CURRENT TOPICS

Haemopoietic colony stimulating factors for preterm neonates

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Bacterial and fungal sepsis is a major cause of morbidity and mortality in neonates. Infection rates are high in infants treated in intensive care units, with the highest rates, of around 30%, occurring in extremely immature preterm neonates.[1–3] A survey of neonatal infection at Yale University, ongoing since 1928,[4] has documented a decline in neonatal septic deaths commensurate with the establishment of neonatal intensive care units and the liberal use of increasingly effective antibiotics. Mortality from sepsis declined steadily until the early 1980s, but since then it has remained constant at near 15%. This plateau of mortality most likely reflects the poor host defences of immature, preterm neonates.[5]

Neutrophil leucocytes are central to the defences against bacterial infection,[6] and in neonates both neutrophil production and function are immature. Neutropenia, defined as a neutrophil count below the normal range for neonates established by Monroe,[7] occurs in up to 35% of preterm neonates[8 9] and in 50% of all infants born to mothers with pregnancy induced hypertension.[10] The development of sepsis together with neutropenia carries a high mortality of 39%, and two out of every three septic infants whose neutrophils fall below 0.5×10^9/l will die.[9]

Kinetics of neutrophil production

It is impossible to measure directly the total neutrophil cell mass in a human neonate. There is a good deal of circumstantial evidence to suggest that neonatal bone marrow has a reduced capacity to produce neutrophils in adequate numbers.[11] Neutrophils develop from multipotent haemopoietic stem cells through lineage-committed progenitors (granulocyte-macrophage colony forming units, CFU-GM). These give rise to a proliferative pool, identified morphologically in the bone marrow as promyelocytes and myelocytes, and a storage pool comprising metamyelocytes, bands, and segmented neutrophils, before being released into the circulation. Mature neutrophils circulate with a peripheral blood half life of 6.3 hours before migrating into the extravascular tissues where they undergo apoptosis.[12] In response to infection, adults release marrow storage pool neutrophils into the circulation, while increasing the proliferative rate of committed progenitors to achieve a sustainable neutrophil leucocytosis.[11 13] During sepsis, neutrophil turnover increases from a steady state of 1.6×10^9/kg/day to 5.0×10^9/kg/day.

What is known of the kinetics of neutrophil production early in development largely comes from studies in rats. These have shown that, in comparison to adult animals, newborn rats have a total body pool of CFU-GM less than 10% of the CFU-GM/g body weight of adults; less than 25% of CFU-GM from neonates are in the resting phase of the cell cycle (G_0), compared with > 75% of the progenitors being in G_0 in adults.[11 14 15] The absolute neutrophil cell mass per gram of body weight in the newborn rat is only one quarter that of adult animals. During the first four weeks of postnatal life, rodent neutrophil cell mass increases to adult levels, with a corresponding increase in the proportion of quiescent CFU-GM.[16] Studies in healthy human neonates born at or near term show a similar pattern of near maximal CFU-GM proliferation rate[17] compared with the large pool of CFU-GM in G_0 observed in adults.[18]

The consequence of this immature pattern of granulopoiesis is that, in the face of overwhelming bacterial sepsis, the neonate has an inadequate reserve of preformed neutrophils and inadequate reserve production capacity, both of which are necessary to mount a rapid and sustained neutrophil leucocytosis. Thus when preterm neonates develop Gram negative or group B streptococcal sepsis, they frequently become neutropenic. When this is associated with marrow neutrophil storage pool depletion, mortality is high.[19 20] The lethal consequence of this inability to mount a rapid and effective neutrophil response is further emphasised by the most recent update from the Yale survey, which found that 72% of septic deaths occur within two days of a positive blood culture, despite appropriate antibiotic treatment.[5]

Neutrophil bactericidal function

The most consistent neutrophil functional defects reported in term neonates are abnormalities of adhesion to vascular endothelium and migration. Phagocytosis and bacterial killing seem to be normal in healthy term infants, but tend to become defective during clinical

