Neonatal Sepsis in the Very Low Birth Weight Preterm Infants: Part 1: Review of Patho-physiology

Khalid N. Haque
92, Grange Road, Guildford, Surrey GU2 9QQ, United Kingdom

Abstract
Background: Over the last fifty years neonatal care has made tremendous progress; increasing survival, reducing morbidity, developing newer modalities of care and therapy for the very low birth weight (VLBW) and premature infant. However, mortality from neonatal sepsis in this group of infants has remained between 18-20% in the developed world and around 80% in the developing world for last three decades with little sign of decline. There is also clear evidence that VLBW infants who survive infection in the neonatal period are at significantly greater risk of neuro-developmental delay; making sepsis the most important cause of mortality and morbidity in this group of infants today.

Objective: The objective of this review is to highlight the reasons for this lack of success in combating neonatal sepsis successfully. These can be attributed to four main reasons: 1) poor host defences, 2) clinician’s inability to diagnose sepsis early and accurately [due to lack of or general availability of highly sensitive and specific markers], 3) clinician’s poor understanding of the ‘process’ i.e. patho-physiology of neonatal sepsis, thus not being able to institute early ‘goal’ directed therapy, and 4) total reliance on killing the pathogen(s) with inadequate attention to correcting the consequences of the inflammatory process itself.

This review presents a brief epidemiological background to neonatal infections in the VLBW infants, discusses host defence systems and how immune compromised VLBW infant combats infection by describing the patho-physiological ‘process’ of sepsis in detail. It is our belief that understanding the heterogeneity and complexity of host response and the defence systems is fundamental in formulating management strategies.

Conclusion: By discussing patho-physiology, current available diagnostic tests and presenting an evidence based management ‘care bundle’ it is hoped to change clinician’s paradigm to use more immune and molecular markers for diagnosis and monitoring of the infection process and in management considering adjunctive therapies that boost host defences.

It is recognised that while this review is static i.e. it presents evidence as we understand it today, sepsis is a dynamic process. Our understanding, ability to diagnose and manage neo-natal sepsis is constantly changing and will continue to change and evolve. By presenting this review it is hoped that over a period of time more of our practices would become evidence based and dogma abandoned.

Keywords: Neonatal sepsis, patho-physiology, diagnosis, management, very low birth weight infant (VLBW).

Correspondence
KHALID N. HAQUE
92, Grange Road, Guildford
Surrey GU2 9QQ
United Kingdom
E-mail: KhalidNH99@yahoo.com

Introduction and Background
Neonatal sepsis remains the unconquered frontier of modern neonatal medicine today, despite advances in knowledge, technology and therapeutic armamentarium available. Blood stream infection rates in hospitals (in the
developed world) range from 10-25% for all neonates to around 50% in preterm very low birth weight (VLBW) infants.\(^{1,2}\) Exact figures for the developing world are not known but are considerably higher. World Health Organisation estimates that of the four million neonatal deaths all over the world every year, over 35% are due to infection in the neonatal period;\(^3\) this translates to approximately two deaths per minute! Whilst most of these deaths take place in the developing world where mortality from sepsis may be as high as 85%, in the developed world neonatal mortality from sepsis has remained around 20% for nearly three decades.

This two part review deals with babies born weighing 1500 Grams or less or earlier than 32 weeks of gestation who have the highest rate of mortality and twenty times greater chance of developing infection (often multiple) between birth and first month of life.\(^{4,5}\) Though under reporting is common, prevalence of confirmed neonatal bacterial infection and or meningitis is reported to be between 1-5/1000 live births but in the preterm and VLBW infants it maybe as high as 1/230 live births.\(^6\) Blood stream infections rates in neonates range from 40% in the community and 10-25% of those admitted to hospital for all neonates and up to 50% in extremely preterm infants.\(^1\) Whilst overall gestation specific survival has consistently improved over the years, mortality from neonatal sepsis in the VLBW infants has not declined from 18-20% for the last three decades in UK, USA or Australia.\(^7\) Worryingly Barbara Stoll et al.\(^8\) in a study of over 6000 infants weighing 1000 Grams or less have confirmed earlier studies\(^9-11\) that VLBW infants who survive at least one proven sepsis episode in the neonatal period have 30-80% increased odds for neuro-developmental impairment and a 30-100% increase in odds for poor head growth (an indirect reflection of poor neurological development) at 18-22 months. This is also true for infants with Coagulase Negative Staphylococcus (CONS) or culture negative sepsis hitherto thought to be benign due to low mortality but who show similar poor neuro-developmental outcome at 18-22 months.

High incidence of both suspected early onset sepsis (EOS) \(\{\text{within 72 hours of birth}\}\) and late onset sepsis (LOS) \(\{\text{infection after 72 hours of birth}\}\) and high levels of mortality and morbidity has led to over 50% of VLBW infants being investigated and treated with antibiotics.\(^5\) Escobar and colleagues\(^6\) have estimated that in United States alone as many as 600,000 infants are screened to ‘rule out’ sepsis while an estimated 130,000-400,000 are treated with antibiotics every year though less than 20,000 actually have proven infection!! This is a serious concern; because it not only promotes development of resistant bacterial flora but also increases length of hospital stay and care of cost.

Newborn infants are normally colonised within 48 hours to first few days after birth by both Gram-negative and Gram-positive organisms and Candida species, this process is much quicker if they require resuscitation at birth or are admitted to neonatal units. EOS most frequently is with organisms like GBS, E.Coli, Staphylococcus aureus, and Klebsiella species whilst LOS is mainly with coagulase negative staphylococcus (CONS), Serratia and Citrobacter species.\(^4\) (Table 1).

Table 1. Most frequent bacteria causing neonatal infection

<table>
<thead>
<tr>
<th>From the Mother</th>
<th>From the Environment</th>
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<tr>
<td>(pre/perinatal)</td>
<td>(postnatal/nosocomial)</td>
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<tr>
<td>Escherichia coli</td>
<td>Staphylococcus aureus</td>
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<tr>
<td>Group B streptococci</td>
<td>Staphylococcus epidermidis</td>
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<tr>
<td>Staphylococcus aureus</td>
<td>Escherichia coli</td>
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<tr>
<td>Streptococcus pneumoniae</td>
<td>Pseudomonas aeruginosa</td>
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<tr>
<td>Listeria monocytogenes</td>
<td>Serratia species</td>
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<tr>
<td>Ureaplasma urealyticum</td>
<td>Citrobacter species</td>
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<td></td>
<td>Enterobacter species</td>
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<td></td>
<td>Salmonella</td>
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It appears that worldwide Gram-negative sepsis is on the increase in VLBW infants, in some reports accounting for more than half of EOS and one third of LOS. It is well known that in developing countries Gram-negative infections form the bulk of both EOS and LOS, but interestingly in a recent cohort of VLBW (< 1000 Grams) infants born at or before 28 weeks of gestation, E.Coli was the most common organism causing EOS in Norway. Most infections occur in infants who have one or more of the known 'risk factors'.

We and others have identified 'risk factors' that predispose VLBW infants to sepsis, these include: prolonged rupture of membranes (> 18 hours), presence of chorioamninitis, repeated vaginal examination in labour, maternal urinary tract infection during pregnancy, need for resuscitation at birth, birth weight less than 1500 Grams and or gestation of or below 31 weeks. Other factors include umbilical catheterisation, long line insertion, total parenteral nutrition (TPN), poor hand washing practices, use of H2 blockers and prolonged or un-necessary use of antibiotics.

Institution of continued surveillance policies in neonatal units have led to better understanding of pattern of sepsis in individual units and in neonatal networks but the critically important comprehensive understanding (of host defences and the patho-physiology of the sepsis cascade) is often not fully appreciated in formulating a management plan. This perhaps is the most important reason for continued high mortality and morbidity in neonatal sepsis.

We have suggested the following as possibly the four main reasons for continued mortality and morbidity from neonatal sepsis;

1) Poor understanding of the host defences of the VLBW infant
2) Inability to diagnose sepsis accurately and early.
3) Imprecise understanding of patho-physiology of sepsis leading to inadequate management strategies such as 'goal directed' therapy which has been successfully applied in adults with sepsis.
4) Total reliance on killing the infecting pathogen/s (with a particular 'course' of antibiotics) while paying little or inadequate attention towards correcting the consequences of the inflammatory process itself and/or boosting host defence.

In first part of this review I shall discuss host defence mechanisms and patho-physiology of neonatal sepsis in detail. In part two we define sepsis and discuss how to investigate and manage sepsis in the VLBW infants according to current evidence concluding by suggesting a pragmatic 'care bundle'.

It should be made clear at the outset that though there are many commonalities between bacterial, viral and fungal infections in the newborn this review deals only with bacterial infection.

**Host Defence in the VLBW Infant**
Main function of the human defence system is to protect the host from invading pathogen/s. For this, the first line of defence are the physical barriers e.g. keratinised skin, mucus membranes and chemicals in the form of enzymes and other substances (e.g. secretory IgA) that inhibit bacterial adhesion to the host or have a direct anti-bacterial action. Epidermal barrier of the skin matures around 32-34 weeks of gestation accelerating rapidly after birth this process can be accelerated by applying oil on premature skin. Mucosal defence is largely dependent on the protective layer provided by secretory IgA (sIgA) that is low in preterm VLBW infants increasing with feeding of colostrum and in response to environmental factors by 2-5 weeks after birth. Use of H2 blockers and continuous naso-gastric feeding (common practice in neonatal care) increase gastric pH thus decreasing bacterial destruction and increasing the risk of infection.

Apart from immunity acquired passively through the placenta there are two defence systems working conjointly that respond to
pathogen/s; 1) the innate immune system and 2) specific or the adaptive immune system. The innate immune system provides the initial immunological response and is responsible for induction of the secondary specific/adaptive immune response. Immune system in mammals develops from cells developed in the yolk sac, fetal liver and bone marrow. These cells then differentiate and proliferate to form components of the innate and adaptive/specific immune system.

**Innate Immune System**

Role of the innate response is to provide for a smooth transition from the normally sterile intrauterine environment to the antigen rich extrauterine environment. Nearly all the cells of the haemopoetic system (granulocytes, macrophage, monocyte, dendritic cell and the natural killer (NK) lymphocyte) along with complement, cytokines and acute phase proteins are involved in innate immunity. This system is characterised by its immediate response, limited diversity, non-specificity and lack of immunologic memory. Pathogen recognition is dependent on pattern recognition through toll-like receptor (TLRs) found on cell surface. TLRs play a central role in recognising and helping the cell to engulf pathogen through 'pattern recognition', and activating other elements of the innate immune system. A number of TLR's have been identified that are specific for recognising bacteria, fungi and viruses.

1a) Cellular Components of the Innate Immune System

1) Macrophage is perhaps the most important cell in the innate immune system. It is derived from blood borne monocytes that first appear in foetal liver and blood during the 5th and 6th week of gestation and in lymph nodes around 12-14 weeks of gestation. Macrophage can discriminate between ‘foreign’ and ‘self’ molecules thus are ideal cells for surveillance and scavenging pathogens. Along with neutrophils, macrophages have receptors for antibodies and complement that enhance their opsonisation and phagocytic ability. This later ability is deficient in the VLBW preterm infants.

2) Alongside activated macrophage interdigitating dendritic cells behave as soluble antigen-presenting cells that up regulate CD80 and CD86 on their surface, induce proliferation of T lymphocytes by secretion of cytokines and endocytose extra-cellular antigens. In neonates data indicates significant deficiencies in this process thus reducing the production of appropriate cytokines in response to infection.

3) Neutrophils are essential for immediate response; they appear in foetal circulation from 10-16 weeks of gestation. In the VLBW infant though they are large in number in the circulating pool (peripheral blood) but the bone marrow storage pool is only 20% of that a term infant. While the neonate can rapidly increase the number of neutrophils in circulation following an infectious stimulus from its bone marrow storage pool it also equally rapidly depletes it, often totally consuming it in severe sepsis (causing severe neutropenia).

4) For neutrophils to get to the site of infection they need to stop rolling along the vascular wall and adhere to the vascular endothelium, deform and pass between endothelial cells. Decreased expression of beta-2-integrins on the neonatal vascular surface leads to diminished adhesion and immobility of neutrophils. For passing through the endothelial cells neutrophils need to deform by formation of actin filaments, this ability is significantly reduced in the neutrophils of VLBW preterm infants, added to this the increased fluidity of their cell membrane results in reduced plasticity/deformability thus delaying transmigration of neutrophils through the endothelial cells. Once outside the vascular compartment, neutrophils move towards the site of infection guided by various chemotactic factors. These chemotactic
factors are also reduced in preterm infants leading to decreased accumulation of neutrophils at the site of inflammation. At the site of inflammation neutrophils ingest and destroy the opsonised pathogen by action of anti microbial proteins and hydrolytic enzymes28 producing a ‘respiratory burst’ i.e. a sudden increase in cellular metabolism of oxygen, leading to production of toxic oxygen metabolites that have bactericidal activity. The capacity to generate ‘respiratory burst’ and activate chemiluminescence is significantly reduced in neutrophils of the preterm infant.30

5) Other cells Unlike neutrophils and macrophages, eosinophils and basophils have only weak phagocytic activity. Natural killer (NK) cells which are large granular lymphocytes destroy infected cells by linking to antibody coated target cells and cytotoxicity are present in adequate numbers. The number of NK cells increases with gestational age reaching adult levels at term, but their capacity for cytotoxicity is much less in the newborn due to phenotypical and functional differences from adult NK cells.31

6) Role of erythrocytes and platelets is often overlooked when discussing sepsis but as they also have complement receptors, they play an important role in clearance of antigen-antibody complexes. Erythrocytes due to their nitric oxide carrying capacity have an important role in maintaining blood flow improving tissue perfusion and oxygen delivery so often compromised in sepsis.

1b) Soluble Factors (Complement, Acute-Phase Proteins and Cytokines)

1) Complement system consists of around 20 proteins produced mainly by the liver of the foetus and the newborn. They first appear in the foetal liver around the 10th week of gestation. Complement system can be activated in three ways; i) classic pathway activated at C1 level by antigen-antibody complexes, ii) alternate pathway by products of microbial cell wall and iii) the lectin pathway by the interaction of microbial carbohydrate with mannos-binding protein in plasma. Complement activation generates immunologically active substances that enhance opsonisation, phagocytosis and release of inflammatory and chemotactic mediators. They form a ‘membrane attack complex’ which perforates the cell membrane of the pathogen causing its death. Newborns in particular preterm VLBW infants have only 10% or less of matenal levels of the terminal cytotoxic components like C3 and C3b that lead to killing of the organism, thus significantly compromising their ability to kill the pathogen. More importantly the VLBW preterm infants have difficulty in activating the rapidly responsive alternate or the mannose binding lectin pathway32,33 compromising chemotaxis, localisation, opsonisation, phagocytosis and killing of the pathogen---- all elements important in the fight against infection.

2) Molecules collectively called acute-phase proteins like C - reactive protein (CRP), proteinase inhibitors, amyloid A protein and various coagulation proteins function to enhance resistance to infection and promote repair. These are deficient both quantitatively and qualitatively in the preterm VLBW infant.34

3) Cytokines are a group of soluble mediators that act as messengers between cells of the immune system and between the cells of the immune system and other systems through an integrated network to regulate host immune response.35 Cytokine response in the newborn infant is related to both gestational age and the environmental milieu. Pro-inflammatory cytokines develop gradually with increasing gestational age while anti-inflammatory cytokines are regulated on an individual basis influenced by the intra-uterine cytokine environment.36
Adaptive/Specific Immune System

Specific/adaptive immune system is dependent on T and B cells and their products like antibodies and cytokines. Specialised T and B lymphocytes are responsible for the very large diversity of this system. This system not only responds to microbial and non-microbial antigens but unlike the innate immune system has the ability to lay down ‘memory’ which enables a quantitatively and qualitatively superior immune response to be mounted on reexposure.

1) T Lymphocytes are specialised cells activated either by directly recognising antigen or being stimulated by antigen-presenting cells. They are capable of producing over a thousand T-cell receptor variable regions as transmembrane molecules responsible for expressing or producing cytokines that regulate the immune system. T lymphocytes that are important for adaptive immunity are CD4+ or the helper Th1 cells whose function is to activate macrophage through interferon gamma (INF$\gamma$) and to encourage B lymphocytes for production of antibodies. Generation of these T lymphocytes is delayed in neonates particularly the VLBW infant. Other important T lymphocyte is the CD8+ or Th2 cytotoxic cell which along with NK cells mediates lysis and eradication of pathogen. In the preterm VLBW infant, both Th1 and Th2 lymphocytes are markedly reduced, exhibiting a slow proliferative response, decreased cytotoxic and cytolytic activity and reduced production of appropriate cytokines.

2) B lymphocytes are responsible for production of immunoglobulin’s/antibodies. Initial response to an antigen challenge is production of IgM. However the capacity to do so in the neonate is only around 10% of that of an adult.

3) Similarly, synthesis, memory and capacity to respond by immunoglobulins like IgA and IgG is limited in the neonate.

Passive (Transplacental) Immunity

Transplacental transfer of immunoglobulin’s starts around 12 weeks of gestation, increasing in a direct linear correlation with gestational age. Initially transplacental transfer is slow and selective, for example, IgG1 and IgG3 (more effective against viral infection) are transferred more efficiently than IgG2 and IgG4 (more effective against encapsulated organisms). IgG2 and IgG4 only reach 50-60% of maternal levels at term though the total level of IgG in infant at term is the same or higher than the maternal levels. We have shown that a serum level of 400 mg/dl of total IgG appears to be critical to prevent the newborn from infection as none of the infants in our cohort who attained level above 400 mg/dl died from infection whilst all the infants who died from sepsis had levels below 400 mg/dl. The foetus normally achieves this level of total IgG around 32 weeks of gestation and it is not surprising that infection is highest before 32 weeks of gestation.

Human milk provides several protective elements like sIgA and lactoferrin (main protein content of mature breast milk) both have antimicrobial and immune stimulatory properties. Oligosaccharides present in breast milk help in development of ‘friendly’ intestinal flora that are essential for reducing the growth of pathogenic bacteria in the gut.

Genetic Influence

It is increasingly recognised that an individual neonate’s response to pathogen depends on its genetic makeup and polymorphisms of its gene coding for proteins involved in recognising and responding to pathogen. Though knowledge concerning genetic polymorphisms is still quite limited, it is known that polymorphisms in TNF locus (TNF$\alpha$-308 and TNF$\beta$-252) for example, correlates with immune dysfunction and increased susceptibility of the host to infection. Lipopolysaccharide (LPS) elicits its response by binding to cell surface through TLR4, due to genetic polymorphism impaired TLR4 pathogen processing leads some neonates to respond poorly to an LPS challenge. The same is true for many other
elements responsible in infection cascade e.g. tumour necrosis factor (TNF), Interleukin-10 (IL-10) and mannose binding lectin (MBL). Thus, in summary: In the preterm VLBW infant, though all molecular and cellular elements necessary for adequate host defence are present; their number/capacity or function is reduced (newborn's immune naivety) accounting for decreased magnitude of immune response. This immune naivety is made worse by sepsis. Unless this is adequately addressed in the management package along with killing of the pathogen/s it is unlikely that mortality rates from sepsis will come down.

