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Neural Tube Defects, Folate, and Immune Modulation

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Abstract

Periconceptional supplementation with folic acid has led to a significant worldwide reduction in the incidence of neural tube defects (NTDs). However, despite increasing awareness of the benefits of folic acid supplementation and the implementation of food fortification programs in many countries, NTDs continue to be a leading cause of perinatal morbidity and mortality worldwide. Furthermore, there exists a significant subgroup of women who appear to be resistant to the protective effects of folic acid supplementation. The following review addresses emerging clinical and experimental evidence for a role of the immune system in the etiopathogenesis of NTDs, with the aim of developing novel preventative strategies to further reduce the incidence of NTD-affected pregnancies. In particular, recent studies demonstrating novel roles and interactions between innate immune factors such as the complement cascade, neurulation, and folate metabolism are explored.

Keywords

complement; C5a; neural tube defects; folate; neurulation

INTRODUCTION

Neural tube defects (NTDs) are a leading cause of peri-natal morbidity and mortality worldwide, affecting approximately 1 in 1000 live births, although prevalence varies significantly with geography and ethnicity (Blom et al., 2006). It is well established that isolated NTDs have a multifactorial origin, with numerous studies demonstrating contributions from both genetic and environmental factors (Campbell et al., 1986; Deak et al., 2008; Finnell et al., 2000). The most significant finding in the field to date has been that dietary supplementation with folic acid during the periconceptional period markedly reduces

the risk of NTDs (Smithells et al., 1976); however, despite decades of subsequent research, the underlying mechanisms by which folic acid exerts its protective effects have remained elusive (Wallingford et al., 2013). Given the ongoing and significant physical and socioeconomic burden of NTDs (Yi et al., 2011), there is a pressing need to investigate novel risk factors and mechanisms by which NTDs may arise to develop new preventative strategies.

The following review reports on both experimental and human clinical data, describing the events linking the immune system, neurulation, and folate metabolism, with the aim of addressing the hypothesis that dysregulation of the immune system plays an etiological role in the pathogenesis of NTDs (Fig. 1). This is an area that has, until now, been relatively unexplored, yet recent evidence suggests that this may be a fertile area for future NTD research.

Neurulation and NTDs

Formation of the neural tube, the embryonic precursor to the brain and spinal cord, occurs during the fourth week of human gestation. Briefly, neurulation begins with the formation of the neural plate from specialized ectodermal cells, the neuroectoderm. The neural plate is subsequently converted into a tube by a two-stage process (Copp et al., 2003). In *primary neurulation*, the dorsal and medial migration of lateral neural plate cells create neural folds that fuse at the midline to form the neural tube. Notably, fusion of the neural folds does not occur simultaneously throughout the embryo, but rather is initiated at several places along the rostral–caudal axis (Van Straaten et al., 1996; Greene and Copp, 2009a).

Following fusion, the neural fold apices remodel to create two continuous epithelial layers: an outer surface ectoderm and an inner neural tube. Following completion of primary neurulation, the neural tube caudal to the mid-sacral region is formed by the process of *secondary neurulation*, in which the neural tube forms from mesenchymal cells that coalesce and undergo epithelialization (Schoenwolf and Franks, 1984; Greene and Copp, 2009a). The epithelium then canalizes and becomes continuous with the primary neural tube.

NTDs reflect a failure of neurulation, which can occur at numerous points along the fusing neural tube. Failure of anterior regions of the neural tube to close results in anencephaly, whereas failure of closure of the posterior regions of the neural tube results in spina bifida, the most common NTD in humans (Botto et al., 1999; Rosano et al., 2000; Boulet et al., 2008). Failure of the neural folds to elevate results in craniorachischisis, which is an open defect along the entire length of what would be the neural tube.

NTDs can further be classified as being “open” or “closed” defects. Failure of primary neural tube closure is associated with defects in the overlying bony structures such that the underlying neural tissue is exposed to the body surface (“open” NTDs). There are also several “closed” skin-covered conditions that involve the neural tube. These conditions are not thought to result from defects in primary neural tube closure, but may result from defects in secondary neural tube development (Copp and Greene, 2010). Table 1 summarizes the various types of NTDs and their clinical sequelae.

