Interferon-γ–Induced Activation of Indoleamine 2,3-Dioxygenase in Cord Blood Monocyte-Derived Macrophages Inhibits the Growth of Group B Streptococci

Colin R. MacKenzie, Ulrich Hadding, and Walter Däubener

Neonatal sepsis is most often caused by group B streptococci (GBS) and is a major cause of death in the neonatal period. The response of the immune system in the newborn child has received much attention and is thought to be deficient in a number of ways. The effector response of neonatal monocyte-derived macrophages (MDM) was investigated. Interferon-γ–induced the activation of indoleamine 2,3-dioxygenase in MDM and inhibited the growth of GBS. Both effects were enhanced by the addition of tumor necrosis factor-α to the culture conditions. The coincident supplementation of L-tryptophan with the bacteria abrogated the bacterial growth inhibition, thus confirming the causative role of L-tryptophan depletion. Control of the extracellular as well as intracellular L-tryptophan levels may thus be one of the effector mechanisms with which the immune system defends the host against GBS dissemination and disease.

Group B streptococci (GBS) are one of the leading causes of neonatal morbidity and mortality [1]. Aspiration of amniotic fluid by the neonate during or shortly before birth may result in pneumonia, which progresses to sepsis and meningitis. In the lungs, the first immune cells that come into contact with the bacteria are the alveolar macrophages and epithelial cells. A determining step in the disease process is thus whether the infection is contained locally or dissemination occurs. GBS disease can occur in adults but is far less frequent and usually related to pregnancy, although an increase in the incidence of GBS disease in nonpregnant adults has been described recently [2]. GBS have been shown to stimulate macrophages to produce a number of cytokines [3, 4] necessary for an adequate immune response to infection. Furthermore, interferon-γ (IFN-γ) has been shown to protect mice from streptococcal disease [5]. Antibacterial functions of macrophages stimulated by cytokines in an autocrine pathway or by IFN-γ released by tumor necrosis factor-α (TNF-α)–stimulated NK cells may therefore be critical. The occurrence of IFN-γ–induced activation of indoleamine 2,3-dioxygenase (IDO) and consequent L-tryptophan depletion has been shown to be the effector mechanism responsible for the inhibition of Toxoplasma gondii growth in glioblastoma cells [6]; however, the mechanism by which human macrophages exert their effect is not known. IDO activation has only been described as an effector mechanism for the intracellular pathogens T. gondii and Chlamydia species [7, 8]. We investigated the effects of IFN-γ activation of cord blood monocyte-derived macrophages (MDM) on the extracellular growth of GBS.

Materials and Methods

Bacteria and culture media. Streptococcus agalactiae was isolated from a clinical specimen received from a neonate diagnosed as having neonatal sepsis. The organism was identified by colony characteristics and agglutination using a diagnostic kit (Strep Plus; Oxoid, Basingstoke, UK) and confirmed by biochemical methods (api20strep; bioMérieux, Lyon, France). Escherichia coli type strain (DSM 1103) was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig) and, in addition, a clinical isolate responsible for neonatal sepsis was also used. All bacteria were grown on brain-heart infusion agar (Difco, Hamburg, Germany) containing 5% sheep’s blood and incubated at 37°C in a 5% CO2–enriched atmosphere. Before adding the bacteria to the cell cultures, a single colony was picked and resuspended in RPMI medium (without L-glutamine or L-tryptophan; Seromed, Berlin). Between 10 and 50 bacteria/well were added to cell cultures. Colony-forming units were calculated by serially diluting the bacteria in RPMI medium and plating 10 μL of the bacterial suspension on agar plates.

MDM preparation and cell culture. Cord blood was obtained from the umbilical cords of healthy newborn babies immediately after clamping and sectioning of the cord. Mononuclear cells were purified from cord blood by gradient centrifugation using Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) and adherence to plastic. After a minimum of 2 weeks of maturation in culture, cells for experiments were resuspended in RPMI medium (Gibco, Grand Island, NY) containing 5% fetal calf serum (BioWhittaker, Walkersville, MD) and plated at 3 x 10^4 cells/well into wells of a 96-well culture plate (Greiner, Solingen, Germany). The MDM were characterized by typical morphology on staining with Giemsa and were >95% nonspecific esterase-positive (Sigma, Deisenhofen, Germany).