Pathophysiology: The Sepsis Cascade
Sepsis disturbs the harmonious balance that exists in healthy state between pro and anti-inflammatory cytokines, coagulant and anti-coagulant elements, and between endothelial integrity and circulating cells. Infection by a pathogen disturbs this balance. Body deals with infection by activating many of host defence systems simultaneously to regain the balance. If the balance is regained then outcome is recovery, but if this balance is either not restored or accentuated then the outcome is poor.

During the inflammatory process, cells of the haemopoetic system and immune modulating mediators are activated to move towards the affected site for destroying the pathogen. Activation of the inflammatory response is initiated by release of endotoxin (LPS) from Gram-negative or exotoxins (peptoglycans) from Gram-positive organism and other cellular antigenic components of the pathogen/s. From then on initiation and maintenance of inflammatory cascade result from a complex array of interactions between pathogen and host defence systems.4,7 Leukocyte activation in particular that of macrophage and mononuclear cells brings about transcriptional changes related to immune activation and signal transduction dependent on genetic predisposition and bacterial characteristics.43

Transcription factors up-regulate the production of pro-inflammatory cytokines such as TNF-α, INFγ, IL-6 and anti-inflammatory cytokines IL-10, IL-18.44 Activation of complement pathway leads to generating C3b that coats the pathogen (opsonisation), production of C5a and chemotactic neutrophils factors along with C3a and C4a that degranulate mast cells causing contraction of smooth muscle increasing permeability of vascular endothelium allowing activated cells to move out of the vessels. Substances released from pathogens and damaged tissues up regulate adhesion molecules on the vascular endothelium arresting and activating rolling neutrophils on to the vascular wall. Activated neutrophils change shape to pass through the vessel wall and move to the site of infection where they phagocytose C3b coated organisms. Mediators like complement, chemokines, products of prostaglandin metabolism, and leukotrienes all contribute towards recruitment of inflammatory cells to the site of infection. As described earlier preterm VLBW infants are either deficient or inefficient in generating these responses in an adequate manner. In particular, poor transmigration of neutrophils and chemotaxis results in lack of localisation of infection hence the neonate is prone to more frequent generalised blood stream infections.

The process of activated inflammatory cells producing range of pro-inflammatory mediators like TNF-α, IL-1, IL-6, and IL-8, platelet activating factor (PAF), leukotrienes and thromboxane A2 accentuate endothelial damage.46 Leak of granulocytes and other mediators through the injured endothelium cause the clinical effects seen in sepsis which can be enumerated by the synonym CHAOS; C = Cardiovascular; changes in the micro and macro-circulation, decrease vascular tone, poor tissue perfusion, hypotension and organ failure.

H = Haemopoetic; anaemia, neutropenia, disseminated intra-vascular coagulation (DIC).
A = Apoptosis; increase in planned cell death.

O = Organ dysfunction; renal, hepatic and cardiovascular system failure.

S = Suppression of the immune system; immune paralysis (usually transitory).

The process of CHAOS take place with varying degree of severity in every infant with sepsis and correction of CHAOS, the imbalance between pro-inflammatory and anti-inflammatory cytokines, hypercoagulation and fibrinolysis apart from killing the pathogen is required for adequate management of sepsis.

Inflammation and coagulation are closely linked in sepsis for example TNF-α, IL-1 and IL-6 activate monocytes that express tissue factors which in turn stimulate the extrinsic coagulation pathway leading to the formation of fibrin clots. Thrombin that normally maintains a balance between coagulation and fibrinolysis also has a pro-inflammatory effect on cells of the endothelium (making them sticky) in addition to making macrophage and monocytes release inflammatory mediators. In sepsis, thrombin generation becomes un-regulated leading to an initial hypercoaguable phase followed by the septic process impairing normal fibrinolysis, therefore, the body becomes less able to remove the microthrombi causing DIC often seen early in neonatal sepsis. During this initial hypercoaguable phase coagulation factors are consumed rapidly leading to fibrinolysis and bleeding also seen in infants with severe sepsis.

Relationship between infection, brain (white matter) injury and neuro-developmental impairment though now established, its pathogenesis is only now being gradually understood. White matter injury due to infection is likely to be the result of multifactorial events involving direct toxin insult, cytotoxic injury and vascular compromise associated with hypoxic/ischemic events. It is also now recognised that hypoxic ischemic brain injury is accentuated in presence of infection and vice versa. The presence of inflammatory cytokines in the brain is known to inhibit proliferation of neuronal precursor cells, activate astrogliosis and stimulate oligodendrocyte death all of which increase white matter injury and hamper recovery.

Thus, it is important to appreciate that whilst microorganisms may initiate the sepsis process, it is our response to their presence that make the disease. We are as much in danger of injury from the bacteria as we are from our own response or lack of it to their presence.

References


Neonatal Sepsis in the Very Low Birth Weight Preterm Infants: Part 2: Review of Definition, Diagnosis and Management

Khalid N. Haque
92, Grange Road, Guildford, Surrey GU2 9QQ, United Kingdom

Abstract
Background: Having presented brief epidemiology of neonatal infection and patho-physiology of neonatal sepsis in the first part of this review we now address the difficulties in defining, diagnosing and treating neonatal sepsis.

Objective: The objective of this part of the review is firstly, to highlight the reasons for lack of consensus on the definition of neonatal sepsis despite a number of international conferences of experts on the subject. Secondly, to discuss the increasing sophistication of available laboratory tests and why they all lack the certainty desired by the clinician and thirdly to discuss the various evidence based treatment modalities available to treat neonatal sepsis.

Conclusion: It is suggested that pragmatic definition of sepsis as suggested by us should be adopted. Greater use of biomarkers and molecular tests should be made to diagnose sepsis early and accurately. Lastly, it is hoped to change the clinician’s paradigm by using evidence based management care bundle/package that includes adjunctive immune-modulatory and host defence boosting drugs.

Introduction
Webster English dictionary describes sepsis as “putrefaction”, i.e. decomposition of organic matter (by bacteria or fungi) resulting from interaction between germ and host. Joseph Carcillo has suggested that ‘sepsis’ is when systemic inflammatory response syndrome (SIRS) occurs in the presence of a “living infection”. Despite numerous consensus conferences there is still no agreed definition of sepsis! The reasons for this are complex, reflecting the marked clinical and biochemical heterogeneity observed in the affected septic individuals due to their genetic variation, environment, state of hosts defence system and characteristics of the pathogen/s involved. Similarly, till very recently there has been no clear definition of blood stream infection in the neonatal period and there is still no consensus as to what constitutes sepsis or septic shock in the newborn, though akin to adults it is not an infrequent problem. Lack of consensus also highlights the fact that sepsis far from being a homogenous condition reflects a continuum from foetal inflammatory response syndrome (FIRS) {akin to systemic inflammatory response syndrome (SIRS) described in adults} to sepsis, severe sepsis, septic shock, multi-organ failure and death (Fig. 1). The infected infant moving from one phase to another in either direction imperceptibly. In a study of 908 out of 1612 infants admitted to our neonatal intensive care unit between 1st January 1996 and 31st December 2000 who were suspected and investigated for sepsis, using regression analysis
we found that the most significant clinical findings for sepsis were presence of tachyapnoea with grunting/chest retraction or apnoea, temperature instability and a capillary refill time of greater than 3 seconds. Of the laboratory tests leucopenia (<4000 × 10⁹ /L) or leucocytosis (>34,000 × 10⁹ /L), C-reactive protein greater than 10 mg/dl and interleukin 8 value of greater than 70 pg/ml were the most important variables. Based on these findings, evidence from literature, and an international consensus conference of experts in 2004 we have suggested a pragmatic and user friendly definition of neonatal sepsis in which we define sepsis as the presence of two or more of clinical features plus one or more of laboratory parameters outlined below with or without positive blood culture:

- Presence of Tachyapnoea (respiratory rate > 60 bpm) plus grunting/retraction or desaturation.
- Temperature instability (< 36 °C or > 37.9 °C)
- Capillary Refill time > 3 seconds
- White Blood Cell count (< 4000 × 10⁹ /L or > 34,000 × 10⁹ /L)
- C-Reactive Protein > 10 mg/dl
- IL-6 or IL-8 > 70 pg/ml
- 16SrRNA gene PCR: Positive

**Diagnosis**

Fischer has very elegantly demonstrated that though the ability of a senior experienced clinician to diagnose sepsis is high, there is still lack of diagnostic certainty at the cot side which is often influenced by the presence or absence of 'risk factors', (described in Part 1 of this review), lack of specific clinical signs and symptoms, differing pathophysiology and crucially the lack of highly sensitive and specific laboratory test.

![Suggested Continuum of Infection in the Newborn](image)

*Presence of two or more of clinical features plus one or more of laboratory parameters.*

Fig. (1). Suggested continuum of sepsis.
Of the known ‘risk factors’ enumerated earlier we have reported that the two most important risk factors are; birth at or before 31 weeks of gestation (OR 3.9, 95% CI 1.4-11.0) and or birth weight less than 1500 Grams (OR 5.7, 95% CI 2.5-15.6).6

Clinical signs and symptoms (Table 1) of neonatal sepsis are non-specific because they are often associated with characteristics of the causative organism and the body’s response to the pathogen/s. These non-specific signs and symptoms are also either common to or associated with other neonatal conditions like respiratory distress syndrome, metabolic disorders, and intracranial bleeding. Signs and symptoms like temperature instability, changes in heart rate or its variability, apnoea, prolonged capillary refill time, hypotension and or decreased urine output, persistent metabolic acidosis, hypo or hyperglycaemia individually have low sensitivity and specificity with none exceeding the likelihood ratio of 15%.9,10 Added to this, are the ever changing metabolic changes due to sepsis that are reflected in the constantly changing signs and symptoms in sepsis. These changes vary from initial phase of hypo-metabolism (temperature and heart rate variability), decreased energy expenditure (lethargy), decreased cardiac output (hypotension, prolonged capillary refill time), lower oxygen consumption and vasoconstriction (peripheral cyanosis, apnoea) to the later phase of hyper-metabolism, increased energy expenditure (irritability, increased oxygen requirement), increased cardiac output (tachycardia) and high oxygen consumption (cyanotic episodes).11

### Diagnostic Tests

Clinicians in search of certainty of diagnosis have long sought biological marker/s that would provide them with early and accurate diagnosis or markers that would also provide guidance to treatment.12,13 There is an increasing array of laboratory tests for diagnosis of sepsis but despite initial enthusiasm most have failed to reach the level of accuracy and consistency or practical utility required by
the practicing clinician. For the most commonly used diagnostic test (Full blood count, Blood culture, CRP) the chances that infection is present are less than 50% when taken on their own. Thus, for a greater degree of certainty clinicians frequently use combination of biological markers to improve their predictive ability. Some newer test, or combinations however provide high positive (PPV) and negative (NPV) predictive values but they are either expensive or not universally available and where they are, clinicians need a paradigm change to use them more frequently. Below we discuss most frequently used current diagnostic methodologies, their advantages and disadvantages and suggest the potential advantages of using tests that measure immune response of the patient to diagnose and monitor sepsis.

Blood culture remains the ‘gold standard’ but is often unreliable when intra-partum antibiotics have been administered to the mother. Blood culture also fails to detect bacteraemia in 27%-92% of preterm VLBW infants. This is often due to the volume of blood inoculated into the blood culture bottle being insufficient or suboptimal processing of the specimen, but perhaps the most important reason is that bacteremia is often transitory or intermittent. Yield from blood culture can be improved not by sending repeated small volume samples but by inoculating a minimum of 0.5-1 ml of blood into the blood culture bottle. Further difficulty with blood culture is its ‘turn around’ time of at least 18-24 hours; this is too long for a test on which clinical decisions have to be made. Recent automated culturing systems based on presence of CO₂, or pH provide higher degree of accuracy and a ‘turn around’ time between 12 and 36 hours.

Biomarkers

There is an on going search for an ideal test or a biomarker that is accurate with high degree of sensitivity, specificity, PPV and NPV that could be delivered in real time. No such test has yet been described. The desire to find a single biomarker is fundamentally flawed and is unlikely to be fruitful because of the complexity and heterogeneity of the sepsis process described above. Moreover, it should be remembered that some tests no matter how sensitive are often negative when taken immediately at birth or before the onset of an inflammatory response. Never the less continued search to find improved methods for diagnosing and monitoring sepsis is exceedingly important when one considers the material and other cost of inappropriate use of antibiotics, drug resistance, increased length of hospital stay due to the uncertainty of clinical diagnosis.

Leukocyte number, character and indices are most frequently utilised to diagnose or monitor sepsis. In nearly 50% of infants with infection their values may be normal at the initial phase of infection only to become abnormal after 12 hours or so. Total leukocyte counts below 4000 x 10⁹ /l or above 30,000 x 10⁹ /l is considered abnormal with sensitivity between 17%-90%, and specificity 31%-100%. Total immature neutrophils count of greater than 1% or immature to total neutrophils ratio of greater than 0.02 has a PPV of only 23% but a NPV of 92%. If platelet count of less than 100,000/cu.mm is added to immature to total neutrophil ratio greater than 0.02 then the PPV increases to 43% and NPV to 96%. An important consideration in resource limited conditions.

Acute Phase Proteins: These are endogenous peptides produced mainly by the liver as a response to tissue injury, or infection. The most frequently used and most studied is CRP.

C-Reactive Protein (CRP). CRP is synthesized by the liver following IL-6 activation; it is involved in coagulation and opsonisation. CRP increases late in infection, with a lag time of 12-24 hours explaining the low sensitivity (60%) early in sepsis that increases to 84% by 48 hours after the onset of sepsis. Specificity and NPV also improve with time reaching 99%-100% by 48 hours of onset of infection. It must be
remembered that neonate’s capacity to produce CRP is lower than that of an adult another reason for its reduced sensitivity.20 We21 and others17-22 have found serial measurements of CRP more helpful in determining duration of antibiotic therapy rather than its ability to diagnose sepsis.

Procalcitonin (PCT): PCT a 14-kDa protein that rises within 4 hours following onset of infection with a half life between 22 and 29 hours sometimes longer in sepsis.23 It is produced by monocytes and the liver. Diagnostic utility of PCT in early onset sepsis is limited due to endogenous postnatal surge of PCT after birth peaking at 24 hours of postnatal age. PCT also has low sensitivity (81.4%), specificity (80.6%) and low NPV of 72% in premature infants.23-25 Stocker et al have suggested serial PCT determinations allow shortening the duration of antibiotic therapy in term and near-term infants with suspected early-onset sepsis. Before routine PCT assessment or PCT-guided antibiotic strategy can be recommended, its usefulness has to be confirmed in a larger cohort of premature neonates.26

Inter-alpha Inhibitor Protein (Iαl): This acute phase protein belongs to the family of serine protease inhibitors that are synthesized in the liver. Unlike other acute phase proteins Iαl is down regulated by inflammation. A recent study27 in nineteen neonates has demonstrated decreased levels of Iαl in infants with sepsis. The numbers studied were small and the values overlapped with those in non-infected infants thus the accuracy of this test needs to be studied further.

Serum Amyloid A (SAA): An acute phase protein induced by IL-1, TNF-α and IL-6. It increases by 8-24 hours after onset of infection and has a sensitivity of 96% with a NPV of 99%.28 Though more robust than many other acute phase reactants larger studies are required to establish this as a routine test in neonates.

Other Acute Phase Proteins: There are a large number that have been studied e.g. neoptin, Lactoferrin, alpha-1-anti-trypsin, anti-thrombin, and others but none have gained popularity due to their poor sensitivity, specificity or technical problems.13,29

Cytokines and Chemokines: Recently there has been a flurry of interest in the possibility of using cytokines and chemokines to diagnose and monitor sepsis both in adults and in neonates. Initial measurements of pro-inflammatory cytokines like TNF-α, IL-2 and Interferon gamma (INFγ) were disappointing due to their very short half life (~17 minutes) leading to high false negative results. Measurements of circulating pro-inflammatory cytokines with longer half life have proven to be more fruitful. Levels above 70 pg/ml of IL-6 or IL-8 have a sensitivity of 77%-97% specificity between 76%-93%, a PPV of 42% and NPV of 99% in sepsis. Kauster et al.30 noted that IL-6 actually increased two days prior to clinical diagnosis of sepsis in neonates suggesting that they may be very early markers of sepsis. Chemo-attractant IL-8 with a sensitivity of 92% and specificity of >70%, NPV of 94% appears to be a better marker of neonatal sepsis than IL-6.5 Current interest is focused on IL-10 an anti-inflammatory cytokine which strongly inhibits pro-inflammatory cytokines like TNFα, interleukin 1, 6, 12 and 18 in additions to inhibiting translocation of nuclear factor-κB (NF-κB).31 Anti-inflammatory cytokine IL-10 in combination with IL-6 and RANTES (regulated on activation normal T cell expressed and secreted) have recently been shown to diagnose disseminated intra-vascular coagulation secondary to sepsis with near absolute certainty (sensitivity 100%, specificity 97% and NPV of 100%).32

Availability of semi-quantitative cot side measurement of IL-6 requiring only 50μl blood and a ‘turn around’ time of 20 minutes has brought the prospect of early cot side diagnosis a little closer but this method warrants robust clinical trial before it can be recommended. We (unpublished) using multiplex bead technology have studied an array of cytokines and chemokines in preterm
neonates with suspected and proven bacterial sepsis. With a drop of blood (<50 µl) collected on a Guthrie card and a two hour turn around time, we have evaluated 20 pro-inflammatory cytokines and chemokines. In this pilot study of 60 infants with culture proven sepsis we found macrophage inflammatory protein (MIP-1β) to be the most useful diagnostic and prognostic marker with sensitivity of 93% and specificity of 87% and a NPV of 98%. This needs to be evaluated further with a larger cohort of infants.

Molecular Markers: There has been a tremendous advance in the use of molecular techniques for diagnosis and monitoring sepsis. These tests are fast and reliable.

Polymerase Chain Reaction (PCR): PCR for bacterial 16SrRNA gene gives a sensitivity of 96%, specificity of 99.4%, PPV of 88.9% and NPV of 99.8%.33 Using microarray hybridization technique PCR not only detects bacteremia but can also identify the offending organism.34 Thus PCR has significant advantages over blood culture in that it has much higher accuracy, a short (4-6 hours) ‘turn around’ time and requires only 0.2-0.3 mls of blood but it is expensive and is not universally available. Commercial companies are developing machines to do PCR within neonatal units.

Soluble Triggering Receptor Expressed on Myeloid Cells (sTREM-1): This cytokine promoter has been evaluated as a potential marker for sepsis with sensitivity between 96%-98% and specificity 89%-90% in adults with pneumonia and other conditions associated with infection.35 We are currently evaluating sTREM-1 in neonates with suspected sepsis.

Cell Surface Antigens: Availability of flow cytometric analysis of cell surface markers has enabled the study of cell surface antigens in sepsis.36 CD64 has sensitivity between 81%-96% and a NPV between 89%-97%.37 Whilst promising, estimation of cell surface markers is limited by the need to process blood samples.

