord nor peripheral blood LK activated adult or neonatal M ϕ , and in limited studies, that neonatal cord LK contained less IFN (\sim 40% as much) than adult LK (3). We have now confirmed this difference and have shown it to be of even greater magnitude both by a more reliable bioassay using IFN γ standards and by a specific RIA. Neonatal MC both from cord and peripheral blood produced \sim 10% as much IFN γ as adult MC in response to ConA or PHA. Others have recently reported similarly decreased IFN γ production by ConA-, PHA-, or OKT3-stimulated neonatal MC (8, 16, 17, 35). Handzel et al. (36) reported modestly decreased production (\sim 60% of adult) of IFN by PHA-stimulated neonatal MC but did not characterize the type of IFN produced.

We performed a series of studies to examine mechanisms for the decreased production of IFN γ . A trivial explanation was excluded: granulocytes and platelets, which contaminate neonatal MC, did not inhibit IFN γ production; neonatal MC did not degrade exogenous IFN γ ; neonatal MC did not contain intracellular IFN γ ; IFN γ was decreased by RIA as well as by bioassay; IFN γ production was decreased in response to a range of ConA or PHA concentrations and at 24–72-h after stimulation.

T cells (both T4 and T8) appear to produce most of the IFN γ under these conditions (34, 37) although NK cells may produce some (38). Production of IFN γ by mitogen-stimulated T cells appears to require interaction with monocytes or M ϕ , the production of IL-1 by monocytes, and is linked to IL-2 production and IL-2 receptor expression by the T cells (23). We found that IL-1 production by neonatal monocytes and IL-2 production and receptor expression by neonatal lymphocytes was similar to that by adult cells. To our knowledge, there are no previous reports of IL-1 production by neonatal cells in which activity was assayed by lymphocyte or thymocyte proliferation. Dinarello et al. (39) assayed production of substances pyrogenic for rabbits, which appear to be IL-1 (40), by neonatal cord blood monocytes. They found normal production by cells of neonates born after maternal labor but not by neonates born by Caesarian section without labor. We found no difference in IL-1 production by neonatal cells in the two infants reported here that were born

without labor compared to those born after labor. The stimulus (LPS) used to induce IL-1 production differed from those used to stimulate IFN γ and IL-2 production. It remains possible that IL-1 production by cells from some neonates may be decreased in mitogen-stimulated cultures. However, supplementation of neonatal MC cultures with exogenous IL-1 slightly increased IL-2 production but did not affect MAF production, suggesting that decreased IL-1 production was not the cause for decreased IFN γ production.

Likewise, under our conditions, IL-2 production by neonatal cord and peripheral MC was similar to or greater than that by adult cells, which is consistent with the results of others with cord MC (35, 41). IL-2 receptor antigen expression on ConA-stimulated adult and neonatal cord and peripheral T cells was also similar using the 2A3 monoclonal antibody as reported by Yokoi et al. (42) using anti-Tac. Furthermore, the number and K_d of high-affinity IL-2 receptors was similar. Proliferation is thought to require binding of IL-2 to high-affinity receptors (22, 23). In contrast to proliferation, IFN γ production by T cells appears to be only partly IL-2 dependent. Under optimal conditions, IL-2 and IFN γ mRNAs are detected at the same time and before detectable product of either appears in the supernatant (30, 43) and cycloheximide does not block synthesis either of IL-2 or of IFN γ mRNA (43). However, Reem and Ning-Hsing (44) found that addition of anti-Tac inhibited IFN γ production by ~ 25 – 75% (44), and we have obtained similar results in preliminary experiments using saturating amounts (0.2 mg/ml) of IL-2R antibody (2A3) both with adult and cord MC (unpublished observations). Production of IFN γ by adult T cells is enhanced by exogenous IL-2 (50 U) when suboptimal but not optimal ConA concentrations are used (37). Under our conditions, addition of IL-2 in amounts equal to the maximum amounts detectable in ConA-stimulated adult supernatants (2,000 U/ml) did not increase IFN γ production by adult or neonatal MC. Extremely high concentrations of exogenous IL-2 (10,000–50,000 U/ml, ≈ 0.7 – 3.5 nM), similar to the concentrations in supernatants of cells stimulated with ConA + PMA, preferentially increased IFN γ production by cord MC compared to adult MC in response to ConA. Such concentrations may act by binding to low-affinity IL-2 receptors since high-affinity receptors should be saturated at $<10,000$ U/ml (21). Our data suggest that IFN γ production by neonatal cells is selectively deficient and is not primarily due to decreased production or binding of IL-2.