Folates and NTDs

Folate is a water-soluble B vitamin present in legumes (e.g., beans, peas, and lentils), leafy green vegetables (e.g., spinach and asparagus), and certain fruits (e.g., banana, cantaloupe, and strawberry) (De-Regil et al., 2010). Folic acid is the synthetic and most stable form of folate. For this reason, it is the form most often used in supplements and food fortification, with a bioavailability 10 to 98% higher than that of folates naturally contained in foods (McNulty and Pentieva, 2004; De-Regil et al., 2010). The 5-methyltetrahydrofolate (5-methylTHF) is the physiologically active form of folate that serves as a cofactor for enzymatic reactions that involve the transfer of one-carbon moieties necessary for purine and pyrimidine synthesis.

Circulating 5-methylTHF can be transported into cells either by the reduced folate carrier, a facilitative anion-exchange carrier, or by an endocytic process mediated by one of two folate receptors, folate receptor alpha (FRA; Folr1; Fbp1; Folbp1) and folate receptor beta (FRb; Folr2) (Beaudin and Stover, 2009). Folr1 is expressed in the neural folds during neurulation (Saitsu et al., 2003). Knockout of the Folr1 gene in the mouse leads to NTDs (Piedrahita et al., 1999; Tang and Finnell, 2003; Spiegelstein et al., 2004), demonstrating the importance of folate uptake to proper neural tube development. High doses of folic acid are able to partially rescue this phenotype in the Folr1 knockout mouse (Spiegelstein et al., 2004).

In humans, dietary supplementation with supraphysiologic doses of folic acid, before and during pregnancy, reduces the incidence of NTDs by up to 70% (Smithells et al., 1976). However, most women who have a pregnancy complicated by a NTD do not have clinical folate deficiency (Giles, 1966), and the protective effect of folic acid supplementation is not dependent on a preexisting overt maternal folate deficiency (Yates et al., 1987). These findings have led to the hypothesis that genetic defects in folate metabolism or transport may account for variations in individual susceptibility to NTD-affected pregnancies. However, although some gene polymorphisms in folate transporters and folate-pathway enzymes have been identified (Christensen et al., 1999; Beaudin and Stover, 2009), studies to date have provided no conclusive evidence that these genetic differences affect folate status (Beaudin and Stover, 2009). Together, these results highlight that folic acid appears to be a corrective, rather than causal factor, in the etiopathogenesis of NTDs (Wallingford et al., 2013).

NTDs and Immune Dysregulation

Several of the many known NTD risk factors have immune features and/or associations, including diabetes mellitus, exposure to pharmaceutical compounds such as valproic acid, and induction of high titers of folate receptor autoantibodies (Wallingford et al., 2013). Furthermore, there is increasing evidence that components of the immune system may directly influence critical morphoregulatory steps during neurulation. Each of these associations will be discussed below.

NTDs and Diabetes

The teratogenic potential of maternal pregestational diabetes is well established and includes a 2-fold to 10-fold increase in the risk of central nervous system malformations (including

NTDs) among the offspring of affected women, relative to the general population (McLeod and Ray, 2002). Moreover, the level of maternal, periconceptional glycemic control has been associated with the risk of NTDs in the offspring of diabetic women (Ray et al., 2001; McLeod and Ray, 2002; Shaw et al., 2003). As human embryos lack pancreatic function until after the seventh week of gestation, the teratogenic effect of maternal diabetes may be due to embryonic exposure to elevated glucose concentrations. Diabetic pregnancy represents a substantial portion of the obstetric population, with maternal diabetes developing in 2 to 6% of all pregnancies, translating to a significant burden on health resources (Kieffer et al., 1999; Moum et al., 2004; Ozumba et al., 2004; Dheen et al., 2009).

Evidence for an underlying immune etiology of NTDs in diabetic women is supported from studies in mouse models of diabetes mellitus, in which nonspecific stimulation of the immune system has been shown to significantly reduce the incidence of NTDs and associated congenital malformations in these diabetic pregnancy models. Torchinsky and colleagues (1997) found that nonspecific immunostimulation with injected rat splenocytes 3 weeks before mating led to a fourfold reduction in the incidence of malformed fetuses in a streptozocin-induced diabetic mouse models. Subsequent studies have demonstrated similar reductions in NTDs and associated congenital malformations using other immune activators including: granulocyte-macrophage colony-stimulating factor (GM-CSF), and interferon- γ (IFN γ) (Hrubec et al., 2006, 2009), which are pleiotropic cytokines known for their roles in inflammation and immune cell regulation (Young and Hardy, 1995; Hamilton and Anderson, 2004).