Table 2. Sensitivity and specificity of various laboratory tests for early diagnosis of neonatal sepsis*

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
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<tr>
<td><strong>Individual Tests:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood Culture</td>
<td>11 – 38</td>
<td>68 – 100</td>
<td>90 – 100</td>
<td>72 - 100</td>
</tr>
<tr>
<td>WBC &lt;4000 or &gt;30,000/cumm</td>
<td>17 – 90</td>
<td>31 – 100</td>
<td>50 – 86</td>
<td>60 - 89</td>
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<tr>
<td>I/T Ratio &gt;0.2</td>
<td>78</td>
<td>45</td>
<td>23</td>
<td>92</td>
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<tr>
<td>CRP &gt;2 mg/dl (EOS)</td>
<td>88</td>
<td>90</td>
<td>99</td>
<td>96</td>
</tr>
<tr>
<td>CRP &gt;2 mg/dl (LOS)</td>
<td>37</td>
<td>86</td>
<td>67</td>
<td>84</td>
</tr>
<tr>
<td>PCT &gt;2 ng/ml</td>
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<td>97</td>
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</tr>
<tr>
<td>IL-8 &gt;70 pg/ml</td>
<td>91</td>
<td>74</td>
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<td>PCR. 16SrRNA</td>
<td>96</td>
<td>99</td>
<td>89</td>
<td>99</td>
</tr>
<tr>
<td>sTREM-1 &gt;60 ng/ml</td>
<td>96</td>
<td>89</td>
<td>86</td>
<td>96</td>
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<tr>
<td>CD 64</td>
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<td>71</td>
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<td><strong>Combination Tests:</strong></td>
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<td></td>
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<tr>
<td>I/T ratio +CRP</td>
<td>89</td>
<td>41</td>
<td>76</td>
<td>94</td>
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<tr>
<td>PCT +CRP</td>
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</tr>
<tr>
<td>IL-8 + CRP</td>
<td>91</td>
<td>90</td>
<td>89</td>
<td>96</td>
</tr>
</tbody>
</table>

WBC: Total white blood count, I/T Ratio: Immature/Total neutrophils count, CRP: C-reactive protein
PCT: procalcitonin, sTREM-1: soluble trigger receptor expressed on myeloid cell, IL-8: Interleukin 8

*Adapted from various sources referenced in the text (mean values).
rapidly before neutrophils die from apoptosis or the surface antigens are down regulated plus the need for sophisticated equipment.

To summarise, diagnostic tests on the whole except perhaps PCR have poor or indeterminate accuracy and or are often not universally available.\(^{38}\) None achieve the desired objectives of being quick, sensitive, and specific with high PPV and NPV. Unfortunately PCR is not commonly available 24 hours of the day in many institutions. Clinicians are therefore obliged to take a pragmatic view on how to use the commonly available tests either individually or as most do, use a combination to assist them in diagnosing and monitoring sepsis. A list of useful tests is given in Table 2.

Urine Examination: Due to difficulties with collection of clean samples and the risks associated with catheterisation or supra-pubic aspiration this important investigation is often not done. Low rate of urinary tract infection in the newborn has lead most authors to recommend against routine urine culture to diagnose sepsis.\(^ {39,40}\) There are inadequate studies evaluating true value of examining and culturing urine as part of every ‘sepsis work-up’. Urine bacterial antigens are no substitutes as their accuracy is poor.\(^ {41}\) Surface cultures (Skin, Ear, and Umbilicus) are not very helpful, their reliability is very poor and their routine use should be abandoned.\(^ {42}\)

Cerebrospinal Fluid (CSF)

There is considerable difference of opinion amongst clinicians and in literature whether CSF should be examined every time a ‘sepsis work-up’ is performed. Due to low rate of meningitis (1% of over 9000 blood culture positive infants\(^ {43}\)) many authors do not recommend routine lumbar puncture in the absence of a positive blood culture or localizing findings.\(^ {44,45}\) Current opinion varies from including CSF examination in every ‘work-up’, to examining the CSF when there are clinical features of meningitis or examining the CSF only when there is a positive blood culture. Data however suggests that as many as 38% of CSF culture-positive meningitis in neonates have negative blood culture taken at the same time.\(^ {44}\) This may have to do with the problems associated with blood culture as enumerated earlier rather than true dichotomy between blood and CSF culture positivity rates. Never the less it remains a fact that meningitis can only be diagnosed or excluded if CSF is examined! We routinely include CSF examination in late onset sepsis evaluation but are selective in doing a lumbar puncture for early onset sepsis, a practice based on on-going surveillance data collected in our unit over last twenty years.

Management

Main objective of managing neonatal sepsis is to prevent it by reducing the source of bacterial entry into the neonate. This is best done by observing good hand hygiene, infection control techniques, avoiding unnecessary breaking of skin, using proper asepsis when skin has to be broken and intrapartum prophylaxis for maternal GBS carriage or PROM. There is some evidence that application of oil on the skin of VLBW infants reduces the rate of infection in these babies.\(^ {46}\)

Once the pathogen has entered the body then the aim of treatment is to kill the offending pathogen/s as quickly as possible, provide initial resuscitation if required, reduce/neutralise/eliminate bacterial toxins, regain the disturbed immunological and coagulation imbalance, boost host defences and most importantly correct the ‘CHAOS’ caused by the sepsis process itself (Fig. 2). This is a tall order and each element is as important as another and none can or should be ignored.

Thus, in an ideal world therapy for managing/treating neonatal sepsis should have the ability to kill the pathogen/s, increase macrophage surveillance, neutralise bacterial toxins, increase the number and function of neutrophils, improve opsonisation, phagocytosis and chemotaxis in addition to activating complement, preventing cytokine induced damage, correct coagulation and immunological disturbance
all at the same time! It is clear therefore, that there is unlikely to be a single ‘magic bullet’ that could cover all these requirements. Hence a composite generic ‘package’ or ‘bundle’ of care needs to be developed that could be adapted to the particular and unique needs of an individual baby. Recommendations offered below are based on evidence where available, personal practice and pragmatism; they cannot replace the wisdom of an experienced clinician who has to make clinical decisions 24/7 based on the available unique set of variables for any given infant.

Initial Resuscitation

Initial standard resuscitation should be initiated as soon as it is recognised that the infant has severe sepsis or impending septic shock (Fig. 1) which is often difficult to recognise early.

Killing the Pathogen (Antibiotic Therapy)

There is strong evidence that intra-partum prophylaxis for GBS or preterm prolonged rupture of membrane reduces the risk of neonatal infection. Use of Ampicillin instead of penicillin for GBS prophylaxis has raised concerns about the rise of Gram-negative and Ampicillin resistant E.Coli infections. Knowledge of local flora and different characteristics of antibiotics are key to effective and safety of antibiotic therapy. In clinical practice, threshold for starting antibiotics on suspicion of infection is justifiably diffuse and low. There is almost universal agreement that initiating early empiric antibiotic treatment on suspicion of EOS with penicillin (or a penicillin derivative like Ampicillin) plus an aminoglycoside (frequently Gentamicin) after obtaining adequate cultures and other samples is important because delay in initiating antimicrobial therapy is known to worsen the outcome. Choice of antibiotic however depends on the known susceptibility pattern, but should have a wide spectrum and be bactericidal in nature. Some initiate mono-therapy with a second or third generation cephalosporin in extremely low birth weight infants due to their relative lack of toxicity and better concentrations in the CSF. It must be emphasised
that use of wide spectrum mono-therapy with third generation cephalosporins has been associated with rapid development of glycopeptide resistant enterococci and selection of beta-lactamase producing Gram-negative organisms. Evidence from randomised clinical trials however does not suggest that any one regimen of antibiotic/s is superior to another in either EOS or LOS. Initial antibiotic therapy should be altered on the basis of microbiological and clinical data once a pathogen/s have been identified.

As stated above no ideal regimen for treating suspected LOS can be recommended either; many clinicians use a combination of glycopeptides (Vancomycin or Teicoplanin) and Ceftazidime (or an aminoglycoside) initially. For suspected Gram-positive Coagulase Negative (CONS) organisms like or S. epidermidis antibiotic regimen consists of either Vancomycin alone or Vancomycin and an aminoglycoside or Tichoplanin is suggested as most strains of S.aureus produce beta lactamase making them resistant to penicillin G, Ampicillin, Carbenicillin and Ticarcillin. Mono therapy with Vancomycin should be avoided due the potential of developing Vancomycin insensitive (VISA) or resistant (VRSA) S. aureus, and Vancomycin resistant enterococci (VRE). Cephalosporins are an attractive alternative due their lack of toxicity and good CSF concentration but their use has been associated with increase in resistance by Gram-negative organisms e.g. Klebsiella pneumoniae. Nafcillin or oxacillins are other useful substitutes to Vancomycin.

For Gram-negative organisms either third generation cephalosporin or an aminoglycoside (Gentamicin or Amikacin) are the usual antibiotics of choice. Emergence of resistance in Gram-negative organisms to these antibiotics is a cause for concern. Aminoglycosides and Vancomycin are both potentially nephrotoxic and ototoxic, therefore should be used with caution and their serum levels monitored.

Extended spectrum beta lactamase (ESBL) producers like Klebsiella, Serratia and E.Coli are resistant to beta-lactam agents. These organisms are best treated with carbapenems (Meropenem, Imipenem) with or without fourth generation Cephalosporins e.g. Cefepime. Meropenem is preferred over other carbapenems in neonates because of its better safety profile. Aztreonam a mono-lactum which is tolerated well by neonates is effective against antibiotic resistant Gram-negative bacilli and aerobic Gram-negative bacilli.

Whilst the choice of antibiotics used is determined by susceptibility of the pathogen to the antibiotic and its pharmacokinetics, there is no consensus as to the duration of antibiotic therapy. Most clinicians stop antimicrobial therapy if the blood culture is negative and the infant is well. In culture proven sepsis clinician often give a ‘course’ which varies from 5 to seven to 21 days (mean 9 days), longer for meningitis (14-21 days) and osteomyelitis (4-6 weeks). These ‘courses’ are not based on any evidence but dogma and personal practice. Except for meningitis and osteomyelitis there is ample evidence that shorter duration of antibiotic therapy (5 days or less) in culture proven sepsis is either as good or better than giving antibiotics for longer periods. Recently Engle and colleagues have shown that cure and recurrence rates in term infants with pneumonia were the same between those infants who received antibiotics for four days or seven days. This is supported by Marc Labenne and colleagues who found that reducing duration of antibiotic therapy does not increase the risk of infection relapse in neonates with early onset sepsis, in fact it decreased the incidence of late onset sepsis in these infants. For the last fifteen years it has been our practice not to give antibiotics for more than four days in culture proven sepsis (except in meningitis or osteomyelitis) and have had no cause to regret this practice. Ideally duration of antibiotic therapy should be guided by infants clinical condition.
and biomarkers like IL-8, MIP-1ß, or PCR. Where these are not available then a combination of C-reactive protein and immature to total neutrophils ratio provide a good monitoring tool with a NPV of 94% (Table 2).

There are no studies comparing different durations of antibiotic treatment in neonatal meningitis thus it is difficult to give evidence based guidance. Our practice is to give antibiotics for two weeks for Gram-positive and three weeks for Gram-negative meningial infection. Textbook recommendation for duration of antibiotic therapy for osteomyelitis is six weeks but recently shorter periods of antibiotics (2-3 weeks) have been advocated with good results.68,69

Intravascular access devices are a major source of sepsis; they should be promptly removed if thought to be infected. Prophylactic antibiotic (low dose Vancomycin) have been shown to have some benefit70 but the potential of developing either Vancomycin resistant (VRE) or Vancomycin insensitive S. aureus (VISA) heavily out weighs the benefit. We do not endorse the use of prophylactic Vancomycin.

Adjunctive Therapy

Neonates more than any group of patients are likely to be exposed to prolonged use of broad-spectrum antibiotics thus are vulnerable to multi-resistant pathogens. Moreover, despite the dramatic increase in both the number of novel antimicrobial agents, antibiotics have proven to be alarmingly insufficient on their own to combat infections in these vulnerable infants with the added problem of increasing drug resistance. This has generated considerable interest in the development of adjunctive immune-modulatory therapies. It is recognized that some antimicrobial agents also have immune-modulatory effect either by directly effecting the immune response or as an indirect consequence of the release of immune modulatory molecules from the bacteria or host cells e.g. depression of phagocytosis (aminoglycosides), anti-inflammatory (macrolides) while some cephalosporin’s enhance immune function but β lactams have no known immune-modulatory effect.

Methods to physically remove toxins by exchange transfusion or replace the depleted storage pool of neutrophils by granulocyte transfusion have not been successful (RR 0.89, 95% CI 0.43, 1.86) in reducing mortality from sepsis and significant pulmonary complications have been reported following granulocyte transfusion.71

Haemopoetic growth factors (GM-CSF, G-CSF) have also not been shown to reduce mortality from sepsis (RR 0.71, 95% CI 0.38, 1.33) except in infants who along with sepsis have severe neutropenia and are growth restricted (RR 0.34, 95% CI 0.12, 0.92).72 Use of TNFα antibodies, soluble TNF receptor, IL-1ra bacterial permeability increasing proteins or nitric oxide inhibitors have failed to reduce mortality from sepsis.73

Activated protein C which reduces neutrophils adhesion to vascular endothelium and restricts TNFα, IL-1 and IL-6 secretions from monocytes has been found useful in adults but in neonates significant bleeding has been reported with its use. Thus, its use is not recommended until further studies in the newborn are available. Low dose steroid therapy has not been investigated in the newborns nor has therapy with pro-inflammatory cytokine INFγ.74,75

Pentoxifylline: A xanthine derivative, carbonic anhydrase inhibitor that inhibits release of TNFα and improves white cell function has been shown to significantly reduce mortality in infants with sepsis in small studies from Poland (RR 0.14, 95%CI 0.03, 0.76).76 Larger trials of this exciting drug are underway in neonates.

Polyclonal Intravenous Immunoglobulin (IVIG): By virtue of their diverse repertoire immunoglobulin’s posses a wide spectrum of antibacterial and antiviral specificities.77 IVIG provide antimicrobial efficacy independently of pathogen resistance. While individual
clinical trials of IVIG in neonatal sepsis have demonstrated dramatic reduction in mortality, number of systematic reviews have yielded contradicting results, in part due to 1) varying study design, 2) failure to include important studies in analyses, 3) inclusion of neonatal, paediatric and adult studies and 4) combining prophylactic and treatment studies together. In our view IVIG continues to represent one of the most promising adjuvant strategies for the treatment of infection in both adults and neonates for the following reasons; Whilst IVIG are polyclonal and heterogeneous serum/plasma derived agents making each preparation distinct and unique. They on the whole have been shown to; increase the number of circulating neutrophils, improve neutrophil migration to the site of infection, prevent depletion of neutrophil storage pool in neonates, increase neutrophil chemo luminescence, opsonic activity, and chemotaxis while also activating complement and inactivating C3b containing complexes thereby reducing C3 activation and complement mediated inflammation.

Kazatchkine has shown that IVIG modulate antibody and cytokine production and activation, interfere with selection of B cell repertoire, control B cell proliferation, neutralize pathogenic auto antibodies, regulate CD8 mediator suppressor or cytotoxic T cell function and super antigens. IVIG down regulate the IL-1 system; contain antibodies directed against IL-1, IL-6 and IFN α, β, and γ that modulate the cytokine cascade. IVIG’s have also been shown to have cytoprotective effect on TNFα induced cell death in fibroblasts. Thus there are many good reasons to consider the use of IVIG in the treatment of sepsis particularly in critically ill and or immune-compromised patients like the VLBW infant.

In a Cochrane systematic review significant reduction in mortality was noted in infants with proven sepsis (RR 0.35, 95% CI 0.23, 0.54) and (RR 0.50, 95% CI 0.34, 0.73) thus, it would seem adding IVIG as adjunct to standard therapy is advantageous. Complication rates reported are extremely low (<0.5%) and mandatory regulations have made IVIG one of the safest blood products available but the extremely low risk of viral or prion transmission of 1/4800, 000 cannot be totally excluded. Recruitment to the International Neonatal Immunotherapy Study (INIS) that has been looking whether addition of polyclonal IVIG to standard treatment reduces mortality in neonatal sepsis has just finished and results of this study are awaited.

Bovine Lactoferrin: In a recent randomized trial bovine lactoferrin supplementation has been shown to reduce the incidence of late onset sepsis in VLBW infants (risk ratio, 0.34; 95% CI, 0.17-0.70; p = .002) but further studies are required to confirm these results.

Supportive Therapy

Fluid Therapy: Fluid resuscitation is the hallmark of treating hypovolemic and septic shock. It does not matter whether colloid or a crystalloid solution is used (though possibly smaller volume of colloid is required for the same effect). There is no evidence from randomized clinical trials to support routine use of early volume expansion in very preterm infants without cardiovascular compromise and insufficient evidence to determine whether infants with cardiovascular compromise benefit from volume expansion. However, in sepsis there is often ‘third spacing’ or pooling of fluid in the vasodilated compartment, for which isolated slow bolus of 10-20ml/kg of fluid given over 20-30 minutes may be helpful. To prevent reperfusion injury it may be preferable to increase the total volume of fluid rather than give boluses. End points of adequate fluid resuscitation in sepsis should be normalization of heart rate, oxygen saturation, serum lactate and pH. It is important to remember that those infants who after adequate fluid resuscitation...
do not self-diurese may need diuretics to prevent fluid overload.

Inotropes: Fluid resuscitation is key and must be achieved prior to instituting vasopressor or inotropic agents. Dopamine acts by its vasoconstrictive action and dobutamine by increasing cardiac contractibility and output. In neonatal sepsis there is initially fall in vascular resistance due to vasodilatation that is followed by decrease in cardiac output. Hence, dopamine is often the first inotrope chosen. In a systematic review93 dopamine was found to be marginally more effective in the short term. Clinically however, it does not significantly alter the outcome which inotrope is used first. There is little experience using other vasopressors in neonates with sepsis.

Coagulation: Sepsis causes the vascular endothelium to become prothrombotic and anti-fibrinolytic. In sepsis anti-thrombotic factors are consumed leading to micro-thrombi formation and DIC, followed by consumption of prothrombic factors leading to spontaneous bleeding. It is important therefore to constantly evaluate coagulation profile of the preterm infant with sepsis. Prolonged prothrombin time/partial thromboplastin time and low fibrinogen levels suggest DIC there is neither consensus nor evidence as to the best method to treat DIC.

Thrombocytopenia is also a feature of severe sepsis and once again there is no consensus when platelet transfusion should be given though most would transfuse platelets if they were less than 50,000/cu mm.5

Anaemia: Anaemia is not an uncommon feature in sepsis due to bleeding, haemolysis and blood loss from multiple sampling. There are no studies guiding transfusion policies in septic newborns. As alluded to above, tissue perfusion and oxygenation are often compromised in sepsis, these must be rectified. In our practice we accept a lower limit of haemoglobin of 10 grams/dl (Hct 33) in a septic preterm neonate below which we would transfuse red cells but have no evidence to support this practice.