Cytokines and reagents. IFN-γ, donated by M. Hündgen (Rentschler, Laupenheim, Germany) was added to the cells in 1:2 serial dilution starting from 200 U/mL. TNF-α (Genzyme,
We have confirmed this depletion of L-tryptophan by measuring supernatants of stimulated MDM using high-pressure liquid chromatography and have found undetectable levels of L-tryptophan and correspondingly elevated levels of L-kynurenine (data not shown).

GBS growth inhibition. It has been shown that IFN-γ protects mice against streptococcal infection [5]; however, which cells are involved and the possible mechanism that may be involved in humans are unclear. To determine the role of cord blood macrophages, we inoculated streptococci into stimulated MDM cultures, and after 8–18 h of incubation, the optical density at 620 nm was determined as a measure of bacterial density. Figure 1 shows that an increasing concentration of IFN-γ produced a dose-dependent GBS growth inhibition in the MDM cell cultures. The results shown in figure 1 are the mean (±SD) of measurements in triplicate wells and representative of experiments repeated three times. In addition to the measurement of optical density, a 10 μL aliquot of the supernatant was removed from selected triplicate wells, and colony-forming unit assays were done by serial dilution. The results in figure 2 show a marked reduction in colony-forming units on stimulation of the MDM with 200 U/mL IFN-γ.

IDO dependence of GBS growth inhibition. Since the induction of IDO catalyzes L-tryptophan catabolism and it is known that L-tryptophan deprivation is responsible for the toxoplasmas-toxoplasmosis observed in IFN-γ–stimulated cells [7], we repleted L-tryptophan in some cell cultures. Addition of L-tryptophan to MDM stimulation and infection with bacteria. MDM in triplicate wells were stimulated with IFN-γ in a 1:2 serial dilution starting at 200 U/mL. In addition, TNF-α at 100 U/mL was added to some wells. As a negative control, some cells were not stimulated with IFN-γ or only with TNF-α. The MDM were incubated at 37°C in 5% CO₂ for 96 h, whereafter the bacteria were added. Bacteria were resuspended in RPMI medium without L-glutamine or L-tryptophan at 1000–5000 cfu/mL and added to the wells in 10 μL. Some cells received, in addition, after inoculation with the bacteria, L-tryptophan in 10 μL to reach a final concentration of 100 μg/mL. After incubation of cells, the optical density at 620 nm was measured and colony-forming units were counted by resuspending the bacteria in the culture supernatant, diluting a 10-μL sample in PBS in a 1:10 serial dilution, and plating 10 μL of each dilution onto agar plates.

Assay of IDO induction. Parallel MDM cultures were stimulated with IFN-γ in the same concentrations, and after 96 h, IDO induction was measured by a method previously described by us [9], which detects the presence of L-kynurenine.

**Results**

**IDO induction.** The induction of IDO by IFN-γ in human cells is an important antimicrobial effector mechanism for Toxoplasma and Chlamydia species, both intracellular parasites. We have previously shown that IFN-γ induces IDO activity in adult MDM and glioblastoma cells [6]. Stimulation of cord blood MDM with IFN-γ also induces an IDO-dependent catabolism of L-tryptophan to L-kynurenine, which is measured in the assay. Figure 1 depicts the dose-dependent IDO induction in cord blood MDM by IFN-γ as well as the enhancing effect of added TNF-α. We have confirmed this depletion of L-tryptophan by measuring supernatants of stimulated MDM using high-pressure liquid chromatography and have found undetectable levels of L-tryptophan and correspondingly elevated levels of L-kynurenine (data not shown).