One possible explanation for this “rescue” of the normal phenotype in these studies is that an overzealous pro-inflammatory response may result in the selective abortion of fetuses affected by NTDs (i.e., terathanasia) (Kalter, 2000; Girardi, 2008; Warning et al., 2011). Immune-mediated terathanasia is theoretically supported by the finding that suppression of various components of the immune system is essential for fetal survival during pregnancy (Xu et al., 2000; Warning et al., 2011). It could thus be hypothesized that abnormal neurulation may trigger a pro-inflammatory response, which has the consequence of aborting malformed fetuses and selecting for the survival of phenotypically normal offspring. However, studies in mice do not support the notion that selective elimination of malformed fetuses is a mechanism of immune based “protection” against NTDs (Copp and Greene, 2000).

NTDs and Valproic Acid

The immune activators GM-CSF and IFN γ have also been shown to be protective against NTDs induced by valproic acid (Hrubec et al., 2006). Valproic acid, used clinically as the formulation of sodium valproate, is a commonly prescribed anti-epileptic and mood-stabilizing drug. Notably, valproic acid has been directly implicated as a potent neural tube teratogen. Among women taking valproic acid (alone or in combination with other anti-convulsant medications), the risk of having a pregnancy affected with spina bifida may be as high as 1% to 2%— at least 10-times higher than in the general population, whereas the risk of anencephaly does not appear to be increased (Robert and Guibaud, 1982; Koren and Kennedy, 1999; Meador et al., 2006; Harden et al., 2009b; Jentink et al., 2010), suggesting

that there may be genetically mediated differences in sensitivity to valproic acid teratogenicity.

Valproic acid has a complex pharmacology, as both an antiepileptic drug and as a reproductive toxicant. Its effects on the immune system are similarly varied with a small number of studies demonstrating that its administration results in alterations in blood levels of proinflammatory cytokines (Ichiyama et al., 2000; Shiah et al., 2005; Mlodzikowska-Albrecht et al., 2007). Of note, the placentas of valproic acid-treated dams did not show any overt histopathology, so the stimulation of these dams with IFN γ or GM-CSF would appear to have a more direct effect on embryonic development (Hrubec et al., 2006). Furthermore, despite numerous suggestions in the literature that this drug alters folate metabolism (Attilakos et al., 2006; Roy et al., 2008), there is no convincing or consistent evidence that valproic acid-induced NTDs are reduced by supplemental folic acid (Craig et al., 1999; Hernandez-Diaz et al., 2000; Kaaja et al., 2003; Morrow et al., 2009; Jentink et al., 2010).

Given the broad-spectrum of possible downstream effects of GM-CSF or IFN γ administration however, these findings are unlikely to translate to any useful clinical benefit until the precise immunoregulatory mechanisms underlying their protective benefit are elucidated.

NTDs and Folate Receptor Autoantibodies

Maternal immunological responses that influence folate transport may also affect neurulation (Botto et al., 1999). Rothenberg and colleagues reported the presence of autoantibodies directed against the folate receptor in 75% of mothers who had given birth to NTD-affected infants, compared with only 10% of mothers with nonmalformed fetuses (Rothenberg et al., 2004). These observations were validated in more robust studies using mid-gestational serum samples (Cabrera et al., 2008; Boyles et al., 2011). Furthermore, administration of antisera against folate receptors to pregnant rats had a dose-dependent adverse effect on embryonic viability and organogenesis (da Costa et al., 2003). Together, these studies led to the hypothesis that these maternal autoantibodies bind to the folate receptor, consequently inhibiting the binding and transport of folates from the mother to the developing fetus compromising embryonic development (Taparia et al., 2007).