Metabolic Control: There is insufficient evidence from randomised controlled trials to determine whether infusion of base or fluid bolus reduces morbidity and mortality in preterm infants with metabolic acidosis secondary to sepsis.94 Acidosis is usually secondary to hypoperfusion or hypoxia that require correction in their own right. Bicarbonate solutions are very hyperosmolar and are associated with intra-ventricular bleeding, thus they should be used sparingly and with caution.

Of greater importance is to maintain a tight glycemic control during sepsis. Hyperglycemia in sepsis by itself is immuno-suppressive and prothrombotic in nature, thus has the potential to make the clinical condition and outcome worse. Hyperglycaemia in sepsis is mainly due to insulin resistance preventing glucose from entering the Kerb cycle. Whilst there is agreement not to allow glucose level to fall below 30 mg/dl, there is no consensus as when to institute insulin therapy. Hyperglycemia is best treated by early initiation of insulin therapy rather than reduction in glucose concentration of infusion. As a matter of good practice rapid fluctuation in blood glucose levels should be avoided.

Nutrition

During sepsis the infant is catabolic (using its own tissue as metabolic fuel) thus its metabolic and caloric needs are increased, this is worse in preterm VLBW infants who has poor muscle mass, body fat and energy reserves. It is essential that catabolic state secondary to sepsis is corrected rapidly by providing the infant with adequate quantities of energy (10% dextrose infusion is adequate to provide 4-8 mg/kg/minute of glucose) minerals, and vitamins.42 Enteral feeding is preferable as it reduces bacterial translocation from the gut into systemic circulation. If enteral feeding is not possible or additional energy source is required then parenteral nutrition (TPN) should
be provided. It should be remembered that the major factor responsible for TPN-induced bacterial translocation and intraepithelial lymphocytes changes is the lack of enteral feeding and not the administration of the TPN per se therefore where ever possible some enteral feed (non-nutritional/trophic) should be provided.

Strategies to prevent Organ function
Lungs: Respiratory failure in severe sepsis or septic shock is common due to acute lung injury caused by infiltration by activated neutrophils, and surfactant consumption leading to rapid fall in functional residual capacity that may require early ventilatory support and surfactant therapy. Care should taken to avoid hyperoxia for fear of retinopathy of prematurity (ROP) and over distention of alveoli which is a potent inducer of IL-6 release predisposing the infant to secondary lung infection i.e. ventilator associated pneumonia.

Kidneys: Ion channels in tubular epithelium are energy/oxygen dependent thus particularly sensitive to hypotension and hypoxia. Two thirds of preterm VLBW infants will develop renal function abnormalities with sepsis these should be looked for and treated conventionally. There is no evidence that renal replacement therapy (haemofiltration or haemodialysis) is of any benefit.

Liver: Liver insult during sepsis is reflected by sharp rise in liver enzymes and worsening coagulation profile. This damage is often self-limiting but should be treated by standard conventional methods.

Gastrointestinal Tract: As alluded to earlier an empty gut may worsen or initiate sepsis due bacterial translocation across inflamed or damaged intestinal mucosa. This is worse in preterm VLBW infants who lack of immunological protection by sIgA. Use of H2 antagonists and continuous feeding should be avoided during this period as they increase the gastric pH allowing bacteria to pass through this barrier.

Prevention of Infection
Time immemorial adage of ‘prevention is better than cure’ is most apt with regard to neonatal sepsis. Principles of preventing infection are universally known and well documented. they include;

a) Obsession regarding hand hygiene.

b) Education and constant re-enforcement of all staff.

c) Avoiding overcrowding.

d) Maintaining adequate nurse/patient ratio.

e) Applying universal precautions on patient contact.

f) Continuous monitoring and surveillance of infection.

g) Closed system of drug delivery.

h) Applying correct disinfectant to clean equipment.

i) Restricting the use of third generation cephalosporins.

Suggested Management Package (Care Bundle)
From the description and evidence provided above it is clear that management of neonatal sepsis requires a thorough understanding of host defences systems of the preterm VLBW infant and of the sepsis process itself to be able to develop a comprehensive package of care.

Such a care package is presented below;

1) Early recognition of sepsis (Risk factors ± signs and symptoms).
Evidence Class A

2) Early institution of appropriate sepsis screen (inclusion of PCR and cytokine measurements if available).
Evidence Class A

3) Early initiation of appropriate antibiotics (consider shorter duration therapy).
Evidence Class A
4) If perfusion is poor AND serum lactate > 5 mmol/l give 10-20 ml of colloid, if still poorly perfused or hypotensive start inotropes. Evidence Class B

5) Maintain Haemoglobin > 10G/dl (Hct < 33). No Evidence

6) Maintain caloric intake > 100Kcal/day entally or > 80 Kcal/day if on TPN add some trophic feeding if possible. Evidence Class B/C

7) Maintain oxygen saturation between 90 and 92%. Evidence Class A

8) Consider adjuvant IVIG (IgM-enriched) therapy. Evidence Class B

Conclusion

It is recognised that while this review is long and static i.e. it presents evidence as we understand it today and sepsis is a dynamic process. Our understanding, ability to diagnose and manage neonatal sepsis is constantly changing and will continue to change and evolve. By presenting this review it is hoped that practices would become rationale, evidence based and dogma abandoned.

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Metabolic Surgery. A New Surgical Discipline?

Nicola Scopinaro

Department of Surgery, University of Genoa, School of Medicine, Italy

Abstract

Metabolic surgery can be considered as part of functional surgery, where the function to be corrected is a metabolic one. The first known example of metabolic surgery is probably the partial ileal bypass for the treatment of hypercholesterolemia. Modern metabolic surgery was preceded and inspired by bariatric surgery, basing on the strong metabolic effect of some obesity surgery operations, especially gastric bypass and biliopancreatic diversion (BPD), mainly active on type 2 diabetes mellitus. A true metabolic operation, primarily aimed at obtaining a favourable metabolic change, should on one side not provoke undue weight loss, on the other side act through specific mechanisms independent of weight changes. BPD, in clinical use since the late 70s, has proven to meet these requirements and is successfully used today in clinical trials for the treatment of the metabolic syndrome. New developed metabolic operations are represented by duodenal-jejunal bypass, endointestinal sleeve, and ileal interposition. All the efforts should be aimed at conceiving an operation equally effective as BPD, yet less invasive.

Introduction

Any type of surgical activity, to be labelled as “discipline”, needs that that specific surgical activity be used with the only aim of obtaining a specific effect, which, in this case, would be a metabolic change. In other words, metabolic surgery can be considered a discipline if one or more types of operation can be used with the only aim of obtaining, as a result, a metabolic change.

Metabolic surgery can be considered as part of the more generally named “functional surgery”, which in turn may be defined as “a surgically-induced anatomic modification which provokes either the reduction or the annulment of the altered function that causes the disease, or a functional change of opposite direction able to counteract partially or totally the originally altered function”. If that function is a metabolic function, that is metabolic surgery. Good examples of the first type of functional surgery are ablation of endocrine tumors, or splenectomy for idiopathic thrombocytopenic purpura, or antrectomy or vagotomy for peptic disease: in all of these cases the surgically-induced anatomic modification which simply reduces or annuls the altered function. A nice example of the second type of action is pyloroplasty associated with vagotomy, where the gastric emptying problems caused by vagotomy are counteracted by the facilitated emptying provoked by pyloroplasty.

Obesity surgery is obviously functional surgery, where the excessive food intake can be reduced or annulled with gastric restriction procedures, or counteracted with the operations which reduce intestinal energy absorption. Bariatric surgery has many beneficial metabolic effects, which, being simply due to the weight loss, do not allow obesity surgery to be...
considered as metabolic surgery, for at least two good reasons: 1) bariatric surgery is primarily aimed at weight reduction, with metabolic effects being only beneficial side effects secondary to weight loss, which are the better the greater the weight loss and would disappear in case of weight regain, while true metabolic surgery should be primarily aimed at the correction of the metabolic alteration, and it should work independently of weight changes; 2) most important, the metabolic disturbances that accompany obesity, like hypercholesterolemia, hypertriglyceridemia, insulin resistance and type 2 diabetes mellitus, can occur also in the absence of obesity, and, in these case, even the best weight reducing operation would be not only ineffective, but also potentially very harmful. On the contrary, a true metabolic operation should be able to resolve one or more of the above conditions independently of the body weight, that is, also in the lean patient, and without causing any undue weight loss. In a few words, when talking about metabolic surgery, we should simply forget about body weight or BMI.

The first known example of metabolic surgery is probably the partial ileal bypass (PIB) for the treatment of hypercholesterolemia.\(^1\)\(^,\)\(^2\) The operation consists of the exclusion from the intestinal flow of the last portion of the ileum, where the bile salts are absorbed. What results is a near total interruption of the entero-hepatic bile salt circulation, with huge loss of bile salt into the colon and consequent greatly increased bile acid neosynthesis by the liver, which occurs at the expense of the cholesterol pool.\(^3\) The same effect on serum cholesterol is obtained by jejunoileal bypass (JIB),\(^4\)\(^,\)\(^5\) where only a few centimeters of the distal ileum are left in-continuity. The difference between the two operations is that the primary aim of JIB is weight loss, and serum cholesterol reduction is a beneficial side effect, while the PIB is a procedure specifically designed for the treatment of hypercholesterolemia, which can be used in any case of high serum cholesterol, independently of the body weight and the body weight changes, that is true metabolic surgery.

Although both JIB and PIB, because of the many side effects, were abandoned, the concept of bariatric surgery was accepted, with gastric banding,\(^6\)\(^,\)\(^7\) gastroplasty,\(^8\) gastric bypass (GBP),\(^9\)\(^,\)\(^10\) and biliopancreatic diversion (BPD)\(^11\)\(^,\)\(^12\) being developed for this purpose. The latter, due to its specific actions on serum cholesterol, which is exactly the same as in PIB, and on type 2 diabetes mellitus,\(^13\) actions totally independent of weight changes, is to be considered the best example both of functional surgery for obesity and of metabolic surgery for the metabolic syndrome.

BPD, by diverting bile and pancreatic juice into the distal ileum, causes a delayed mixing between food and biliopancreatic secretions resulting in a limited digestion, and thus a limited absorption which is selective for fat and starch, responsible for weight loss and indefinite weight maintenance.\(^14\)

In 1984 the powerful specific metabolic actions of BPD\(^15\) were well known, but at that time bariatric surgery in general and BPD in particular were far from being widely accepted, therefore BPD was continued to be used only for morbid obesity therapy. About ten years later, in the mid nineties, Walter Pories,\(^16\)\(^,\)\(^17\) followed by many others,\(^18\)\(^-\)\(^20\) described the powerful action of gastric bypass on the resolution of type 2 diabetes. In GBP a very small proximal gastric pouch (15-30 ml) causes rapid gastric emptying which, on the one hand, provokes an intense and long lasting postprandial syndrome, and on the other, allows food to reach the ileum, where the production of anorexigenic gut hormones like GLP-1 and PYY is stimulated.\(^21\)\(^-\)\(^23\) Both these actions, provoking reduced food intake, act in tandem to cause weight loss.

As in the case of BPD, GBP action appeared to be a specific one, which was independent of weight loss, since the effect became apparent a few days after the operation. At that moment, bariatric surgery became a discipline
that was accepted worldwide. BPD and GBP had been in clinical use for more than 20 years, and they both showed very important antidiabetic activities. It had become possible to consider surgery for the treatment of diabetes and metabolic syndrome, even if that was not yet true “metabolic surgery”- that is operations that could be considered specifically or primarily aimed at obtaining beneficial metabolic effect- independently of the presence of obesity. Surgical treatment of obesity is indicated for patients with a minimum BMI of 35 kg/m², but since more than 90% of type 2 diabetic patients have a BMI in the range of 25-35, treatment of diabetes then becomes the real target of metabolic surgery.

A first meeting on “true” diabetes surgery was held almost secretly (we were not more than 15 people) in Strasbourg, June 2006, thanks to the initiative of Francesco Rubino, a young researcher, author of wonderful experimental studies in rats, and a second one, more official and with larger participation, though disguised as a meeting on animal model surgery, was held in Boston, in October of the same year. But the main event was the large international consensus conference called Diabetes Surgery Summit, held in Rome in March 2007, the first endorsing body being the American Diabetes Association. The goal of the meeting was to reach a consensus on the essential guidelines for the use of surgery to treat type 2 diabetes. After two days of presentations on the subject, the about 50 more prominent world researchers in the field of endocrinology, diabetology and gut hormones reached some important agreements, the most important, approved unanimously, being that “in patients with BMI lower than 35, determining the appropriate use of gastrointestinal surgery for the treatment of type 2 diabetes is an important research priority”. The statement had been carefully constructed, because, while opening the door to the use of surgery for the treatment of diabetes in patients not morbidly obese, a totally new population, the word “research” clearly indicated that this surgery would be allowed only within carefully designed clinical trials, after the approval of an Ethics Committee.

Therefore, even though only as part of an investigation in this phase, surgery could be used to treat type 2 diabetes in the BMI range for which the use of bariatric surgery is not indicated, that is, independently of BMI. What is immediately evident is that the operations performed in patients with BMIs in the lower range, especially simple overweight (BMI 25-30), should be able to achieve two aims: 1) to cause little or no weight loss in case there is little or no excess weight to lose; 2) to act on type 2 diabetes through specific actions, independent of weight loss. Once we have surgical procedures that meet these two requirements and can be used solely to obtain metabolic changes, only then will we be able to talk about a “new discipline”.

Specific metabolic surgery can be found among those currently used for the surgical therapy of obesity, or new operations can be developed which possess the above two requirements. The two well established bariatric procedures which have proved to possess specific mechanisms of action independent of weight reduction are BPD and GBP. However, only BPD can be considered true “metabolic surgery”, as it can be used with the unique aim of diabetes treatment also in lean people. In fact, in GBP the effect of diabetes resolution cannot be separated from that of weight loss, so that the operation, obligatorily causing weight loss, cannot be used in normal weight people. This does not apply to BPD because BPD does not make one lose weight, it simply leads the operated subject to the weight commensurate with the amount of calories that is able to be absorbed after the operation, so that if the patient’s weight is equal to or lower than that weight, there is no reason for weight loss. Therefore, BPD, as it causes weight loss only if there is an excess weight to lose, can be used with the only aim of diabetes treatment at any body weight, and thus can be considered a true “metabolic operation”. Actually, while GBP for type 2 diabetes...
was used only in patients in the BMI 30-35 range. BPD was successfully employed in both the mild obesity and simple overweight patient ranges. We have recently completed the first year follow-up of a prospective study of 30 type 2 diabetes patients equally distributed between BMI 25 and 35 submitted to BPD, obtaining 83% of control (HbA1c ≤7% on free diet and with no antidiabetic therapy), and 17% of improvement (unpublished data).

How do BPD and GBP work? Let us mention first a peculiar mechanism of action of BPD, which is based on the minimal fat absorption, causing intramyocellular fat depletion with consequent return to glucose utilization as a source of energy and disappearance of insulin resistance. The two other specific (i.e. independent of weight loss) mechanisms of action which have been hypothesized to explain the effects of GBP and BPD are based on two anatomo-functional features shared by the two operations, that is the bypass of the duodenum and the food stimulation of the ileum. These mechanisms are related to a family of gut hormones, called "incretins," characterized by the property of stimulating insulin production by the beta-cell, and mainly represented by the gastric inhibitory polypeptide (GIP) and the glucagon-like peptide-1 (GLP-1), respectively released by the duodenum and the ileum. The first mechanism, which is known as the "foregut hypothesis" is based on the bypass of the duodenum, which is considered responsible for the type 2 diabetes causation. Particularly, Pories hypothesized that an excessive response of the duodenum to food stimulation causes excessive incretin secretion, and thus insulin release, the insulin resistance representing a mechanism of defense. Rubino speculated the existence of "anti-incretins", produced by an ill duodenum on food stimulation, which would interfere with normal incretin action. In both cases, the bypass of the duodenum would solve the problem.

On the contrary, according to the "hindgut hypothesis", the beneficial effect of GBP and BPD would be based on the food-stimulated release by the ileal mucosa of a powerful incretin, the GLP-1, which has proven to be able not only to improve beta-cell function, but also to stimulate beta-cell proliferation and decrease beta-cell apoptosis. An increased production of GLP-1 was demonstrated both after GBP and after BPD.

With the exception of omentectomy, which has proven to be totally ineffective, the newly developed operations specifically designed for type 2 diabetes treatment were inspired by the two above hypothesis. The foregut hypothesis generated the duodenal-jejunal bypass (DJB) surgery, consisting of transecting the duodenum 1-2 cm distal to the pylorus, and then fashioning a short (30 + 50 cm) Roux-en-Y reconstruction with pyloro-jejunal anastomosis. The experiences reported so far by Cohen and Ferzli are rather disappointing. It seems (personal communication by Dr. Ricardo Cohen) that better results can be obtained by adding to this operation a sleeve gastrectomy (SG, a subtotal longitudinal gastrectomy leaving a gastric tube along the lesser curve of no more than 100 ml capacity, which results in much more rapid gastric emptying), but this evidently represents a mix of foregut and hindgut mechanisms. Moreover, sleeve gastrectomy is a weight loss operation, with the consequent risk of excessive weight reduction if used in simply overweight diabetic patients.

Another procedure suggested by the foregut hypothesis is the so called "endobarrier" surgery, consisting of a tubular prosthesis 60 to 100 cm in length which is inserted endoscopically in the duodenum and anchored to the muscular layer distal to the pylorus. What results is a lack of contact between food and duodenal mucosa, but also a shortening of food pathway to the ileum, thus again mixing the two mechanisms. The results, reported by Galvao-Neto after a 12-week implant, are good both in terms of weight loss and of diabetes improvement.
Finally, the procedure exploiting the hindgut mechanism, that is ileal interposition, has been extensively studied in animals in the past for the effect on food intake,\textsuperscript{50-51} and recently for the beneficial influence on type 2 diabetes,\textsuperscript{52-54} and pioneered in man by De Paula\textsuperscript{55,56}. After a disappointing experience with ileal interposition alone (personal communication by Dr. Aureo Ludovico De Paula), De Paula had much better results by adding a sleeve gastrectomy to the procedure, with or without the bypass of the duodenum, the former procedure being more effective. Again, the presence of a weight loss component entails the unpleasant side-effect of undue weight loss. Moreover, it is a formidable major surgery operation, entailing the presence of 4 to 7 staple lines at risk of dehiscence, with no demonstrated advantages compared to the much safer and more effective BPD.

In summary, metabolic surgery can be considered today a true new discipline, which includes all the operations that can be used with the only aim of treating type 2 diabetes, and/or severe hypercholesterolemia, and/or the other components of the metabolic syndrome, independent of BMI. All of these operations belong today to major abdominal surgery, so all future efforts must be aimed at designing new operations equally effective but less invasive.