While our studies were in progress, two other groups proposed different mechanisms for decreased IFN γ production by neonatal cells. Wakasugi et al. (27, 28) suggested that, in contrast to adult cells, neonatal IFN γ production (~ 4 U/ml to PHA and ConA, vs. ~ 150 U/ml for adults), but apparently not proliferation or IL-2 production, was excessively sensitive to endogenous PGE $_2$ suppression. This was based primarily on the finding that low-dose-irradiated (500 rads) but not control neonatal MC produced IFN γ that was suppressed by PGE $_2$ $> 10^{-10}$ M. However, points of evidence against this being the major mechanism were: (a) the modest effect of indomethacin—8 of 13 neonatal cultures contained increased IFN with indomethacin and the mean IFN (~ 20 U/ml) was still much less than by similarly treated adult MC (~ 200 U/ml); (b) that preculturing for 24 h, as a putative mechanism for eliminating PGE $_2$ -sensitive cells (26), increased neonatal IFN production slightly (from ~ 4 to 80 U/ml) but had similar effects on adult cells (from ~ 150 to 700 U/ml); (c) SEA, a potent stimulus for T cell IFN γ pro-

duction (33, 34), effectively stimulated neonatal IFN γ production although PGE $_2$ production was unchanged. We have not found evidence of PGE $_2$ -induced suppression because indomethacin (at similar concentrations) and preculturing did not effect IFN γ production by neonatal MC. We also did not find evidence of excess suppression of IFN γ production by neonatal cells either by coculturing neonatal MC with adult MC or by depleting T8-positive cells from neonatal MC. It is possible that in the studies of Wakasugi's et al. (27, 28), irradiation, which increased the neonatal response to $\sim 30\%$ of the adult response, acted by another mechanism, (e.g., perhaps by affecting regulatory regions of genes for IFN γ in neonatal cells) and also induced increased PGE $_2$ sensitivity.

Taylor and Bryson (45) recently reported that neonatal T cells produced IFN γ when adult but not cord blood monocytes that had been cultured in vitro for 7 d were added; however, the mechanism for this was not further defined. These results differ from those of Wakasugi et al. (27, 28), who found that the addition of adult monocytes to neonatal cord MC did not affect IFN γ production. We explored this both by supplementing neonatal MC or purified T cells with PMA, which can totally replace monocyte-M ϕ requirement for mitogen-stimulated IFN γ production (29, 30), and by adding adult monocytes to cord T cells. Purified adult T cells + PMA produced 573–2,359 U/ml of IFN γ , whereas neonatal cells produced <1 –329 U/ml. The amount of IFN γ released was higher than reported by Taylor and Bryson (45) or Wakasugi (27, 28) and appears to reflect the maximum capacity of T cells to produce IFN γ under these conditions. The data indicate that neonatal IFN γ production can be augmented more than that of adults by purification of T cells and addition of PMA, but that the response is still markedly less than that by adult cells. We have also added adult monocytes to neonatal T cells with results similar to but somewhat lower than with PMA. In contrast, production of IFN γ by adult T cells plus either adult or cord monocytes was less than that by whole MC and similar with cord and adult monocytes. Because these conditions were clearly suboptimal for adult cells but near optimal for neonatal cells, our data are consistent with differences in monocyte regulation of T cell IFN γ production between adult and neonatal cells. Nevertheless, under conditions that stimulated maximally, adult cells produced much more IFN γ than neonatal cells. Our data suggest that decreased IFN γ (and consequently MAF) production by neonatal T cells may be both intrinsically limited and diminished by abnormal regulatory interactions. Interestingly, Salahuddin et al. (46) found that none of eight HTLV-1 transformed cord blood T cell lines produced IFN γ , whereas 9 of 12 adult T cell lines did. This suggests that cells capable of producing IFN γ may be decreased in number in neonatal blood.

The ability of SEA to induce IFN γ production by neonatal MC in our studies and those of Wakasugi et al. (27, 28) is unique and suggests that it may act by a different mechanism or on a different cell population. Others have found that IFN γ production by adult T cells in response to SEA peaks later than that in response to ConA or PHA and is also more dependent on proliferation. We found that SEA-stimulated adult cells produced IFN γ by 24 h, whereas neonatal cord cells produced little. By 72 h cord cells in microwells at low cell densities produced $\sim 25\%$ as much IFN γ as adult cells. This may reflect a greater need for cord cells to replicate or differentiate into IFN γ producer cells in vitro. Further study of differences in the mechanisms of IFN γ

induction by ConA and SEA in adult and neonatal cells should provide insight into intracellular and intercellular mechanisms of IFN γ regulation.

Production of IL-2 and IFN γ by adult T cells appears to be regulated pretranslationally (43, 47). However, Salahuddin et al. (46) found that IFN γ was regulated posttranslationally in certain HTLV-I-transformed T cell lines. We have recently found that decreased IFN γ production by neonatal cells appears to be due to a pretranslational deficiency, since Northern blots of RNA from ConA-stimulated cord MC that were hybridized to an IFN γ probe revealed bands that were <5% as intense as with RNA from comparable adult cells (48).

IFN γ is a much more potent inducer of M ϕ activation than IFN α or β (3, 45, 49). In contrast to IFN γ , IFN α , and β production by neonatal cells is similar to that by adult cells (50, 51). Thus, decreased production of IFN γ may be an important factor that predisposes the neonate to severe infection with intracellular pathogens.

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