The mechanism by which the folate receptor becomes a self-antigen is unknown; however, one hypothesis is that it undergoes posttranslational modification in high homo-cysteine (i.e., low folate) conditions in a process termed “homocysteinylation.” Homocysteine is a sulphur-containing amino acid that accumulates under conditions of folate deficiency (Jakubowski and Glowacki, 2011). Homocysteinylation describes a process by which a highly reactive homocysteine metabolite, homocysteine thiolactone, becomes incorporated into proteins with a high concentration of lysine or cysteine residues. Studies by Jakubowski and colleagues over the last decade have shown that homocysteinylation of circulating proteins such as albumin and hemoglobin occurs in humans in proportion to the amount of circulating homocysteine and, furthermore, that this leads to the generation of auto-antibodies that selectively recognize the homocysteinylation (cf. native) form (Undas et al., 2004; Jakubowski, 2005).

The folate receptor also contains several putative surface lysine residues, which may be susceptible to homo-cysteinylolation. Indeed, the recombinant human folate receptor can be homocysteinylated in vitro by low and physiological concentrations of homocysteine thiolactone, a reaction that is irreversible at physiologic conditions (Cabrera and Finnell, unpublished data). This demonstrates the potential for the formation of a neo-antigen from the modified folate receptor, and generation of autoantibodies against this altered receptor protein. Whether such reactions occur in vivo, or whether the altered receptor has a compromised function or altered immunogenicity has not yet been elucidated, but is a current focus of research in our laboratories. Indeed, it would be of significant interest to re-evaluate the initial studies by Rothenburg and colleagues and characterize the autoantibodies against the maternal folate receptor and determine if they were directed against the native form, or against a homocysteinylated species. Additionally, it may be hypothesized that autoantibodies formed against a homocysteinylated form of the folate receptor may have a degree of cross-reactivity with the native folate receptor at high serum titers; this is another potential avenue of investigation in future studies.

Immune Factors as Regulators of Neurulation

Finally, it is proposed that immune factors may be directly involved in neurulation, independent from their expression and function in immune cells. This hypothesis is supported by observations in animal models of neurulation in which various immunoregulatory proteins have been found to be expressed in the developing neural tube (Gendron et al., 1991; Wride and Sanders, 1993; McGrath et al., 1999; Lomaga et al., 2000; Regnier et al., 2002; Tissir et al., 2004; Phelan et al., 2005; McLin et al., 2008). The chemokine, CXCR4, is expressed in E7.5 embryonic ectoderm and E8.5 embryos (McGrath et al., 1999), and is localized to the neural tube and neuroepithelium of E9.5 embryos (Tissir et al., 2004; Lieberam et al., 2005). Of interest, the *Cxcr4*^{-/-} mouse has no reported NTDs, although there are reports of defects in neural development (Zou et al., 1998; Lieberam et al., 2005). However, genetic ablation of adaptor factors for tumor necrosis factor receptors have been found to exhibit a high incidence of spina bifida and exencephaly (Lomaga et al., 2000; Regnier et al., 2002). The underlying mechanism of NTD generation in these models is uncertain, although a disruption to cytokine-mediated apoptosis has been postulated (Lomaga et al., 2000).

Toll-like receptors (TLRs) are important pathogen-associated molecular pattern receptors that operate as part of the innate immune system. TLR2, 3, and 4 have varied roles in adult and embryonic neurogenesis (Rolls et al., 2007; Lathia et al., 2008), but TLR3 is notably expressed in the floor plate of the developing zebrafish neural tube (Phelan et al., 2005). Indeed, several downstream components of the TLR signaling pathway have been linked to neural tube development. Inactivation of transforming growth factor- β -associated kinase 1 (TAK1) leads to neural tube closure defects in the mouse (Shim et al., 2005), and a dominant-negative acting myeloid differentiation primary response gene 88 (MyD88) leads to defects in notochord and cephalic development in *Xenopus* (Prothmann et al., 2000).