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stress.[21–22] In preterm neonates of less than 32 weeks gestation there are additional functional defects. Phagocytosis of *Escherichia coli* by neutrophils from uninfected, clinically stable preterm neonates is less efficient than that by cells from term babies and there is similar impaired phagocytosis of larger *Candida* cells.[23 24] Preterm neonates have a smaller population of metabolically active cells in assays of the respiratory burst.[25] Infants born earlier than 28 weeks also tend to have reduced peak chemiluminescence (reflecting the bactericidal activity of the respiratory burst),[26] but this seems to be more related to the clinical complications of prematurity rather than immaturity itself.[27 28] Septic preterm neonates seem unable to develop the enhanced oxidative metabolism that occurs in the neutrophils of infected adults.[29 30] There have been very few studies examining postnatal maturation of neutrophil function in preterm neonates, but we have shown that the abnormalities of chemotaxis, phagocytosis, and cell membrane complement (CR3) and Fcy (FCRIII) receptor expression persist for three to four weeks after birth before 32 weeks of gestation[23 31–33] compared with the quite rapid postnatal maturation of chemotaxis observed in term babies.[34 35]

Colony stimulating factors

Granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) are naturally occurring proteins that regulate haematopoiesis by stimulating the proliferation and differentiation of myeloid progenitor cells.[36] Since they were cloned in 1986 and 1985, respectively,[37] and became commercially available in pharmacological quantities, these two growth factors have become part of standard clinical treatment in adult and general paediatric medicine for stimulating neutrophil production to correct disease related or iatrogenic neutropenia.[38] Both are in routine use for shortening the period of neutropenia following chemotherapy for solid tumours and leukaemia, and after bone marrow transplantation. G-CSF, in particular, has been effective in correcting neutropenia associated with the congenital neutropenias, Kostmann's syndrome, and cyclical neutropenia.[39]

The cellular targets and activities of G-CSF and GM-CSF are different. The effect of G-CSF is restricted to the neutrophilic granulocyte lineage, whose committed progenitors it stimulates to proliferate and differentiate, which shortens their transit time through the marrow and enhances the survival of the mature cells in the circulation. GM-CSF is an earlier and more widely acting cytokine, which induces growth and differentiation of multilineage haematopoietic progenitors as well as progenitors committed to both the granulocyte and macrophage lineage.[40 41] GM-CSF also has a much greater effect on the bactericidal functions of mature neutrophils than G-CSF[42 43] as well as functionally enhancing monocytes, which are unaffected by G-CSF.[44 45] The effects of GM-CSF on mature phagocyte bactericidal function occur at lower concentrations than are necessary for induction of progenitor proliferation. Most of these functional effects are indirect and require secondary stimuli, such as chemotactic factors or immune sera, to trigger the full enhanced response. This indirect, or priming, nature of the GM-CSF effect may be physiologically useful to the host, in that endogenous release of GM-CSF would then only result in activation of neutrophils/monocytes at sites of injury and inflammation, where the secondary stimuli are concentrated.[46] The normal physiological interplay between G-and GM-CSF remains to be fully elucidated. One possible model is that G-CSF and GM-CSF are both elaborated during acute infections, but that G-CSF circulates and stimulates neutrophil proliferation and maturation, while GM-CSF remains localised at the site of infection to help retain and activate arriving effector cells.[46]

In current clinical practice G-CSF is more commonly used to shorten the period to recovery from chemotherapy induced neutropenia. However, recent interest has focused on the potential clinical benefit of the functional enhancement induced by GM-CSF, particularly for the treatment of invasive fungal infections.[45 47–49]

Colony stimulating factor physiology in neonates

The potential for G- and GM-CSF to reduce the incidence and severity of infection in preterm neonates is supported by the nature of the abnormalities displayed by the immature phagocyte immune system, the efficacy of growth factors at enhancing neutrophil and monocyte immunity in adults, and the in vitro and animal experiments that have specifically examined these cytokines in relation to neonate immune function.