References


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Islet Transplantation in Bone Marrow

Lorenzo Piemonti
San Raffaele Diabetes Research Institute (HSR-DRI), San Raffaele Scientific Institute, Milan, Italy

Abstract
Islet transplantation can be an important therapeutic option for adults with unstable type 1 diabetes—individuals who, despite their best efforts, have wide, unpredictable fluctuations in blood sugar levels or who are no longer able to sense that their blood sugar is dangerously low. For these people, transplantation may be a viable solution. Since the first report of successful pancreatic islet transplantation to reverse hyperglycaemia in diabetic rodents, there has been great interest in determining the optimal site for implantation. Although the portal vein remains the most frequently used site clinically, it is not ideal. About half of the islets introduced into the liver die during or shortly after transplantation. Complications associated with intraportal islet injection and the progressive functional decline of intrahepatic islets encourage the exploration of alternative sites. In tests in animals, scientists from the Diabetes Research Institute in Milan showed that bone marrow is a promising alternative site for islet transplantation. This review considers bone marrow as site of islet transplantation and metabolic, immunological and technical aspects are discussed.

Introduction
Despite the substantial improvements in insulin therapy thanks to new commercial drugs and the adoption of intensive treatment regimens able to improve glycemic control, exogenous insulin administration cannot avoid the long-term complications of diabetes and the life expectancy of diabetic patients is still shorter compared to that of the general population. In principle, the treatment for type-1 diabetes and many cases of type-2 diabetes lies in the possibility of finding a beta cell mass substitute capable of performing two essential functions: assessing blood sugar levels and secreting appropriate levels of insulin in the vascular bed. Currently, the only available clinical therapy capable of restoring beta cell mass in diabetic patients is the allogenic/autologous transplantation of beta cells (somatic cell therapy with pancreas, Langherans islets or beta cell transplantation).

Replacement of the whole gland re-establishes long-term normoglycemia, with a success rate of 80% and is especially successful for patients who undergo simultaneous pancreas and kidney transplantation. However, because of the risk of surgical complications, this procedure will never be a viable option for most type 1 diabetic patients. Those offered this treatment are patients who have already developed many of the secondary complications, including end-stage renal failure, and still have a quality of life that is adequate for undergoing such a difficult treatment. Since the breakthrough made by Shapiro et al. islet transplantation has emerged as an attractive alternative to whole pancreas transplantation. Despite advances in recent years allogenic somatic therapy is still problematic.
A non-specific immune response mediated predominantly by innate inflammatory processes related to mechanics and site, and pre-existing and transplant-induced auto- and allo-specific cellular immune responses (possibly promoted by the initial inflammation) play a major role in the loss of islets and islet function from the liver. Although significantly improved by the implementation of the Edmonton protocol, our capacity of achieving long-lasting insulin independence in patients with T1D undergoing portal vein islet transplantation remains limited. This indicates that the detrimental impact of innate and adaptive immune responses is not fully contained by the Edmonton protocol-associated regimen of generalized immunosuppression (i.e. induction with daclizumab [anti-IL-2Rα mAb] and maintenance with rapamycin [mTOR activation blocker] plus tacrolimus [calcineurin inhibitor] in a steroid-free treatment).

Prolong intrahepatic islet survival by increasing the potency of such regimen is not practicable, due to the likelihood of enhancing susceptibility to cancer and infections, and the toxicity that some of these drugs may have towards kidney functions and transplanted islets. Rather, it is intuitive that alternative strategies aimed at selectively inhibiting undesired islet-specific or non specific immune responses represent an ideal step towards a better management (i.e. weaning/withdrawal of generalized immunosuppression) and outcome (i.e. long-lasting insulin independence) of islet transplanted T1D patients.

The liver was suggested as an optimal site for islet transplantation by Lacy et al., using a rat model of diabetes. By the 1980s, successful transplantation of islet autografts was reported in humans by using infusion of cells into the patient's liver through the portal venous circulation. Subsequently, the publication of the first case of insulin independence in a diabetic patient after infusion of islets through the portal vein consecrated the liver as the site of choice for the islet transplantation in humans. Because of this early success, the subsequent clinical experience of islet transplantation has been developed almost exclusively using the intra-hepatic infusion through the portal vein. In the last years, however, it has becoming increasingly recognized that the liver may not be the optimal environment as a recipient site for pancreatic islets, owing not only to immunologic but also anatomic and physiologic factors that likely contribute to the decline of islet mass after implantation. Intrahepatic islet infusion in man is associated with an immediate blood-mediated inflammatory reaction, thrombosis and hepatic tissue ischemia with elevated blood liver enzymes. Loss of as many as 50-75% of islets during engraftment in the liver has been suggested to be a prime factor necessitating the very large number of islets needed to achieve normoglycemia. Furthermore, the necessity for cannulation of the portal system to seed the islets produces an increase in portal pressure proportional to the islet mass administered by infusion thus restricting the total mass that can be implanted. As a consequence, a highly purified suspension of islets is needed to transplant sufficient cells to achieve insulin independence. Because the purity of the suspension is inversely proportional to the islet yield per donor, fewer islets can be isolated from the already scarce donor pool, further limiting broad clinical applicability of pancreatic islet transplantation. The recognition of these problems has renewed the interest in the search for an alternative site for implantation such as the intramuscular site and the omental pouch.

Bone marrow (BM) may represent an ideal alternative site for pancreatic islet transplantation, thanks to its protected and extravascular (but well-vascularized) microenvironment. Because of its broad distribution and easy access, BM has the potential to overcome not only the physiologic loss of islets, but also the technical limitations and complications encountered with the intraportal infusion. To address the potential of BM as an alternative site for pancreatic islet transplantation...
tion, we recently implanted syngeneic pancreatic islet isografts (C57BL/6 islets to C57BL/6 mice) into BM of diabetic recipients and assessed short- and long-term graft survival, function and safety in comparison with the liver site. The results show that the BM is a more suitable site than the liver for the implantation of islets. In our study, both the percentage and the timing in reversal of hyperglycemia were superior after BM infusion as compared to intrahepatic infusion using the minimal mass model. Moreover, with the exception of a small delay in gaining normal glucose tolerance after OGTT, the quality of glucose metabolism in mice that reached normoglycemia via intra-BM islet infusion was similar to that achieved by islet transplant into the liver for all the parameters evaluated (fasting and not fasting glycemia, blood insulin, HOMA-B and glucose tolerance after IVGTT). Based on our results we can conclude that the BM site for islet transplantation has a higher probability to reach euglycemia (2.5 fold increase) than the liver without compromising the quality of glucose metabolism. This is relevant because the process of intra-hepatic infusion was traditionally considered optimal due to the supposition that insulin is delivered more physiologically after intraportal transplantation.

However this argument has recently been challenged by experimental studies showing that intra-portal transplanted islets respond to glucose stimulation only when perfused via the hepatic artery; no response is observed after challenge via the portal vein. There are also reports on alterations in islet function after intra-portal islet transplantation, such as a defective glucagon response to hypoglycemia and defective glucose-stimulated insulin release.

Since it was suggested that hyperinsulinemia might contribute to cancer development through the growth-promoting effect of elevated levels of insulin, it is possible that intra-BM islet transplantation could increase the risk of proliferative disease. For this reason we evaluated the impact of islets on hematopoietic activity of BM. After islet infusion the cellularity, the histological appearance, the analysis of cell subpopulation and the progenitor cell frequency were unaffected by the presence of islets in the BM. We also took into consideration the consequences of BM islet infusion on the capacity to respond to virus-induced aplasia and the bone structure. Islets in BM of LCMV infected mice did not affect hematopoietic activity consequent to aplasia nor CTL-mediated viral clearance. These results also suggest that islets in BM are capable of sustaining those metabolic changes that are likely to occur during the rapid expansion of a very robust adaptive immune response (i.e. by day 8 post-infection secondary lymphoid organs of LCMV-infected mice are much larger in size and about 50%-70% of all CD8+ T cells are LCMV-specific).

In conclusion, we show that pancreatic islets can be engrafted into the BM, thus opening a research line with potentially significant clinical impact not only for the treatment of diabetes but for other diseases amenable to treatment with cellular transplantation. Because the BM as a site for pancreatic islet grafts can be clinically applicable and, in theory, can solve many of the problems encountered with the intrahepatic location, further research is warranted by the initial findings presented here to determine whether the results can be reproduced in large animals and eventually in humans.

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ISLET TRANSPLANTATION IN BONE MARROW

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Studies of Immune Mechanisms of Diabetes and Treatment with Isolated Pancreatic Islets

Clyde F. Barker and Ali Naji

Donald Guthrie Professor of Surgery
University of Pennsylvania School of Medicine
Philadelphia, PA 19104 USA

J. William White Professor of Surgery
University of Pennsylvania School of Medicine
Philadelphia, PA 19104 USA

Abstract
Reviewed here are studies in animal models of type I diabetes that have led to better understanding of the disease and progress to its cure by of pancreatic islet transplantation and/or manipulations of the immune system. These studies include: 1) the first complete and permanent cure of chemically induced diabetes in rodents; 2) demonstration of autoimmune recurrence of diabetes in islet recipients; 3) induction of intrathymic tolerance to islets; 4) prevention of autoimmune diabetes by thymic manipulation; and 5) demonstration of the importance of B lymphocytes in autoimmune diabetes and islet rejection. These experimental studies over the last four decades are the basis of a successful ongoing human islet transplant program.

Keywords: Diabetes, autoimmune, islet cell transplantation.

Introduction
In the early 1970’s Dr. Clyde Barker at the University of Pennsylvania and the late Paul Lacy at the Washington University in St. Louis were the pioneers in exploring the concept of islet transplantation as a means to cure diabetes. In 1972, Ballinger and Lacy reported amelioration of diabetes in islet recipient rats.1 In 1973, Barker and Reckard were the first to show that islet transplantation could completely and permanently restore normoglycemia in rodent models of chemically induced diabetes.2 They found that islets, rather than being immunologically privileged tissue and thus partially exempt from rejection as others had assumed, are unusually vulnerable to destruction by immune mechanisms which could be avoided only with potent immunosuppression based on anti T cell antibodies.3 Transplanted islets also sensitized their hosts, inducing accelerated rejection of subsequent donor strain grafts of islets or other tissues. Barker’s basic early findings heralded the challenges still being encountered in human trials of islet transplantation.

Barker and Naji next investigated the fate of islet transplantation in BB rats, the only animal model at that time of spontaneous genetically determined diabetes and closely reminiscent of human type I diabetes.4 Their experiments yielded two remarkable findings. First, BB rats rendered immunologically tolerant by neonatal inoculation of allogeneic bone marrow from normal rats were usually protected from
developing diabetes. The disease prevention afforded was shown to be attributable to the generation of hematopoietic cellular chimerism and restoration of self tolerance to beta cell autoantigens.\(^5\) This finding was the first conclusive evidence that diabetes was an autoimmune disease, at a time when different etiologies such as viral infections were being strongly considered. It also represented the first immunotherapy for the prevention of autoimmune diabetes. Second, those immunologically tolerant BB rats that did develop diabetes were found to permanently accept skin allografts yet mount a vigorous response to islet transplants. That this recurrent anti-beta cell autoimmunity destroyed transplanted islets but could be prevented by immunosuppression were crucial findings that several years later were confirmed in human pancreas transplants between identical twins.\(^6\) The biologic threat of recurrent autoimmune damage of transplanted islets is probably highly relevant to current clinical outcomes in human type I diabetic patients.

In view of their finding that autoimmune diabetes is a consequence of the failure in self-tolerance to beta cell antigen(s), Barker and Naji explored the impact of intrathyemic inoculation of islets on the restoration of central immune tolerance. When a small number of islets were transplanted into the thymus of newborn BB rats, it was found that this exposure to putative islet autoantigens completely prevented the development of diabetes, indicating the restoration of tolerance to these antigens by ‘educating’ developing thymocytes during T cell ontogeny.\(^7\)

In addition, these experiments showed the thymus to be a previously unrecognized privileged site, as evidenced by the permanent survival of allogeneic islets transplanted into the thymus.\(^8\) Akin to the impact on autoimmunity, exposure of developing thymocytes to alloantigens of islets or other cells implanted to the thymus led to a state of donor specific immunological tolerance via deletion or inactivation of T lymphocyte clones.\(^9\) This prevented rejection of donor strain grafts transplanted outside of the thymus. Though intrathyemic islet transplantation has yet to be utilized in patients because of biologic problems related to atrophy of the adult human thymus, these studies provided important proof of concept for induced central tolerance.

By the early 1980s, it was evident that type I diabetes is the result of the selective destruction of insulin-producing islet beta cells by autoreactive T lymphocytes. The presence of islet-specific autoantibodies in the serum of human subjects however suggested that the loss of B lymphocyte tolerance to islet autoantigens may also be involved in the destruction of beta cells and the development of diabetes. This concept was tested in a series of studies by Naji and Barker to assess the role of B lymphocytes in the pathogenesis of autoimmune diabetes.\(^10-12\) With Hooman Noorchashm, they reported that depletion of B lymphocytes prevents insulitis and the onset of diabetes in non-obese diabetic (NOD) mice. Insights gained from these basic studies have led to a current clinical trial of B cell depletion by rituximab (i.e., anti-CD20) for the reversal of recent onset type I diabetes.

Subsequently, they reported that the dysregulation of B cell tolerance is linked to a failure to eliminate from the developing B cell repertoire autoreactive B lymphocytes with beta cell autoantigen specificity.\(^13\) Physiologically, B cell tolerance occurs via two dominant mechanisms, central deletion of autoreactive B cell clones in the bone marrow or functional inactivation in the peripheral lymphoid system. The stringency of selection at the latter tolerance checkpoint is dominantly regulated by the TNF-related B cell survival factor, B lymphocyte stimulator (BLYS) which is the limiting survival factor required for successful B cell maturation. Naji is currently focusing on the processes that govern the tolerance checkpoint of immature transitional B cells in the peripheral immune system and the effect of modulating this checkpoint on the development of diabetes.
In this regard, Naji recently demonstrated in NOD mice that neutralization of BlyS leads to marked depletion of follicular and marginal zone B lymphocytes in the spleen, abrogating production of insulin autoantibodies, and offering protection from progression to spontaneous diabetes. As such, BlyS inactivation may be a logical and novel target of immunotherapy for the prevention of islet beta cell destruction of type I diabetic patients. Dr. Naji’s work also provided a mechanistic rationale for testing the efficacy of the anti-BlyS antibody belimumab as a novel immunotherapeutic agent for the prevention or reversal of type I diabetes in the clinical setting.

Based on their findings on the requisite role of B lymphocytes in autoimmune diabetes, Barker and Naji have investigated the efficacy of B cell depletion therapy in the induction of immunological tolerance to islet allografts in non-human primates. These studies revealed that B cell depletion at the time of islet transplantation promotes prolonged islet allograft survival without the need for chronic immunosuppression. An immunosuppressive regimen including the anti-B cell agent rituximab allows survival of islet allografts transplanted to diabetic cynomolgus monkeys for as long as 4 years.

Convinced of the progress they made over two decades of research to develop effective immune intervention strategies to prevent the rejection of islet allografts, Barker and Naji developed a clinical islet transplantation program at the University of Pennsylvania. The program, established in 1999 includes a state of the art cGMP islet isolation facility that produces high-quality human islets both for clinical transplantation and basic research. The facility has been selected as an Islet Cell Resource Center in the United States, not only for transplants in their own program but also for distributing high-quality human islets to other centers for transplantation and basic research in islet biology. The outcome of the initial series of 18 human islet transplants performed at the University of Pennsylvania between 2001 and 2005 was similar to that of the widely reported simultaneous series at Edmonton. All patients who completed the protocol of 2-3 islet infusions became normoglycemic without requirement for insulin. But over the next 4-5 years, there was a progressive loss of islet allograft function and recurrence of diabetes indicating vulnerability of islets grafts to recurrent autoimmunity or rejection just as predicted by the earlier experiments of Barker and Naji in BB rats.

Encouraging results from the pre-clinical studies on the efficacy of B cell depletion therapy to promote tolerance to islet transplants was one factor leading to the selection by NIH of the University of Pennsylvania’s human islet transplantation program as one of only three U.S. centers to be a member of the Clinical Islet Transplant Consortium. These consortium centers are charged with the mission to improve islet transplant outcome and avoid late failures by exploiting several new immunosuppressive protocols. A central aim of Naji and Barker's program at the University of Pennsylvania is implementing novel immunotherapies including the one based on Naji’s primate studies. It targets B lymphocytes with the aim of achieving transplant tolerance. The first two islet transplants performed under this NIH supported trial were performed by Naji at the University of Pennsylvania in 2008. To date, the subjects show outstanding degrees of glycemic control and have remained insulin independent for more than a year.

References


Modulation of Serine Proteases-Mediated Platelet Activation by Novel Direct Thrombin Inhibitors

Sarfraz Ahmad
Florida Hospital Medical Center, Cancer Institute, Orlando, FL 32804, USA

Abstract
Novel direct thrombin inhibitors (DTIs), such as bivalirudin, are replacing heparin in several clinical scenarios. In particular, DTIs are indicated for the treatment and thromboprophylaxis of patients with heparin-induced thrombocytopenia (HIT). In interventional cardiology, DTIs have several advantages over heparin, and offer a clinical benefit equivalent to that of a combination of heparin and antiplatelet agents. We hypothesize that this benefit results from the ability of DTIs to inhibit platelet activation by activated serine proteases. This study represents the development of a modified 14C-serotonin release assay (SRA) to investigate the relative inhibitory effects of three DTIs (argatroban, bivalirudin and hirudin) on thrombin- and factor Xa-mediated 14C-serotonin release (SR) in plasma-free systems. Washed platelets were isolated from blood of healthy volunteers. The 14C-SRA test was similar to that used to detect heparin-PF4 antibody-mediated platelet activation, except that it was used to evaluate the ability of DTIs to modulate protease-induced SR responses. The inhibitory effects of DTIs were determined at protease concentrations that induced >50% SR. Serine proteases induced SR from platelets in a concentration-dependent manner. Human thrombin was found to be more potent than bovine thrombin (2-3 times for 50-80% SR). Bovine factor Xa (>0.2 nKat/ml) produced a comparable (50-80%) SR response. All three DTIs effectively blocked serine protease-mediated platelet activation in a concentration-dependent manner. The optimum inhibitory concentrations of bivalirudin on SR was ~100 nM for human thrombin and bovine factor Xa and almost double for bovine thrombin; well below plasma concentrations necessary for effective anticoagulation for percutaneous coronary interventions. Wide variations in the inhibitory effects of each DTI on thrombin- and factor Xa-mediated platelet activation were noted, which was partly dependent on the donor platelets and stability of the proteases/inhibitors. It is concluded that DTIs can directly inhibit serine proteases-mediated platelet activation responses.

Keywords: Hirudin, thrombin, factor Xa, serine proteases, thrombogenesis.

Introduction
Serine proteases, such as thrombins and Factor Xa, play key role(s) in thrombogenesis and are capable of activating platelets through various mechanisms. Thrombin is not only a catalyst in the conversion of soluble fibrinogen into an insoluble fibrin clot, but is also an extremely potent platelet activator. Thrombin mediates platelet agonist effect through a unique and specific proteolysis of cell surface receptor known as PARs (protease-activated receptors). Two of the known PARs (namely, the PAR-1 and PAR-2) are expressed by human platelets. The PAR-1 has been designated as having a higher affinity for thrombin than PAR-4. Recently, it has also been shown that thrombin binds to the platelet glycoprotein (GP) Ib/IX/V complex, which supports a role for GPIbα in thrombin-induced platelet activation/aggregation.