Signaling from several innate immune receptors, including TLR4, tumor necrosis factor receptor, interleukin-1 receptor and complement C5a receptor (C5aR), culminates in nuclear

factor kappa B (NF- κ B)-dependent transcriptional activation. Of interest, several genes in this pathway have been linked to NTDs including the mouse $IKK\alpha^{-/-}$ $IKK\beta^{-/-}$, $TRAF6^{-/-}$, $Bcl10^{-/-}$, and *Xenopus* dominant negative NF- κ B, animal models (Li et al., 2000; Lomaga et al., 2000; Lake et al., 2001; Ruland et al., 2001). Furthermore, NF κ B2, NF κ BIA, NF κ BIB, RelA, and upstream regulators IRAK1 and MyD88 have been found in a microarray surveying human neural crest cells (Thomas et al., 2008). Together, these findings support a possible role for these factors in neural crest survival and proliferation.

The innate immune complement system (Woodruff et al., 2010), has also been demonstrated to play roles in embryogenesis. Studies in *Xenopus* have demonstrated that numerous components of the complement system (C1qA, C1qR, C3, C3aR, Properdin, and C9) are expressed in the neural plate and neural crest during development (McLin et al., 2008). Furthermore, a recent study by Carmona-Fontaine and colleagues demonstrated that a key component of the innate immune system, complement component 3a (C3a), is a key mediator of neural crest cell chemotaxis in *Xenopus* (Carmona-Fontaine et al., 2011). Our laboratories have also recently demonstrated the presence of complement component C5 and related complement components in the murine neural tube throughout the period of neurulation (Coulthard et al., 2010; Denny et al., 2013). Expression of the principal receptor for complement component 5a (C5a), C5aR, was additionally demonstrated in the neuroepithelium of early human embryos (Denny et al., 2013). Furthermore, we have demonstrated an increased incidence of various NTDs in mice lacking a functional C5aR, or dosed with the selective C5aR antagonist PMX53 (Woodruff et al., 2005), under conditions of dietary folate deficiency (Denny et al., 2013). Together, these studies suggest a novel mechanism by which complement may be involved in neurulation, independent of its classical role in immune cell function, and may reflect a clinically relevant model for NTD susceptibility.

Ongoing research in our laboratories aims to additionally elucidate: whether other components of the complement system can similarly influence neurulation; the temporal window in which C5a may exert its protective effect during neurulation; whether the alternate C5a receptor, C5L2 (Li et al., 2013), can also mediate neural tube closure; the mechanism(s) of complement factor activation in the early embryo under both folate-replete and folate-deficient conditions; and if administration of complement activators or selective C5a agonists can rescue defective neurulation in mammals. We believe that these experiments will facilitate an understanding of the precise mechanisms by which complement and folate interact to ensure healthy neurulation in humans.

The precise role of these many different immune factors during neurulation is largely unknown. Nevertheless their immune roles may provide some insight as to what cellular processes these factors may control. For example, C3aR was recently shown to play an important role in neutrophil mobilization from the bone marrow (Wu et al., 2013), and so it is not surprising that C3aR is also involved in cell migration of neurons in rat cerebellum (Benard et al., 2008), and chemotaxis of neural crest cells in *Xenopus* (Carmona-Fontaine et al., 2011). Several studies have investigated gene expression in neural crest populations and revealed a range of immune factors to be expressed in these migratory cell populations, such as interleukin 2 receptor, NF κ B2, RelA, genes relating to tumor necrosis factor signaling,

and complement factors (Thomas et al., 2008; Makki and Capecchi, 2011; Krupp et al., 2012). The role for each of these factors in neurulation will need to be examined on a case-by-case basis. Ultimately, studying the role of immune factors in neurulation is opening the door for new insights into NTDs.

Folates and Modulation of Immune Factors

In support of a role for an interaction between complement and folate status in humans, a recent proteomics study of human plasma has revealed strong relationships between circulating folate levels and proteins associated with the activation and regulation of complement (Duthie et al., 2010). Furthermore, a study by Jensen and colleagues (Jensen et al., 2006) found that a maternal polymorphism in the promoter region of another innate immune factor, monocyte chemoattractant protein 1 (MCP-1), is associated with an increased risk (1.5-fold) of spina bifida in humans. MCP-1, also termed CCL2, is a member of the CC chemokine subfamily that acts by means of interaction with its G-protein coupled receptors, CCR2 and CCR4, to induce the chemotaxis of monocytes and basophils (Yadav et al., 2010). Intriguingly, folate insufficiency has been demonstrated to induce the synthesis of MCP-1 both in vitro (Brown et al., 2006; Lu et al., 2009) and in vivo (Li et al., 2007). More recently, Hammons and colleagues demonstrated that the 677C>T polymorphism in, 5,10-methylenetetrahydrofolate reductase (MTHFR), a key enzyme in the folate metabolic pathway, is strongly associated with increased concentrations of MCP-1 in females (Hammons et al., 2009). Therefore, it is not surprising that supplementation with folic acid reduces concentrations of MCP-1 and other pro-inflammatory cytokines (interleukin-8 and C-reactive protein).