Both G- and GM-CSF are present in measurable concentrations in the cord blood of both full term and preterm neonates.[50 51] In one study,[52] G-CSF concentrations measured in the immediate postnatal period correlated with gestational age and values were higher in infants with clinical signs of infection, suggesting an appropriate physiological response. However, several in vitro studies have shown reduced production of both G-CSF[53 54] and GM-CSF[55 56] by stimulated mononuclear cells isolated from term neonate cord blood, compared with production by adult cells. The neonatal cells accumulate less G- and GM-CSF mRNA, which may be secondary to an alteration in the mRNA post-transcriptional stability.[57] Mononuclear cells from preterm infants produce even less G-CSF than term neonates.[53 58] G-CSF production does not increase appropriately in response to experimental sepsis in newborn rats.[59] Nor did it increase in response to neutropenia in a small series of neutropenic human neonates, who had serum G-CSF values no different from those of non-neutropenic controls.[53] This is in contrast to the high circulating G-CSF concentrations seen in septic or neutropenic adults. The weight of evidence suggests, therefore, that colony stimulating factor production does not

increase appropriately in response to physiological stimuli in the newborn and that this deficiency is more severe in premature infants.

However, while considering the potential efficacy of therapeutic colony stimulating factors, an important finding has been that the number and affinity of both G-CSF and GM-CSF receptors on cord blood neutrophils are identical to those of adult cells.[54 55] In vitro functional experiments have confirmed that GM-CSF can prime term and preterm neonate neutrophils for enhanced chemotactic and respiratory burst responses to appropriate stimuli.[25 60 61] Similarly, granulocyte and granulocyte-macrophage committed progenitors isolated from neonate blood samples respond well to the proliferative stimuli of exogenous G- and GM-CSF, respectively, in vitro.[62] In our laboratory, progenitors from preterm neonates (median gestation 31 weeks) were at least as responsive to GM-CSF as those from adults.[63] It has also been shown that the in vitro proliferative response to G-CSF is independent of the infant's endogenous plasma G-CSF concentration.[62]

Colony stimulating factor treatment in newborn rats

Further encouragement to attempt to enhance the ability of human neonates to resist infection using colony stimulating factors has come from studies in rodents. Following an initial study showing that a single dose of either G-CSF or GM-CSF given to 1 day old newborn rats could induce a significant neutrophilia,[64] Cairo's group administered recombinant human G-CSF (RrHu G-CSF, Amgen), 5 μg/kg/day intraperitoneally, to newborn rats for seven consecutive days. By the seventh day the absolute peripheral blood neutrophil count had increased by 750% and the marrow neutrophil storage pool by 100% over untreated animals. The marrow proliferative pool remained unchanged, showing that there was no depletion of early myeloid progenitors. They were then infected with a lethal dose of type III group B streptococci (GBS) and randomly allocated to receive antibiotics 24 hours later. Seventy two-hours after infection all the animals pretreated with G-CSF and given antibiotics survived, whereas only 50% given antibiotics without G-CSF survived. G-CSF on its own did not protect against septic death.[65] Subsequent studies have shown that a single dose of G-CSF given immediately before inoculation with GBS could similarly enhance survival (91% survival: G-CSF with antibiotics; 28% survival: antibiotics alone). However, if the G-CSF was given 12-18 hours after GBS infection, it had no beneficial effect.[66]