Despite a remarkable therapeutic spectrum, the use of heparins is known to have medical complications such as bleeding and the onset
of a potentially catastrophic syndrome known as heparin-induced thrombocytopenia (HIT) associated with or without thrombosis.\textsuperscript{4,5} Heparin as an indirect thrombin inhibitor increases the ability of antithrombin III (AT) to neutralize thrombin and other serine proteases of the coagulation cascade. Platelets, vascular surfaces, plasma proteins, and fibrin are all key factors in this anticoagulant effect of heparin. Heparin’s indirect mechanistic limitations are: i) its inability to inactivate thrombin bound to fibrin, therefore, protecting it from inactivation by the heparin-AT complex;\textsuperscript{6} and ii) its inability to inactivate platelet bound factor Xa, therefore, sheltering it from the heparin-AT complex while secreting platelet factor 4 (PF4), a heparin neutralizing protein.\textsuperscript{7} These limitations make heparin a competitive inhibitor for circulating non-bound thrombin, but unfortunately leave room for a desirable alternative for clot-bound thrombin anticoagulants.\textsuperscript{8,9}

Because of their pharmacokinetic and biological advantages, direct thrombin inhibitors (DTIs), such as argatroban, bivalirudin and hirudin, have recently been developed as a heparin substitute for various clinical indications.\textsuperscript{10-12} In particular, these agents are widely used as the alternate anticoagulant management of heparin-compromised patients (e.g., HIT, where massive thrombin generation occurs in symptomatic patients), requiring therapeutic or interventional anticoagulation.\textsuperscript{13,14} The DTIs have shown a clear advantage due to their ability to inhibit both clot-bound and circulating thrombin. These agents prevent thrombin from interacting with its substrates by binding to either or both the active site and the exosite-1. These two sites are responsible for the modulation of thrombin-substrate interactions. The active site is responsible for cleaving the scissile bond, while the proper orientation of the substrate is determined by exosite-1.\textsuperscript{15}

Thus, DTIs have several advantages over heparin and offer a clinical benefit equivalent to that of a combination of heparin and antiplatelet agents. We hypothesize that this benefit results from the ability of DTIs to modulate platelet activation by activated serine proteases. In this study, a modified \textsuperscript{14}C-serotonin release assay (SRA) was developed to study the relative inhibitory effects of various newly developed DTIs (in plasma-free system), on the platelet activation induced by serine proteases (thrombin and factor Xa).

**Materials and Methods**

**Materials**
The materials used in this study and their sources are given in parentheses: human thrombin and bovine factor Xa (Diagnostica Stago, Gennevilliers, France), bovine thrombin (Pacific Hemostasis, Middletown, VA), 5-hydroxy-\textsuperscript{14}C-tryptamine creatinine sulfate (Amersham, Piscataway, NJ), argatroban (GlaxoSmithKline, Philadelphia, PA), bivalirudin (The Medicines Company, Parsippany, NJ), hirudin, apyrase Grade III from potato, and scintillation fluid/universal LSC cocktail (SigmaAldrich, St. Louis, MO). Other reagents used in this study were of analytical grade and from the best commercial sources available.

**Preparation of Washed Platelets**
The platelet isolation procedure was similar to that described earlier.\textsuperscript{16} Briefly, human whole blood (WB) from normal healthy volunteers was collected by venipuncture into acid-citrate-dextrose (ACD) anticoagulant, pH 4.5 (1 part ACD: 5 parts WB). The first 3 ml of WB were discarded to avoid any pre-activation of platelets. Platelet-rich plasma (PRP) was obtained by centrifugation of blood at 300 \(g\) for 15 min at room temperature. The PRP was incubated with \textsuperscript{14}C-serotonin 0.1 \(\mu\)Ci/\(\mu\)l (2 \(\mu\)l of \textsuperscript{14}C-serotonin for per ml PRP, having specific activity of 2.07 GBq/mmol 56.0 mCi/mmol) for 45 min at 37\(^o\)C. Centrifugation of the \textsuperscript{14}C-labeled-PRP at 600 \(g\) for 10 min at room temperature yielded a platelet pellet. Platelets were washed with 10 ml calcium-albumin-free (CAF) buffer, pH 6.2, containing apyrase as described above. Finally, the pellet was resuspended in albumin-free-Tyrode’s (AFT) buffer, pH 7.4, at a platelet concentration of 250,000 - 300,000/\(\mu\)l for the \textsuperscript{14}C-SRA experiments.
14C-Serotonin Release Assay (SRA)
The 14C-SRA test system used in this study was similar to that utilized in the heparin-PF4 (HIT) antibody-mediated platelet activation responses,17 except that instead of patients' plasma and exogenous heparin, we evaluated the effects of serine proteases to cause platelet activation (serotonin release as an end-point index) under different experimental conditions, and subsequently determined the modulation of platelet activation by various DTIs. The first assay condition evaluated the effect of varying concentrations of serine proteases (bovine and human thrombins, and bovine factor Xa) to achieve the optimal platelet activation responses. Once the optimal serine protease concentration was established, we investigated the modulating effects of DTIs (such as argatroban, bivalirudin, and hirudin) on the serine protease-mediated platelet activation responses.

Briefly, the serine proteases (10 μl in saline, at varying concentrations) were incubated in a round-bottom 96-well plate with 14C-serotonin-labeled washed platelets (70 μl) while shaking gently for 60 min at room temperature. The platelet activation process was terminated by the addition of 100 μl of EDTA (4% solution in saline). The content was centrifuged at 1,600 g for 5 min at room temperature and 50 μl of the supernatant was transferred to a scintillation vial, pre-filled with 2.5 ml of scintillation fluid (universal LSC cocktail). The radioactivity of each sample was determined on a \( ^{14} \text{C} \)-counter connected with a printer (Wallac, Inc., Gaithersburg, MD). The AFT buffer and 10% Triton X-100 solution (30 μl each) were run simultaneously, which served as 0% and 100% controls of the serotonin release responses, respectively.

To determine the effect of various DTIs concentration necessary to inhibit the serine proteases-mediated serotonin release responses, we utilized the similar approach as described above, except that varying concentrations of DTIs (10 μl in saline) were incubated with washed-radiolabeled platelets (70 μl) and proteases (10 μl at a pre-determined concentration where ≥50% serotonin release response was achieved).

Data Analyses
The following formula was used to calculate the percent serotonin release: % serotonin release is equal to the release of the test sample minus the background (the AFT buffer response), divided by the total radioactivity (the Triton X-100 response) minus the background (AFT), multiplied by 100. A ± SEM (standard error mean) was also calculated to account for the certainty of sample means among the data obtained from multiple donors' platelets. Statistical significance was declared at p value <0.05.

Results
The optimal concentration of each serine protease to produce the maximum platelet activation (percent serotonin release) was determined by taking appropriate concentrations of the proteases. Fig. (1A) displays the percent serotonin release response upon activation of platelets by varying concentrations of human thrombin. Human thrombin at a concentration of 0.15 U/ml resulted in an average of 80 ± 3.9% serotonin release while 0.03 U/ml of human thrombin gave an average of 50 ± 7.8% serotonin release. Concentrations < 0.015 U/ml of human thrombin were found to produce < 40% serotonin release, which was considered to be less than optimal. The fixed (optimum) concentration for human thrombin to achieve a range of 50-80% serotonin release from activated platelets, was therefore, set at 0.1 U/ml.

Fig. (1B) shows the concentration-dependent response on platelet activation by bovine thrombin. As can be seen, an optimal concentration range of 0.08-0.4 U/ml of bovine thrombin was sufficient to cause a platelet activation response (79 ± 3.5% serotonin release). The maximum serotonin release of 91 ± 3.3% was, however, found to be achieved at a concentration of nearly 1.0 U/ml of bovine thrombin. There was a steep decline in serotonin release response when bovine thrombin was used at concentration below 0.1 U/ml (only 4-30% release).
Data obtained from the bovine factor Xa-mediated serotonin release response is shown in Fig. (1C). Clearly, about 0.5 nKat/ml of bovine factor Xa was found to be sufficient to produce 73 ± 3% serotonin release. Although, bovine factor Xa at nearly 0.2 nKat/ml resulted in about 55 ± 4.2% serotonin release but any further lowering of factor Xa concentration resulted in the sharp decline of platelet activation responses.

The inhibitory effects of various DTIs were determined at the fixed proteases concentrations that induced >50% serotonin release (usually in the range of 50-80% release, as optimized above). Fig. (2) shows the results obtained on the concentration-dependence of argatroban to inhibit various proteases-mediated platelet activation responses. Bovine thrombin-mediated platelet activation appeared to be most sensitive to argatroban
As only 5-10 nM (which is equivalent to 2.5 – 5.0 ng/ml) of argatroban was sufficient to inhibit significantly (< 25 ± 11% serotonin release), whereas human thrombin- and bovine factor Xa-mediated platelet activation required relatively much higher concentrations of argatroban (at least 19 nM) to achieve a comparable inhibitory response. While platelet activation modulation by argatroban varied for human thrombin, bovine thrombin, and bovine factor Xa, all serine proteases-mediated activations were almost completely inhibited at 190 nM of argatroban (resulting only in < 10 ± 1% serotonin release).

Fig. (3) shows the effect of serine proteases-mediated platelet activation response and its modulation by bivalirudin. Bivalirudin showed a very strong concentration-dependence on

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**Fig. (2).** Effect of argatroban concentration to modulate the serine proteases-mediated platelet activation responses (14C-serotonin release). Washed human platelets were activated at fixed (optimum) concentration of human thrombin (0.1 U/ml), bovine thrombin (0.1 U/ml), and bovine factor Xa (0.5 nKat/ml). High percent of 14C-serotonin release (>50%) can be seen at lower concentrations of argatroban, which is greatly inhibited at higher concentrations of the drug. Each data point represents the average results obtained from different donors’ platelets (n=5) and the data are reported as mean ± SEM of the percent 14C-serotonin release upon platelet activation.

**Fig. (3).** Effect of bivalirudin concentration to modulate the serine proteases-mediated platelet activation responses (14C-serotonin release). Washed human platelets were activated at fixed (optimum) concentration of human thrombin (0.1 U/ml), bovine thrombin (0.1 U/ml), and bovine factor Xa (0.5 nKat/ml). High percent of 14C-serotonin release (>50%) can be seen at lower concentrations of bivalirudin, which is greatly inhibited at higher concentrations of the drug. Each data point represents the average results obtained from different donors’ platelets (n=6) and the data are reported as mean ± SEM of the percent 14C-serotonin release upon platelet activation.
the inhibition of proteases-mediated platelet activation responses. The optimal inhibitory concentration of bivalirudin on serotonin release was determined to be ~100 nM, which is equivalent to < 0.1 μg/ml (for human thrombin and bovine factor Xa). However, for bovine thrombin-mediated platelet activation, a relatively higher concentration of bivalirudin was required to achieve such response. These concentrations of bivalirudin were still well below the plasma levels of the drug necessary for effective anticoagulation during percutaneous coronary interventions (PCI).

Similarly, we tested the comparative inhibitory effects of hirudin (another potent and widely used DTI) on the proteases-mediated platelet activation responses. Fig. (4) shows that hirudin also caused a concentration-dependent inhibition on all the proteases-mediated platelet activation responses. Again, as low as 14 nM (equivalent to ~0.1 μg/ml) of hirudin was sufficient to modulate the proteases-mediated serotonin release by ≤10%.

Discussion
In recent years, several new DTIs have emerged as alternate anticoagulants to heparin in various clinical situations including treatment and thromboprophylaxis of patients with HIT and prevention of acute coronary events and thrombosis. In this study, we compared the thrombin inhibitory effects of some novel DTIs on specific serine protease-mediated platelet activation (serotonin release) responses in plasma-free systems. Our results clearly indicate that despite some variable inhibitory effects of the DTIs (presumably due to their differential biochemical properties and mechanism of action), all these agents, particularly bivalirudin, are capable of modulating the thrombin- and factor Xa-mediated platelet activation responses, at well below the plasma concentrations necessary for effective anticoagulation in cardiovascular indications, such as PCI.

Serine proteases are known to play central role(s) in the coagulation cascade and have
long been known to participate in thrombogenesis and cause platelet activation through direct or indirect mechanisms. In this investigation, we implemented a novel approach by selecting three widely explored serine proteases (namely, human and bovine thrombins and bovine factor Xa) to evaluate their relative ability to activate washed human platelets and thus specifically quantified one of the final released products upon platelet activation (such as 14C-serotonin). The modulatory effects of DTIs on these radio-labeled platelet serotonin release responses were systematically investigated.

Table 1 compares some of the biochemical and clinicopharmacological properties of the three DTIs with that of unfractionated and low-molecular-weight heparins. The discussion below focuses on additional characteristics of the three DTIs that we used in this investigation that are clinically relevant and on its relationship with experimental observations.

Argatroban (Novastan<sup>®</sup>), one of the first synthetic DTIs, is a small molecular weight (526.66 D) compound derived from L-arginine with reported K<sub>i</sub> values of 19-39 nM<sup>18</sup>. This reversible thrombin inhibitor is readily metabolized in the liver and has a molecular formula of C<sub>23</sub>H<sub>36</sub>N<sub>6</sub>O<sub>5</sub>S-H<sub>2</sub>O. Thrombin inhibition occurs by directly blocking the active site on thrombin.<sup>19</sup> The argatroban-thrombin complex has two hydrophobic side chains that create a U-shaped configuration connecting with the S2 and the ary1-binding pocket, both hydrophobic domains of thrombin.<sup>20,21</sup> The highly specific inhibition of thrombin is allowed by a Tyr-Pro-Pro-Trp loop found in the pocket,<sup>22</sup> which allows argatroban to have a highly selective nature towards thrombin in comparison to other drugs. Because of its solubility, this drug is normally administered via an intravenous bolus followed by infusion. The advantages of this agent lie in its small molecular weight and the ability to directly interact with the active site (Table 1). It is also notable that argatroban lacks the generation of any clinically significant antibodies.<sup>23</sup> As shown in Fig. (2), although argatroban had the most potent inhibitory effect on bovine thrombin-mediated platelet activation, human thrombin- and bovine factor Xa-mediated serotonin release response was also modulated by this agent at a relatively lower and concentration-dependent manner.

Bivalirudin (Angiomax<sup>®</sup>), a novel specific and reversible DTI, which is a recombinant protein based on hirudin, and composed of a 20 amino acid peptide analogue of the carboxy-terminal region of hirudin, linked via four

Table 1. Comparison of some of the molecular characteristic properties of direct thrombin inhibitors with unfractionated heparin and low-molecular-weight heparins

<table>
<thead>
<tr>
<th>Agents</th>
<th>Size (MW)</th>
<th>Mechanism of Action</th>
<th>Reversibility</th>
<th>Metabolized</th>
<th>Advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argatroban</td>
<td>527 D</td>
<td>Direct: Active Site</td>
<td>Reversible</td>
<td>Liver</td>
<td>Small Molecular Weight</td>
</tr>
<tr>
<td>Bivalirudin</td>
<td>22 aa</td>
<td>Direct: Active Site and Exosite</td>
<td>Reversible</td>
<td>Liver, and 20% Renal</td>
<td>Strength plus Reversibility</td>
</tr>
<tr>
<td>Hirudin</td>
<td>65 aa</td>
<td>Direct: Active Site and Exosite</td>
<td>Non-reversible</td>
<td>Kidney</td>
<td>Strength of Inhibition</td>
</tr>
<tr>
<td>UFH</td>
<td>12,000 - 15,000 D</td>
<td>Indirect</td>
<td>Reversible</td>
<td>20-50% Excreted Unchanged, Some Hepatic Metabolism</td>
<td>Non-anticoagulant Effects</td>
</tr>
<tr>
<td>LMWH</td>
<td>2,000 - 8,000 D</td>
<td>Indirect</td>
<td>Reversible</td>
<td>20-50% Excreted Unchanged, Some Hepatic Metabolism</td>
<td>Less Monitoring and Immunogenic Response</td>
</tr>
</tbody>
</table>

Size and/or molecular weight (MW) is measured either in Daltons (D) or amino acid(s) (aa) peptide sequence length. The non-anticoagulant effects for heparins [both unfractionated heparin (UFH) and low-molecular-weight heparin (LMWH)] include the release of cytokines, anti-inflammatory mediators, tissue factor, and adhesion molecules. The LMWHs have the same benefits as UFH, while it has less of an immunogenic response such as in heparin-induced thrombocytopenia.
Gly residues to D-Phe-Pro-Arg-Pro, which interact with the active thrombin site (both circulating and clot-bound thrombin). Characteristically, bivalirudin directly inhibits thrombin by binding both to the catalytic site and to the anion-binding exosite (derived from residues 53-64 of hirudin), thereby blocking serine proteases-mediated platelet activation and/or aggregation. Bivalirudin's bivalent mechanism of action occurs in a ratio of 1:1 and involves thrombin's cleavage of fibrinogen and its activation of factors V and VIII.\textsuperscript{15,24,25} The clearance of bivalirudin is thought to be mainly metabolic, which may include clearance by the liver or proteolysis at other sites including the vascular compartment. Only 20% of bivalirudin is removed renally.\textsuperscript{26} Bivalirudin has been shown to have clinical advantages such as reducing the risk of ischemic complications and the reduced risk of bleeding.\textsuperscript{27,28} To date, no confirmatory report exists about the immunogenic response in patients treated with bivalirudin. As shown in Fig. \textsuperscript{3}, this experimental observation clearly demonstrates that bivalirudin is a highly potent inhibitor of serine proteases-mediated platelet activation at a much lower concentration usually required for anticoagulation during PCI.

Hirudin (Refludan\textsuperscript{8}), another potent and high molecular weight DTI obtained from Hirudo medicinalis, a medicinal leech located in the salivary glands, is a 65 amino acid compound and is administered as a bolus.\textsuperscript{29} Hirudin can be used in two forms, both native and recombinant. While the two forms may differ characteristically, their clinical advantages of being a DTI are by and large the same. It is an extremely potent DTI in that it forms an irreversible complex with thrombin in a stoichiometric ratio of 1:1. This strong bond occurs on multiple sites and eliminates the need for circulating antithrombin.\textsuperscript{30} Hirudin's mechanism of action is via both the active site and the exosite-1. This inhibition of thrombin occurs by the aminoterminal domain inhibiting the active site, while exosite-1 binds to the acidic carboxy-terminal domain.\textsuperscript{7,8} Hirudin is metabolized in the kidneys, which poses a limitation for this agent, and because of this limitation, patients being treated for impaired renal function can not use hirudin.\textsuperscript{15,29} Furthermore, recent reports suggest that >40% of hirudin-treated HIT patients develop drug-specific antibodies that enhance/suppress the anticoagulant activity of hirudin.\textsuperscript{31-33} Despite these limitations, reports have shown some clinical advantages of hirudin over heparin, i.e., reduction of the risk of death or myocardial infarction at 24 and 48 h post-treatment in GUSTO IIb study,\textsuperscript{34} and in unstable angina patients.\textsuperscript{7} Further research has indicated that hirudin is more effective in preventing new ischemic events, revascularization procedures, and new myocardial infarction than heparin as in OASIS studies.\textsuperscript{35,36} In our laboratory studies, we indeed found that like other DTIs, hirudin is also an equally good inhibitor modulating the serine proteases-mediated serotonin release responses in a concentration-dependent manner.