Taken together, studies on MCP-1 and complement provide compelling evidence for an interaction between folates, immune factors, and the risk of NTDs in humans. However, a mechanistic explanation as to how folate status differentially modulates immune function remains to be elucidated. Furthermore, how these findings may translate to the clinical setting remains a major endeavor in NTD-related research.

CONCLUSION

There are a variety of emerging studies, both in vivo and in vitro, demonstrating a relationship between folate metabolism and the immune system. The link, yet not the mechanism, between folate status and NTDs in humans has been clearly established. What is not clear, and requires a diverse battery of approaches in future studies, is whether folate metabolism and the immune system interact during embryonic development to adversely affect neural tube formation. Recent experimental results in our laboratories suggest the plausibility of this notion.

These studies highlight the complexities of both linking various aspects of fetal survival, fetal health, and immune functioning. At present, it seems prudent to recognize that our profound ignorance of immune surveillance in healthy fetal development (either occasioned by maternal or fetal factors) is a major impediment to progress in the field. The immune system and fetal development are both highly complex, and attempting to link the two directly together will be a difficult, but potentially fruitful, endeavor. All we can say, at

present, is that this is an area of burgeoning interest, and the emerging evidence is that immune surveillance is important for the healthy appearance of the developing neural tube. We have recently demonstrated that the innate immune complement system, when confronted with maternal folate deficiency, provides a homeostatic safety net for the developing neural tube. This finding provides a novel platform on which to approach the study of NTDs, and one that should provide new insights into neural tube development.

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Figure 1.

The connecting links between neural tube defects (NTDs), immune factors, and folate. Many lines of evidence now link two of the three hubs, while recent evidence shows that knockout or inhibition of the innate immune complement receptor, C5aR, leads to neural tube defects under conditions of folate deficiency (Denny et al., 2013). Notes contained in blue boxes relate to positive relationships, notes in red boxes relate to inverse or negative relationships. ¹ Denny et al. (2013). ² Girardi et al. (2008). ³ Lomaga et al. (2000); McGrath et al. (1999); McLin et al. (2008); Phelan et al. (2005); Regnier et al. (2002); Tissir et al. (2004). ⁴ Torchinsky et al. (1997); Hrubec et al. (2006). ⁵ Greene et al. (2009b). ⁶ Spiegelstein et al. (2004). ⁷ Smithells et al. (1976). ⁸ Duthie et al. (2010). ⁹ Cabrera et al. (2008). ¹⁰ Hammons et al. (2009). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table 1

Types of Neural Tube Defects and Their Clinical Sequelae (Botto et al., 1999)

Neural Tube Defect (NTD)	Description and Clinical Sequelae
Anencephaly	An open NTD in which the brain and calvaria are absent. Universal neonatal lethality.
Craniorachischisis	Anencephaly accompanied by a continuous bony defect of the spine and exposure of neural tissue. Universal neonatal lethality.
Spina Bifida	A bony defect of the posterior vertebral arches accompanied by herniation of neural tissue and meninges. The defect may be open (not covered by skin) or closed (covered by skin). High rates of mortality and morbidity. Common complications include neurologic deficits (deformity of the limbs, paralysis, bladder and bowel dysfunction) and infection.
Encephalocele	Often considered to be a closed NTD in which the brain and meninges herniate through a defect in the calvaria. High rates of mortality and morbidity. Common complications include spastic quadriplegia, developmental delay, ataxia, seizures and infection.
Iniencephaly	A closed NTD that is more common in Asian populations than in Caucasians. Dysraphia in the occipital region is accompanied by severe retroflexion of the neck and trunk. Very poor prognosis with high (>95%) mortality.