**Figure 1.** Indoleamine 2,3-dioxygenase (IDO) activation and GBS growth inhibition in cord blood MDM stimulated by interferon (IFN)-γ alone or IFN-γ plus tumor necrosis factor (TNF)-α (100 U/mL). Left y axis shows GBS growth inhibition by IFN-γ–activated MDM measured by optical density (OD) at 620 nm (squares). IDO activation measured at 492 nm is shown on the right y axis (triangles). Open symbols for both sets of data represent values with TNF-α (100 U/mL). Data are mean ± SD of triplicate wells.

**Figure 2.** Inhibition of interferon (IFN-γ)–induced bacteriostasis in macrophages by supplemental L-tryptophan. GBS growth inhibition by IFN-γ and IFN-γ– plus tumor necrosis factor (TNF)-α–stimulated MDM is abrogated by supplemental L-tryptophan added at time of infection. In contrast, growth of Escherichia coli is not inhibited by stimulated MDM. Data are mean ± SD of triplicate values.
reach a final concentration of 100 µg/mL abrogated GBS bacteriostasis completely, thus proving that IFN-γ–mediated, IDO-induced l-tryptophan depletion results in bacteriostasis in MDM (figure 2). The antimicrobial effects induced by IFN-γ are clearly enhanced by TNF-α, as seen in figure 2. This synergism of TNF-α with IFN-γ in terms of bacteriostasis correlates well with the data shown in figure 1 regarding IDO induction by these cytokines. The addition of l-tryptophan completely abrogated the IFN-γ– plus TNF-α–induced bacterial growth inhibition. As a control for the l-tryptophan dependence of this growth inhibition, we performed the same experiments with E. coli, an organism that is likewise a neonatal pathogen, is not known to be an intracellular parasite, and is not l-tryptophan–dependent. The growth of E. coli in the cell culture supernatant in an experiment parallel to that with GBS is shown in figure 2. As can be seen, no inhibition of bacterial proliferation was detectable after stimulation of the MDM with IFN-γ and TNF-α. The results shown are the mean of triplicate wells (±SD).

Discussion

IFN-γ–induced IDO activation has to date only been described as an effector mechanism in host defense against the intracellular parasites T. gondii and Chlamydia species [8, 10], and no role in the growth inhibition of extracellular parasites has been reported. We show herein that IDO is inducible by IFN-γ in cord blood MDM and that this effect is enhanced by TNF-α. Extracellular GBS are able to stimulate macrophages to produce cytokines such as TNF-α and IL-6 [3, 4], of which TNF-α in turn induces the secretion of IFN-γ by NK cells. These NK cells may then act as a source of IFN-γ, which induces IDO activation in macrophages. This possible cascade of events represents the first line of defense in a GBS infection and may be a determining factor in the outcome of the disease. We were therefore interested in the possible effect of IDO in IFN-γ–stimulated MDM on the growth of GBS.

Here we show for the first time that IFN-γ–induced IDO activation of MDM in vitro causes an l-tryptophan–dependent growth inhibition of GBS in the cell cultures. This growth inhibition was reversible if l-tryptophan was repleted at the time of infection with GBS. We had shown in prior experiments that E. coli was able to grow in l-tryptophan–free medium and, as expected, no inhibition of the growth of E. coli was seen in the cell cultures. To our knowledge, the function of IDO in the control of parasites outside the cell membrane has not been previously described. Furthermore, we are not aware of any reports describing an effector mechanism in macrophages that is completely antagonized by l-tryptophan, whereas a partial antagonism by l-tryptophan of the antiparasitic effect induced by IFN-γ has been reported [11]. In mouse macrophages, GBS are able to stimulate a nitric oxide (NO) response that is dependent on the complement receptor 3 [12]. NO production is not inducible in human macrophages; however, IDO is readily induced by IFN-γ and, as we have demonstrated herein, the resulting l-tryptophan depletion in vitro has an antibacterial effect on GBS.

The role of the GBS capsule in evasion of phagocytosis has been reported as a virulence factor [13], and therefore, the extracellular inhibition of bacterial growth has definite advantages for the host. Since GBS can stimulate macrophages to produce large amounts of TNF-α [3], which then has a synergistic role in IDO induction, it can be assumed that the intracellular concentrations of l-tryptophan approach zero and that the bacteria are thus unable to proliferate and will in time be destroyed by the macrophages. This inhibitory effect on GBS may be of particular importance in neonates, who are reported to have a deficient macrophage antibacterial activity [14], and therefore bacterial growth inhibition may be seen as a way of “buying time” for the immature immune system.