While all the DTIs - just like any other antithrombotic, antiplatelet, or thrombolytic agents - have some advantages and disadvantages over heparin in specific clinical settings, particularly in cardiovascular indications. Bivalirudin and related DTIs investigated in this study clearly show that they could effectively modulate the serine proteases-mediated platelet activation at concentrations well below the plasma levels generally required for effective anticoagulation in cardiovascular interventions. Such effects may be of particular benefits to the management of patients with HIT as well as other patients (e.g., with diabetes, hypertension, inflammation and shock), where platelet activation is often associated with massive thrombin generation and hypercoagulable state. 

\textbf{Acknowledgements}

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References


Refusal to Participate in Blood Testing in a Study of Childhood Immunizations and Atopic Disorders: Characteristics of Non-Participants and Assessment of Possible Bias

Roos M.D. Bernsen1,2, Harry A. Aardoom3, Nico J.D. Nagelkerke1, Johannes C. van der Wouden2

1Department of Community Medicine, Faculty of Medicine and Health Sciences, United Arab Emirates University
2Department of General Practice, Erasmus MC – University Medical Center Rotterdam, The Netherlands
3District Health Service ‘Zuid-Holland Zuid’, Dordrecht, The Netherlands

Abstract
Aim: Assessing characteristics of non-participation in epidemiological studies is often complicated by lacking information. The aim was to assess characteristics of non-participants in blood testing and possible non-participation bias in our previous study on the impact of vaccinations on atopic disorders.

Methods: In a previously conducted study on vaccinations and allergy we now used multivariable logistic regression to assess characteristics of non-participants in blood testing, an optional part of the study protocol. Possible bias due to this non-participation was assessed by an analysis weighted with the inverse of the probability of being a participant and by a sensitivity analysis.

Results: Having refused consent to consult vaccination registration data (OR: 4.7, CI95% 2.9-7.6), not having disclosed income, lower school class, lower birth order, not having a history of pertussis, and eating less vegetables were significant determinants of non-participation in blood testing. Weighted analysis and sensitivity analysis yielded results similar to those in the original study.

Conclusions: We found that refusal to participate in blood testing was related to reluctance to disclose private information in general and to sensitivity on the subject of vaccinations in particular. Also, parents of younger children with less older siblings, without a history of pertussis, and consuming less frequently vegetables, were more likely to be a non-participant. Selective participation in blood testing may have affected our assessment of the reliability of the reported vaccination status, but leaves our conclusion from the original study, that there is no positive association between the DTP-IPV vaccination and atopy, unaffected.

Keywords: Non-participation bias, childhood vaccinations, atopic disorders.

Introduction
Non-participation should always be addressed in studies involving volunteers. Frequently participants and non-participants differ in aspects that are related to the outcome and/or to main determinants in the study and this could lead to biased estimates. For example, in a survey assessing the prevalence of Diabetes Mellitus in the United Arab Emirates (UAE), special techniques had to be applied to adjust for considerable non-response among se-
lected households\(^1\). Another cross-sectional study carried out in the UAE had a response rate of only 30%. The authors of this study, conducted to determine the prevalence of established cardiovascular risk factors, concluded that this low response rate did not affect their findings, as they had enough information on non-responders to assess that this group was similar to the responders in terms of variables relevant to cardiovascular disease\(^2\). However, in general such a detailed comparison between participants and non-participants is not possible because non-participants are by definition less visible to researchers than participants, and comparisons between these groups are limited to the (often few) characteristics available for both. Consequently, studies with low response rates are possibly subject to bias of an unknown extent and this phenomenon could be one of the explanations for heterogeneity in findings among studies investigating the same research question.

In a study\(^3\) on the association between the diphtheria-tetanus-pertussis-(inactivated) poliomyelitis vaccination (DTP-IPV) in the first year of life and reported atopic disorders at age 8-12 years in Orthodox Reformed (Protestant) primary school children in the Netherlands, our main conclusion was that there was no association between childhood vaccinations and atopic symptoms and even a negative association with objective allergy. This study was mainly based on questionnaires but we also requested a blood sample for assessment of allergy by specific IgE and for validation of their vaccination status. Participation in this blood test was not a requirement for participation in the study. Therefore assessment of bias due to selective participation in blood testing is extremely important since the conclusion of no, or even a negative, association with vaccinations with reported symptoms and/or objective allergy heavily impacts the public attitude towards vaccinations and thus potentially the health of millions.

In the present study, our objective is to explore which characteristics were related to non-participation in blood testing and their potential impact on possible participation bias.

2. Methods

2.1. Study Area, Population and Design

The original study was designed to assess the relationship between DTP-IPV vaccination and reported atopic disease\(^3\). Briefly, in 2003 and 2004, we sent questionnaires to 4480 children (aged 8-12 years) of 38 Orthodox Reformed (Protestant) primary schools in the Netherlands in three different regions (one in the eastern, one in the western, and one in the Southwestern part of the Netherlands). Many parents of children attending these schools refuse vaccinations for religious reasons, making this an appropriate group for exploring this relationship. A total of 1872 questionnaires (42%) were returned and suitable for analysis of which 671 pertained to reportedly DTP-IPV-unvaccinated children. A subsequent non-responder (to participation in the questionnaire part of the study) study found no evidence of selection bias: vaccination coverage in responders and non-responders was almost equal (63.1% and 63.8% respectively)\(^3\).

3. Data Collection in the Original Study

3.1 Questionnaire

The questionnaire asked questions on symptoms of atopic diseases (a Dutch translation of the ISAAC questionnaire\(^4\)), whether the child had received childhood vaccinations, demographics and other relevant variables (see Table 1).

3.2 Blood Measurements

In order to get an objective measurement of allergy (specific IgE) and to validate the risk factor (DTP-IPV vaccination), we invited participants to give a blood sample. Because of the cost of blood tests and limited available funds we planned to collect blood samples from 100 children only. After first recruiting schools in the Western and Eastern parts of the Netherlands, we had already enrolled 948 participants of whom 683 (72%; 74% of the vaccinated, and 70% of the unvaccinated) had consented to blood collection. As we only needed 100 blood samples we omitted the
invitation for blood sampling from the questionnaire distributed in the third region (Southwestern part of the Netherlands). From these 683 children we selected a stratified random sample of 100 children from whom blood was taken. The sample was stratified by DTP-IPV vaccination (ratio 1:4 for vaccination yes/no), atopic symptoms (1:1 yes/no) and primary school class (equal distribution over 4 school classes).

3.2.1 Specific IgE, Objective Measurement of Allergy
We performed RAST tests using the Pharmacia® RIA method to determine specific IgE to five of the most common aero allergens in the Netherlands (house dust mite, cocksfoot pollen, common silver birch pollen, cat epithelium dander and dog dander) in the sera of the 100 children to obtain an objective measurement of allergy. Allergy was defined as at least one RAST class 2 or higher (i.e., IgE >=0.7 IU/ml). This objective IgE based definition of allergy had a 66% agreement with reported asthma or hay fever (current symptoms or ‘ever had’). On the basis of these objective allergy data of 100 children and their relationship to reported allergy as well as other relevant variables, we then imputed the objective allergy variable for the remaining 1772 children. Analysis of these imputed data yielded a statistically significant negative association between DTP-IPV vaccination and allergy.

3.2.2 IgG Antibodies, Validation of the Risk Factor
Vaccination status was validated in the original study in two ways: by comparing the reported DTP-IPV vaccination status with the tetanus toxoid IgG and diphtheria IgG antibodies (in 80 reportedly unvaccinated children from the sample of 100 mentioned above) and by comparing the reported vaccination status with the official vaccination registry in a random sample of 120 children (drawn from those children in the total study population who gave consent to consult these data). Children with titres of at least 0.6 IU/ml were considered as having been vaccinated for the pathogen concerned.

3.3 Statistical Analysis
SPSS version 15.0 was used for all analyses. A two-sided p-value of 0.05 or less was considered significant. Different analyses were carried out. The aims of these analyses were to 1) determine characteristics of non-participants 2) assess whether adjustment for non-participation in blood testing would result in a similar agreement between atopic symptoms and objective allergy and 3) determine with a sensitivity analysis (by assuming that all subjects who refused participation to both blood testing and inspection of vaccination records had misrepresented their vaccination status) whether non-participation to blood testing might have affected the validity of the vaccination status.

3.3.1 Factors Related to Non-Participation in Blood Testing
In order to determine which characteristics were related to non-participation in blood testing, we first determined which of the variables in Table 1 were univariately related (p<0.10) to non-participation and then performed logistic regression (backward elimination, Wald method; p to remove = 0.10) with this subset of variables as independent variables (predictors) and non-participation in blood testing as the outcome variable. Using this logistic regression function we also calculated for each participant the predicted probability of non-participation given his/her values of predictors.

3.3.2 Specific IgE, Reliability of Reported Symptoms
In order to assess whether selective participation in blood testing could have biased our original result of 66% agreement, we repeated the original analysis after weighting the data with the inverse of the predicted probability of being a participant in blood testing, using the logistic regression analysis described above (inverse probability weighting). We reasoned
that cases with a high predicted probability of non-participation effectively also represent several cases who did not participate. For example, if a subject had a probability of 90% of...
being a non-participant, this category of subjects (with this particular set of predictors) had a probability of 10% of being a participant, and thus the participants among this category of subjects have to be weighted with a factor 10 to make up for the non-participants.

3.3.3 IgG Antibodies, Validation of Vaccination Status
In order to assess whether selective participation could have affected our validity assessment, we performed a sensitivity analysis (worst case scenario) by replacing the actual vaccination status of those children who refused to consent to both blood testing and consulting their vaccination records, with the alternative value (i.e., we considered those who reported to be vaccinated as not vaccinated and vice versa).

Results
A total of 948 participating children were invited to give consent for blood testing. Of these, 265 (28%) refused.

Factors Related to Non-Participation in Blood Testing
The variables marked with an asterix in Table 1 were univariately related (p < 0.10) with non-participation in blood testing. Including these variables in the logistic model, backward variable elimination yielded a model with refusing

<table>
<thead>
<tr>
<th>Variables</th>
<th>Non-Participation n/N (%)</th>
<th>aOR*</th>
<th>95%CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refusing consent for consulting official vaccination data</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>212/860 (25)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yes</td>
<td>53/88 (60)</td>
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<td>2.9-7.6</td>
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<tr>
<td>Refusing to answer the question on income</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>No</td>
<td>138/602 (23)</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>127/346 (38)</td>
<td>1.8</td>
<td>1.4-2.5</td>
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<td>School class</td>
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<td>5</td>
<td>82/241 (34)</td>
<td>0.8**</td>
<td>0.7-1.0</td>
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<td>6</td>
<td>68/254 (27)</td>
<td></td>
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<tr>
<td>7</td>
<td>62/222 (28)</td>
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<tr>
<td>8</td>
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<td>Birth order</td>
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<tr>
<td>1</td>
<td>87/265 (33)</td>
<td>0.9**</td>
<td>0.8-1.0</td>
</tr>
<tr>
<td>2</td>
<td>57/200 (29)</td>
<td></td>
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<tr>
<td>3</td>
<td>36/151 (24)</td>
<td></td>
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<tr>
<td>4+</td>
<td>85/332 (26)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequent consumption of vegetables</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no</td>
<td>128/400 (32)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>yes</td>
<td>137/547 (25)</td>
<td>0.7</td>
<td>0.5-1.0</td>
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<td>Having a history of pertussis</td>
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<td></td>
<td></td>
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<tr>
<td>no</td>
<td>211/700 (30)</td>
<td>-</td>
<td>-</td>
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<tr>
<td>yes</td>
<td>54/248 (22)</td>
<td>0.5</td>
<td>0.4-0.8</td>
</tr>
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</table>

* Odds ratios (OR) with 95% confidence intervals (95%CI) are adjusted for all other variables in the table
** OR for increase of one position
consent to consult vaccination registration data, refusing to answer the question on income, being in a lower school class, having less older siblings, eating less vegetables, and not having a history of pertussis, being positively related to non-participation in blood testing. Adjusted odds ratios (OR) and 95% confidence intervals (95%CI) are shown in Table 2. The strongest association was with refusing consent to consult vaccination records: OR=4.7 (CI95%: 2.9-7.6).

Objective Measurement (IgE) of Allergy, Reliability of Reported Symptoms
Our weighted analysis to assess the agreement between reported symptoms of asthma/hay fever and allergy as defined by increased specific IgE to at least one out of five aero allergens resulted in a 2x2 table similar to the (unweighted) one in the original study with 64% agreement (66% in the original study).

IgG Antibodies, Validation of Vaccination Status
There were 53 participants (5.6%) without consent for both procedures (blood testing and disclosure of official vaccination records). Our sensitivity analysis resulted in an adjusted OR of any atopic disorder (vaccinated/unvaccinated) of 1.1 (CI95% 0.9-1.4), in the original data this was 1.0 (0.8-1.2).

Discussion
This study found that non-participation to a non-obligatory nested sub-study of a cross-sectional study on vaccinations and atopic disorders was related to refusal of consent to consult official vaccination records, not answering a question on income, a lower school class, having fewer older siblings, infrequent consumption of vegetables and not having a history of pertussis. Non-participation in blood testing was not related to the main variables of the original study (vaccination and allergy). These results raise two questions, viz. 1) how to interpret the associations found and 2) whether selective participation might have biased results in the original study where blood measurements were involved.

Interpretation of Factors Related to Non-Participation in Blood Testing
Refusing consent for blood testing was clearly related to less openness and cooperativeness. Vaccinations are a sensitive issue in this religious group: both acceptors (parents who agree to vaccinate) and decliners (those who don’t agree) are under pressure: acceptors deviate from the beliefs and practices of the more traditional segment of the Orthodox Reformed Church, while the decliners are often portrayed by the lay press as backward and gambling with the health of their children. Perhaps, although confidentiality was assured and participation was voluntary, some parents may even have misrepresented the vaccination status of their child. Also, non-participation was more common in younger children, who are in general more afraid of medical examinations, in children with lower birth order, who possibly don’t have older siblings as role models and whose parents, being less experienced health care seekers, may be less willing to cooperate with physicians. Non-participation was also related to the child not having a history of pertussis. Maybe (as we believe to be the case with lower birth order) these parents lacked experience with physicians treating their child for a serious infection. Another predictor of non-participation was a lower consumption of vegetables, which is presumably an indicator of lower health awareness in parents. These parents may have motivated their children less to participate in blood testing.

Biased Results in Original Study?
Objective Measurement (IgE) of Allergy, Reliability of Reported Symptoms
Selective participation seems not to have biased the estimated agreement between symptoms of allergy and the objective measurement of allergy.

Validation of Vaccination Status
Our comparison of children’s reported vaccination status with their IgG antibodies in our original study resulted in 4% disagreement (3 out of 80 reportedly unvaccinated children had IgG antibodies), while our comparison
with the official registry yielded an almost 100% match. However, these comparisons include only children with parental consent to these validation procedures. If parents who refused consent for both validation procedures reported a false vaccination status, then a much larger percentage of reported vaccinations would have been misclassified. Clearly, such a misclassification could have biased several of our findings. However, large biases are unlikely because 1) there were only 53 participants (5.6%) without consent for either of the two procedures, and 2) the prevalence of any atopic disorder in these 53 children was similar to that in the study population, 3) 37 (70%) out of these 53 also did not answer the question on income, so these were presumably people who are just sensitive about disclosing private information, 4) our sensitivity analysis yielded an OR similar to the original result, 5) people who did not want to tell the true vaccination status of their child could have refused any participation in the study.

Imputation of IgE Positivity

Bias due to selective participation is not likely because all variables, including those that appeared in the present study to be predictors of non-participation to blood testing, were used to impute IgE positivity. Nevertheless, this assumes that non-participation to blood testing is completely determined by the available variables. This MAR (missing at random) assumption however can only be made plausible, never proven.

Conclusions

In this study of characteristics of non-participants in blood testing we found that refusal to give a blood sample was related to reluctance to disclose private information in general and to sensitivity on the subject of vaccinations in particular. Additionally, parents of younger children with less older siblings, without a history of pertussis, (probably having less experience with health care for their children), and with a lower consumption of vegetables (maybe indicating less health awareness in the parents), were more likely to be non-participants. Selective participation in blood testing may have affected our assessment of the reliability of the reported vaccination status, but leaves our conclusion from the original study, that there is no positive association between the DTP-IPV vaccination and atopy, unaffected.

Abbreviations

DTP-IPV = Diphtheria-tetanus-pertussis-(inactivated) poliomyelitis vaccination
UAE = United Arab Emirates
RAST = Radioallergosorbent test
RIA = Radioimmunoassay
SPSS = Statistical Package for Social Sciences
OR = Odds ratio
MAR = Missing at random

Acknowledgement

AstraZeneca Ltd provided the Emla® anaesthetic patches, but had no role in the design and conduct in the study, neither in the collection, analysis and interpretation of the data, nor in the preparation, review and approval of the manuscript.

The first author, who is independent of any commercial funder, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. None of the authors has any conflict of interest.

References


The NF-kB Inhibitor IkBα Negates Colon Cancer Cell Migration, Invasion, Proliferation and Tumor Growth

Samir Attoub1,6, Rabah Iratni2,5, Suhail Al-Salam3, Khouloud Arafat1, M.A.H Al Sultan1, Nadia Al Marzouqi1, Eric Bruyneel4, Marc Bracke4, Olivier De Wever4 and Christian Gespach6

1Department of Pharmacology & Therapeutics, Faculty of Medicine & Health Sciences, UAE University, P. O Box 17666, Al Ain, United Arab Emirates. 2Department of Biology, UAE University, P. O. Box: 17551, Al Ain, United Arab Emirates. 3Department of Pathology, Faculty of Medicine & Health Sciences, UAE University, P. O Box 17666, Al Ain, United Arab Emirates. 4Laboratory of Experimental Cancer Research, University Hospital, De Pintelaan 185, B-9000 Gent, Belgium; 5Institut Albert Bonniot, INSERM U823, University Joseph Fourier, Grenoble 1, Site Santé, 38042, Grenoble, Cedex 6, France and 6INSERM U 673 and U938, Molecular and Clinical Oncology of Solid Tumors; University Pierre et Marie Curie Paris VI, Hôpital Saint-Antoine, 75571 Paris Cedex 12, France

Abstract
It is now well accepted that the NF-κB pathways are involved in inflammatory diseases, cancer development and progression in human solid tumors. The NF-κB signaling element IkBα was shown to inactivates NF-κB activity through sequestration of this transcription factor in the cytoplasm. In the present study, we investigated the impact of the IkBα on the invasive growth of human colon cancer cells HCT8/S11 stably transfected by this endogenous NF-κB inhibitor. We report that IkBα ectopic expression inhibited NF-κB promoter activity induced by the Y527Fsrc oncogene, and reduced HCT8/S11 cell migration in wound healing assays. Our data show that IkBα abrogated collagen type I invasion induced by the trefoil factors TFF1 and TFF3, but was ineffective on the invasive phenotype determined by leptin. Moreover, IkBα reduced HCT8/S11 cell proliferation in vitro and the growth of their corresponding tumor xenografts established in the athymic mice. Taken together our data demonstrated that the intrinsic NF-κB inhibitor IkBα negates several transforming functions in human colon cancer cells. Our data provide the rationale for further preclinical and clinical studies based on therapeutic interventions targeting NF-κB pathway.