GM-CSF can also increase neutrophil production and reduce mortality in experimentally infected newborn rats. Cairo's group administered recombinant murine GM-CSF (rmGM-CSF, Immunex, 4×10^7 units/mg protein) at a dose of 75 μg/kg/day for seven days before inoculating with GBS on day 8. Mice receiving rmGM-CSF had a 75% increase in circulating neutrophils and a 50% increase in the marrow neutrophil storage pool by day 7, compared with control animals. But the GM-CSF group had the same mortality as the control group (57% *vs* 59%), both groups having also received antibiotics.[67] In two other studies, where much lower doses of GM-CSF were given, there was improved survival. Frenck[68] gave a single dose of rmGM-CSF before inoculating newborn rats with *Staphylococcus aureus* 6 hours later. Titrating the dose over a wide range, rats given rmGM-CSF (Immunex, 4×10^7 units/mg protein) at a dose of 0.03 μg/kg had 52% survival at three days compared with the control group survival of 10%. But at higher doses, survival declined (29% at 10 μg/kg). The increased survival was entirely due to GM-CSF, as no antibiotics were given. At these low doses there was a transient (50%) increase in the neutrophil count at 6 hours, but over the subsequent 36 hours there was no increase in the neutrophil count, marrow neutrophil storage, or proliferative pools compared with untreated animals. This suggests that the enhanced survival achieved by the single dose of GM-CSF was entirely due to bactericidal functional priming. Similar results were found by Wheeler[69] when intraperitoneal rmGM-CSF (Sandoz, 6.2×10^7 units/mg protein) at a dose of 0.2 μg/kg was given 7-19 hours after intraperitoneal inoculation with GBS. At this optimal dose, mortality was reduced to 37% (compared with 67% in controls), again without antibiotics. There was no induced neutrophilia, but neutrophils recovered from the peritoneum 3.5 hours after GM-CSF administration showed significantly enhanced respiratory burst activity. These data suggest that the beneficial effect of GM-CSF is related to enhanced neutrophil/monocyte function. Higher doses, which increase neutrophil numbers, may in fact be detrimental through over-activating the cells, making them hyperadherent and interfering with migration into infected sites.[70 71]

Colony stimulating factor therapy in human neonates

The first documented human neonate to receive a therapeutic colony stimulating factor was a 654g infant born at 30 weeks gestation, to a mother with severe pregnancy induced hypertension. After birth he remained persistently neutropenic and had five episodes of septicaemia (including one with *S aureus* and three with GBS). At four weeks he started G-CSF, rapidly established a normal neutrophil count, and had no more septic episodes.[72] Since then, Christensen and Cairo have reported two phase I/II studies of CSF treatment in newborn infants with suspected sepsis. These showed that both G- and GM-CSF could effectively increase the neutrophil count, without evidence of toxicity.[73 74] In the first study, 35 infants of 26-41 weeks gestation were given G-CSF (Amgen) within 72 hours of birth. G-CSF was administered by one hour intravenous infusion for three consecutive days at a dose of 1, 5, or 10 μg/kg/day, or 5 μg/kg or 10 μg/kg/12 hourly. A significant increase in neutrophil count was seen at 48 hours with 5 μg/kg/day and 10 μg/kg/day, but no additional

increase was seen with the 12 hourly dosing schedule. Gestation did not seem to affect the response. All infants had tibial bone marrow aspirates at 72 hours, which showed a dose dependent increase in the neutrophil storage pool (metamyelocytes, bands, and segmented neutrophils), but no significant change in the number or morphology of early committed granulocyte progenitors.[73]

In the GM-CSF study, 15 infants of 24-33 weeks gestation were given GM-CSF (Immunex) by two hour intravenous infusion for seven consecutive days at doses of 5 μg/kg/day, 5 μg/kg/12 hourly or 10 μg/kg/day, starting within 72 hours of birth. All doses produced a significant neutrophilia, which persisted for five days after the last dose, as well as a monocytosis. The 5 μg/kg/day dose also caused a significant rise in platelet count, compared with placebo treated controls.[74] In both studies there was evidence of in vivo neutrophil activation, as indicated by an increase in the expression of the neutrophil adhesion molecule C3bi (CR3, CD11b) on the cell membrane. In neither study could clinical benefit be assessed as there were few positive blood cultures.

In an uncontrolled British study,[75] 12 critically ill, neutropenic neonates with presumed sepsis (seven confirmed) were given G-CSF 5-10 μg/kg/day. In all infants the neutrophil count increased, even though all but one had greatly increased endogenous G-CSF concentrations before treatment. Six of these infants survived.