Neonates are infected either in utero during late pregnancy, by a retrograde infection after premature rupture of membranes, or during birth by passage through the birth canal, and the primary site of infection is the lungs. In the alveoli, the first cells that come into contact with the bacteria are lung epithelial cells and alveolar macrophages, both of which induce IDO on IFN-γ stimulation. Some neonates develop pneumonia, which may progress to disseminated disease and sepsis, including meningitis. Not all newborn babies born to mothers who carry GBS become ill, and many factors may determine which neonates either progress to disease or contain the infection and overcome the bacteria at an early stage. Among these factors may be the inoculum and the balance between proliferation of the bacteria and the host cellular defense. Local inhibition of bacterial growth by creating unfavorable conditions is an important defense mechanism and is used, for example, by reducing iron availability for pathogens [15]; we believe that l-tryptophan depletion may be one factor tipping the balance in favor of the neonate in GBS disease. Nutrient deprivation may be an important part of the macrophage defense system, by allowing time either for macrophage micbicidal mechanisms to take effect or for effective mobilization of the cellular and humoral immune system.

Acknowledgments

We acknowledge Peter Wernet and Gesine Kögler (Bone Marrow Donor Center, Heinrich-Heine-University, Düsseldorf) for supplying cord blood and Christian Willberg for performing the high-pressure liquid chromatographic analyses.

References


3. Goodrum KJ, Dierksheide J, Yoder BJ. Tumor necrosis factor alpha acts as an autocrine second signal with gamma interferon to induce nitric
Anticapsular Polysaccharide Antibodies and Nasopharyngeal Colonization with Streptococcus pneumoniae in Infant Rats

Richard Malley, Anne M. Stack, Michelle L. Ferretti, Claudette M. Thompson, and Richard A. Saladino

To evaluate the effect of passive immunization with anticapsular antibodies on nasopharyngeal carriage, two models of Streptococcus pneumoniae colonization were developed in infant rats. In a direct inoculation model, 3- to 4-day-old infant rats were intranasally inoculated with \(2 \times 10^3\) cfu of S. pneumoniae type 3 or 6 of S. pneumoniae type 23F. In an intralitter transmission model, 2 infant rats were intranasally inoculated with \(10^6\) cfu of pneumococcus type 3 or type 19F and placed in a cage containing 10 infant rats. Pretreatment with bacterial polysaccharide immune globulin led to a significant reduction in colonization of contact animals with S. pneumoniae type 3 or 19F in the intralitter transmission model \(P < .05\). No effect of immune globulin could be demonstrated in the direct inoculation model. These results indicate that systemic anticapsular antibodies conferred significant protection against nasopharyngeal acquisition by intralitter spread of S. pneumoniae type 3 and 19F.

The nasopharynx is the main reservoir for polysaccharide encapsulated bacteria, such as Streptococcus pneumoniae, Haemophilus influenzae type b (Hib), and Neisseria meningitidis. Exposure to these organisms, as in day care centers or after close contact with infected persons, has been shown to be a risk factor for the development of invasive disease. Invasive S. pneumoniae disease usually occurs within 1 month of acquisition of a new serotype [1]. Immune-mediated prevention of nasopharyngeal colonization may therefore lead to an important reduction in the incidence of invasive pneumococcal disease.

A dramatic reduction in the incidence of invasive Hib disease was noted after the introduction of conjugate Hib vaccines in 1988. This effect has been attributed to protection not only from bacteremia but also from nasopharyngeal carriage, thereby protecting unimmunized children from exposure (“herd immunity”). Two possible mechanisms have been postulated for the reduction of colonization. First, IgA-mediated mucosal immunity may directly prevent colonization. Children immunized with conjugate Hib vaccine express secretory IgA Hib polysaccharide antibodies in saliva [2]. In infant rats, intra-