Keywords: NF-κB, promoter, src, TFF, leptin, wound healing, collagen type I, Ki-67.

Introduction
Cancer is the third leading cause of death in the United Arab Emirates (UAE) and other major World regions. Despite recent progress in the treatment of human solid tumors and leukemia, new strategies leading to the design of targeted anticancer drugs are expected to improve cancer patient survival. Nuclear NF-κB is a key transcription factor involved in normal development, inflammatory diseases, and cancer progression. In many types of cancers including breast and colon cancer, the transcription factor NF-κB is constitutively activated. The activity of NF-κB is regulated by the IkB proteins, which bind NF-κBand retains it in the cytoplasm. Thus, IkBα-inhibitor of NF-κB, induces the formation of the inactive heterotrimeric complex comprising p50/p65.
and IκBα. Upon cellular stimulation, IκB is phosphorylated by the IκB kinase (IKK), ubiquitinated and degraded by the proteasome, allowing the nuclear translocation of the p50/p65 NF-κB complex to regulate transcription of diverse genes encoding cytokines, growth factors, cell adhesion molecules, and regulators involved in survival and apoptosis\textsuperscript{5,6}. Consistently, NF-κB activation is associated with transient and chronic inflammatory states linked to tumor promotion, progression and metastasis in several mouse models and clinical situations\textsuperscript{7}.

Loss-of-function mutations in IκB-α, resulting in high constitutive nuclear activity of NF-κB, are characteristic of Reed–Sternberg cells in Hodgkin lymphoma\textsuperscript{8,9}. This implies a tumor suppression function for IκB. Moreover, defective IκBα were found in several solid tumors such as colon, breast, ovarian, pancreatic, bladder, prostate carcinomas and melanoma\textsuperscript{10}. While ubiquitination-mediated proteolysis of IκBα by the 26S proteasome leads to NF-κB activation\textsuperscript{11}, proteolysis of IκBα by caspase 3 inhibited NF-κB activation instead. This proteolysis generated an N-terminal truncated protein that can still bind to NF-κB and is resistant to TNFα-induced phosphorylation and degradation\textsuperscript{12}. Interestingly, phosphorylation of IκBα by IKK or replacement of Ser32 and Ser36 with glutamates to mimic their phosphorylation prevented caspase-mediated proteolysis\textsuperscript{13}. It appears then that phosphorylation of IκBα by IKK serves for proteasome-dependent proteolysis of IκBα, and avoids generation of a repressor-like fragment of IκBα by caspases.

In the present study, we investigated the impact of IκBα ectopic expression on several transforming functions explored in the HCT8/S11 human colon cancer cell line. We examined the behavior of IκBα-transfected HCT8/S11 cells on cell proliferation, migration and invasion assays monitored in vitro with the proinvasive agents trefoil factors TFF1, TFF3, and leptin\textsuperscript{14,15}. In addition, we examined the influence of IκBα on the growth of HCT8/S11 tumor xenografts established in immunodeficient mice.

**Materials and Methods**

**Cell Culture, NF-κB Gene Reporter Assays, and Stable Transfections**

Human colorectal cancer cells HCT8/S11 were cultured in RPMI 1640 (Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal bovine serum (Roche Molecular Biochemical’s, Meylan, France). For transient transfections, HCT8/S11 colonic epithelial cells were seeded in six-well plates at a density of 75,000-200,000 cells/well. After overnight adhesion, cells were transfected with the Luciferase reporter constructs driven by NF-κB-dependent promoter, using the LipofectAMINE Reagent (Invitrogen). Where indicated, the transfection mixture was combined with plasmid vectors encoding src and IκBα. The src expression vector pSGT-srcY527F encoding the src oncogene and the corresponding control empty plasmid pSGT were provided by Dr. Serge Roche (CNRS UPR-1086 CRBM, Montpellier, France). Before assaying for luciferase activity, cells were washed with cold phosphate buffered saline (PBS) and scraped in the luciferase lysis buffer. Luciferase assays were performed using the Luciferase Assay System (Promega, Madison, USA). Values shown are mean ± SEM of at least three independent experiments, each performed in triplicate.

For stable transfections, HCT8/S11 cells were plated for 24h in 60 mm culture dishes and transfected by lipofection (LipofectAMINE Reagent Plus, Invitrogen), according to the manufacturer's instructions. Cells were transfected with 4μg of the expression vector pECPF-IκBα carrying the neomycin resistance gene. The expression vector pECPF-IκBα was a gift from Dr. Johannes A. Schmid (Department of Vascular Biology and Thrombosis Research, University Vienna, Austria). The next day, cultures were split into two 100-mm-diameter dishes and selected for ten days in the culture medium supplemented with 400 μg/ml of G418. Resistant colonies were ring-cloned as individual clones or pooled. Expression of the
transgene was determined by Western blot-ting using the IκBα mouse monoclonal antibody H4 raised against the GST-IκBα human fusion protein (Santa Cruz, CA, USA).

Wound Healing and Invasion Assays

Cells were grown in six-well culture dishes until confluence and then incubated for 10 min in Moscona buffer (137 mM NaCl, 2.68 mM KCl, 11.9 mM NaHCO3, 9.43 mM glucose, 0.176 mM NaH2PO4), pH7.4. The buffer was sterilized by filtration through a 0.22μm filters and stored at -20°C until use. A scrape was made through the confluent monolayer with a plastic pipette tip of 1mm diameter, the Moscona buffer was removed, and the dishes were washed twice and incubated at 37°C in fresh RPMI containing 10% fetal calf serum. At the underside of each dish, a mark was made at three arbitrary places where the width of the wound was measured with an inverted microscope (objective x 4). Migration was expressed as the average ± SEM of the difference between the measurements at time zero and the time points 4h, 6h, and 48h. The complete healing of the wound corresponds to 100%. The healing index corresponds to the % of the wound that is repaired per unit of time (h).

For invasion of collagen gels by HCT8/S11 and HCT8/S11-IκBα cells, six-well tissue culture dishes were filled with 1.35 ml of neutralized type I collagen (Upstate Biotechnology, Lake Placid, NY) and incubated overnight at 37°C to allow gelling. Parental and HCT8/S11-IκBα cells were harvested using Moscona buffer and trypsin/EDTA, and seeded on top of the collagen gels at the density of 300,000 cells per dish. Cultures were incubated for 24h at 37°C, in the presence or absence of the indicated agents. The number of invasive and superficial cells were counted in 12 fields of 0.157 mm² using an inverted microscope. The invasion index corresponds to the ratio of the number of cells invading the gel over the total number of cells counted in each field16. Recombinant human TFF1 and TFF3 were provided by Dr. Lars Thim and Pr. Bruce Westley.

Cell Proliferation and Tumor Growth Assays

Parental and stable HCT8/S11-IκBα cells were plated at the density of 25,000 cells into six-well tissue culture dishes supplemented with 10% FBS. At the time indicated, cancer cells were trypsinized, collected in 1 ml of medium, and counted by a Cell Coulter. Six-week-old athymic NMRI female nude mice (nu/nu, Elevage Janvier, France) were housed in filtered-air laminar flow cabinets and handled under aseptic conditions. Procedures involving animals and their care were conducted in conformance with Institutional guidelines that are in compliance with FMHS, national and international laws and policies. Parental and stable HCT8/S11-IκBα cells were injected s.c. into the lateral flank of athymic nu/nu mice (3 X 10^6 cells in each xenograft, 7 to 8 animals in each group). Tumor volume (V) was calculated using the formula: V = 0.4 x a x b², with "a" being the length and "b" the width of the tumor. Tumor dimensions were measured with calipers every week for 9 weeks. Then, animals were sacrificed and the tumors excised, weighed and fixed for immuno-histochemical analysis. For the immunohistochemical determination of the proliferating, five micrometer paraffin-embedded tissue sections were deparaffinized, microwaved during 5 min for antigen retrieval, and then incubated with the mouse mAb against Ki-67 antigen (DAKO, Copenhagen, Denmark, clone MIB-1, dilution 1:50) for 1h at room temperature. The samples were then washed and incubated with secondary antibody for1h at room temperature, followed by incubation with the streptavidin-peroxidase complex. Ten high-power fields (0.159 mm²) per section from 4-5 tumor specimens of each group were examined microscopically. The average number of cells that stained positive for Ki-67 was evaluated in established control and IκBα -transfected HCT8/S11 xenografts.

Statistical Analysis

Data are means ± SEM for the number of experiments indicated. The statistical signifi-
cance between experimental values was assessed by the unpaired Student’s t-test and P < 0.05 was considered to be statistically significant.

RESULTS

Functional Expression of the IκB-α Transgene in HCT8/S11 Cells

First, we examined the impact of the NF-κB inhibitor IκBα on the activity of the NF-κB reporter gene in HCT8/S11 human colon cancer cells. Transient transfections were performed using the pSGT-srcY572F vector encoding the oncogene src, together with the luciferase gene reporter construct driven by the NF-κB-dependent promoter. We have previously shown that this HCT8/S11 cell line exhibits a low level of src activity17. Consistently, introduction of the src oncogene induced a robust NF-κB activation (6.3-fold) in HCT8/S11 cells, as compared with co-transfections performed with the control empty vector pSGT (Fig. 1A).

As expected from previous studies showing the implication of src in NF-κB activation induced by the stress signals hypoxia and reactive oxy-

![Graph A](image1.png)

![Graph B](image2.png)

Fig. (1). *IκBα* down-regulates NF-κB promoter activity and wound healing in HCT8/S11 cells.

**A.** Reversion by IκBα of src-induced NF-κB promoter transactivation. Human cancer cells HCT8/S11 were transiently transfected by luciferase reporter constructs driven by the NF-κB-dependent promoter, alone or combined with expression vectors encoding src alone or combined with the intrinsic NF-κB inhibitor IκBα. Data are means ± SEM of 3 independent experiments. **B.** Wounds were introduced in confluent monolayers of parental and stably transfected HCT8/S11-IκBα cells. Cells were then cultured at 37°C for 4, 6 and 48 h. The mean distance that cells traveled from the edge of the scraped area was measured in a blinded fashion, using an inverted microscope (4 x magnifications, insets). Data are means ± SEM of 3 independent experiments.
ogen species, as well as cellular adhesion to extracellular matrix components, over-expression of \( \kappa B \alpha \) abrogated the direct activation of NF-\( \kappa B \) induced by the src oncogene in HCT8/S11 cells.

Thus, our data validate the functional insertion of \( \kappa B \alpha \) in our HCT8/S11 model and encouraged us to establish a stably transfected HCT8/S11 cell line expressing high levels of the endogenous NF-\( \kappa B \) inhibitor \( \kappa B \alpha \). The resulting cell line was designated HCT8/S11- \( \kappa B \alpha \). Expression of the tagged \( \kappa B \alpha \) transgene was confirmed by western blot (not shown).

**Impact of \( \kappa B \alpha \) on Cancer Cell Migration, Invasion, Proliferation, and Tumor Xenograft Growth**

We next examined the effect of \( \kappa B \alpha \) ectopic expression on cellular migration in HCT8/S11 cells. Using wound-healing experiments performed on sub-confluent cell cultures, we show that \( \kappa B \alpha \) inhibited by 29-53% the migration of HCT8/S11- \( \kappa B \alpha \) cells during the 4-6h short incubation time considered (Fig. 1B, left) suggesting that the inhibition of cellular migration is not due to inhibition of cell proliferation. Parental HCT8/S11 cells were able to achieve a complete wound healing within 48h, while \( \kappa B \alpha \) cells were unable to fully colonize the wounds at this time period (Fig. 1B, right).

Although trefoil factors contribute to the immunity and mucosal protection in the normal gastrointestinal tract, it is now widely accepted that these secreted regulatory peptides are strongly induced during inflammatory diseases and cancer progression. In agreement, TFFs play a pejorative role in several transforming functions associated with the neoplastic progression in cancer cells and tumors, including cellular scattering and invasion, survival and protection against anoikis-apoptosis, adenoma-adenocarcinoma transition, and angiogenesis. Indeed, we have previously shown that trefoil factors (TFF) and leptin induce the invasive phenotype in HCT8/S11 cells cultured on collagen type I gels. As shown in Fig. 2, the leptin invasive phenotype was not affected by \( \kappa B \alpha \), while this NF-\( \kappa B \) inhibitor selectively impaired the invasive responses elicited by the trefoil factors TFF1 and TFF3. To test the ability of \( \kappa B \alpha \) to interfere with cancer cell proliferation, parental HCT8/S11 cells and their \( \kappa B \alpha \) transfected counterparts were compared for their growth rates. As shown in Fig. 3A, HCT8/S11- \( \kappa B \alpha \) cells

![Fig. (2). \( \kappa B \alpha \) ectopic expression selectively negates cellular invasion induced by pS2/TFF1 and intestinal trefoil factor/TFF3 in HCT8/S11 cells.
Cellular invasion induced by leptin (100 ng/ml), TFF1 (100nM) and TFF3 (100nM) was measured in parental HCT8/S11 cells (control) and stably transfected HCT8/S11- \( \kappa B \alpha \) cells. Data are means ± SEM of 3 independent experiments.](image-url)
exhibit slower proliferation rates, as shown at days 4 and 5 in culture (24 and 31 % inhibition, respectively). This interesting data prompted us to investigate the impact of \( \text{I}\kappa\beta \) ectopic expression on the growth of HCT8/S11 human colon tumor xenografts in immunodeficient mice. As shown in Fig. (3B), \( \text{I}\kappa\beta \) ectopic expression led to significant inhibition (45%) of tumor growth at week 9 following heterotransplantation. To test the role of \( \text{I}\kappa\beta \) on tumor cell proliferation in HCT8/S11 human colon xenografts, we performed a complementary study on the nuclear antigen Ki-67 by immunohistochemistry. This nuclear Ki-67 antigen, which is present in all phases of the cell cycle, except the G0 phase, is considered as a classical marker in cells engaged in the cell proliferation cycle. There was a significant decrease in the mean number of Ki-67-positive cells in HCT8/S11-\( \text{I}\kappa\beta \) human colon cancer xenografts (*P < 0.05 versus HCT8/S11 xenografts)
be due, at least in part, to a direct effect on tumor cell proliferation.

**Discussion**

Deregulated and constitutive activation of cell proliferation and survival signals constitute a critical mechanism underlying tumor development and cancer progression. Evidence suggests that aberrant activation of NF-κB and its downstream signaling pathways is responsible for the initiation of tumorigenesis including evasion from apoptosis, malignant transformation, sustained cell proliferation, metastasis, and angiogenesis. A sustained activation of NF-κB contributes to the expression of proto-oncogenes c-myc and cyclin D1, which are responsible for both transformation initiation and tumorigenic proliferation.

In the present study, we have shown that ectopic expression of the intrinsic NF-κB inhibitor IκB strongly inhibits NF-κB transactivation directly induced by src oncogene in HCT8/S11 human colon cancer cells. The src oncogene is known to be activated during the early and late stages of the neoplastic progression in human colon tumors. Consistently, we demonstrated that blockade of the NF-κB pathways by IκBα led to the invalidation of several transforming functions in HCT8/S11 cells. We have shown that IκBα ectopic expression reduced the ability of HCT8/S11 cells to proliferate in vitro and in vivo as tumor xenografts in immunodeficient mice. Convergent data and reports also revealed that alterations in the adhesive properties of cancer cells are asso-

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**Fig. (4).** **Diagram summarizing the NF-κB pathway in a cancer cell before (A)** and following transfection with IκBα (B). Over-expression of IκB by stable transfection titrates IKKs out, leaving some IκB in the ternary complex unphosphorylated. This will prevent NF-κB nuclear translocation and thus maintaining a constitutive repression of the NF-κB responsive genes.
associated with invasive growth and tumor malignancy (data summarized in Fig. 4). Cellular adhesion to ECM components via their integrin receptors, intercellular adhesion, and activation of the actomyosin system in the cytoskeleton are known to play pivotal roles in the integration of the mechanisms leading to directed cellular movements during wound healing assays. Loss of E-cadherin and other intercellular adhesion molecules also correlate with cancer cell scattering, migration, and tumor cell invasion. This loss of cell-cell adhesion appears to be a key event in acquisition of the invasive potential, because re-expression of E-cadherin suppresses the invasive growth of human tumors. Consistently, it has been shown that NF-κB pathway can suppress E-cadherin expression via specific transcription repressors such as Snail, an NF-κB target gene. Snail-induced E-cadherin loss and epithelial-mesenchymal transitions (EMT) are associated with the invalidation of the epithelial cell polarity, stimulation of cancer cell proliferation, and exacerbation of the invasive and metastatic potential in clinical human epithelial tumors. Similarly, integrin ligation to ECM components was shown to activate the transcription factor NF-κB in multiple cell types. Given the role of the NF-κB inhibitor IκBα in inhibiting HCT8/S11 cell migration in the present study, one can postulate that this pathway has potential roles in the regulation of intercellular and cell-matrix adhesion mechanisms, as well as activation of the cytoskeleton during cancer cell migration and invasion. In coherence with this prediction, we have shown here that IκBα ectopic expression selectively negated HCT8/S11 cancer cell invasion induced by TFF1 and TFF3, but not by leptin, in collagen type I gels. Consistently, several studies indicate that both TFF expression and biological activities are controlled by NF-κB since several NF-κB binding sites are localized in the promoter regions of the three TFF genes. In contrast, blockade of the NF-κB pathways is not associated with the reversion of the leptin invasive potential in our study, suggesting that alternative proinvasive pathways induced by this cytokine are still operational under NF-κB blockade. Indeed, several studies have identified the multiplicity of the leptin receptor signaling networks connected with cancer cell invasion and tumor angiogenesis. These include the PI3-kinase/AKT axis, MAPK ERK1/2 cascade, the Rho-GTPases, the JAK2/STAT3 transcription signals, as well as several crosstalks engaged with other transmembrane receptors. These canonical and alternative leptin pathways are inherent to cancer cell proliferation, survival, invasion and metastasis, as well as tumor angiogenesis and metastasis.

We propose that the NF-κB oncogenic pathway can be targeted to halt tumor invasive growth and progression to the metastatic stages. Several studies have addressed the design of putative NF-κB inhibitors as pharmacologic therapeutic agents in cancer patients. Anti-inflammatory drugs and natural compounds, such as curcumin and transveratrol, inhibit NF-κB by interfering with IKK activity. Finally, proteasome inhibitors were also shown to prevent NF-κB activation by blocking the degradation of IκB. In summary, the results of our study provide new insight into the development of therapeutic strategies against colon cancer promotion and progression, using NF-κB interfering drugs in combinations with other anticancer agents targeting selected oncogenic pathways or genotoxins that disrupt the functional integrity of the DNA.

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References