Potential colony stimulating factor toxicity in neonates

Neonatal paediatricians have been slow to introduce the haemopoietic colony stimulating factors into clinical practice. Even though their extensive use in adults has shown them to be safe drugs when used in appropriate therapeutic doses,[76] there is understandable anxiety about toxicity from these powerful modulators of granulopoiesis and phagocyte function on the neonate's developing immune system. Some of this anxiety stems from early reports of a "first dose" effect with GM-CSF when high doses given intravenously caused transient pulmonary sequestration of leucocytes and the induction of various cytokine cascades via neutrophil and monocyte activation.[77] Such toxicity is avoided and the therapeutic effect enhanced by giving G- and GM-CSF by slow intravenous infusion or by the subcutaneous route.[78] At doses ≤ 10 μg/kg and when high peak blood concentrations are avoided by slow infusion or subcutaneous administration, the only adverse effects commonly encountered are mild fever and occasional bone pain.[76] Their perceived safety in adults is best exemplified by the growing practice of giving G-CSF to healthy haemopoietic stem cell transplant donors, in whom safety is a paramount concern, to allow harvesting of multipotent haemopoietic stem cells from the peripheral blood, rather than subject them to the discomfort and risks of a general anaesthetic for a conventional marrow harvest.[79]

There are specific anxieties related to giving therapeutic colony stimulating factors to neonates: GM-CSF, in particular, could exacerbate acute and chronic lung injury through its powerful activating effects on monocytes and neutrophils. Their stimulation of granulopoiesis could result in a "lineage steal" effect, with a reduction in erythropoiesis and thrombopoiesis. Such an association has been observed in some neonatal erythropoietin (Epo) studies when infants given Epo developed neutropenia.[80–82] However, neutropenia has not developed during other studies.[83 84] Thrombocytopenia has been observed in some septic infants given G-CSF, but this might well have been a consequence of the sepsis itself.[75] GM-CSF, on the other hand, tends to increase the platelet count, through its known, but mild, effect on megakaryopoiesis.[74 85] An additional anxiety, that exposing newborn babies to G-CSF in infancy may predispose to leukaemia in later life, arises from the experience in Kostmann's syndrome. This congenital neutropenia can be corrected by long term treatment with G-CSF, but small numbers of children have developed leukaemia on treatment.[86] However, there is evidence that Kostmann's syndrome is in itself a pre-leukaemic condition and the development of leukaemia may relate to the structural abnormality of the neutrophils and their G-CSF receptor, as well as the longer survival achieved by preventing early neutropenia-related septic deaths.[87 88]

The evidence to date from clinical trials is that short term administration of G-CSF or GM-CSF to neonates undergoing intensive care is safe with, in particular, no evidence of pulmonary or haematological toxicity.[73 74] Even more encouraging is that 21 of the infants enrolled in the G-CSF pilot study have now been followed up at two years and had normal haematological, immunological, and neurological development.[89]

Future directions

What, then, is the current status of colony stimulating factor treatment for neonates at high risk of sepsis? There is good theoretical evidence for its beneficial use, supported by carefully conducted experiments with infected newborn rats. In human newborn infants the treatment can raise the peripheral blood neutrophil/monocyte count, even in the presence of Gram negative sepsis. Above all, its use seems to be safe in the short term, with no long term sequelae.

However, much work now needs to be done to establish how best to use them to achieve the greatest effect. Should colony stimulating factors be used as an adjunct to antibiotics in acute life threatening sepsis or would greater benefit be achieved by prophylactic use over several weeks, in an attempt to accelerate the maturation of phagocyte immunity and so prevent infection? Which colony stimulating factor is more appropriate in which circumstances? And what doses should be used? Selecting the correct dose of GM-CSF may be particularly important in the light of the rodent model

experience where higher doses seem to afford less protection against death.[68]

In the presence of so much uncertainty, there is a strong argument that the use of these powerful biological response modifiers should be restricted to randomised clinical trials. These should be designed to address these questions with both laboratory and clinical endpoints. A small number of such trials are already underway in the USA and the UK. However, others have argued that when faced with a critically ill, septic, and neutropenic infant, given the evidence to date and high mortality with conventional antibiotic treatment, it would be unethical to withhold G-CSF, even in the context of a randomised trial. Though we have some sympathy with this view, we feel that these concerns can and should be taken into account through careful trial design. G-CSF is already entering into use in neonatal intensive care units in an uncontrolled way and, unless this research is actively promoted, we may lose the opportunity to obtain firm evidence of efficacy.

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