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ONTOGENY OF THE FETAL IMMUNE RESPONSE TO MATERNAL SMOKING IN RELATION TO ALLERGIC ASTHMA

By

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ABSTRACT

ONTOGENY OF THE FETAL IMMUNE RESPONSE TO MATERNAL SMOKING IN RELATION TO ALLERGIC ASTHMA

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The relationship between maternal smoking and the prenatal origins of allergic disease was studied in 78 unselected mothers and their corresponding newborns with a sensitive test for interleukin-4 (IL-4), interferon-γ (IFN-γ), and cotinine as a biological marker to validate smoking data. For both maternal [$F(3, 74) = 26.43$, $p < .0001$] and cord blood cotinine [$F(3, 74) = 25.2$, $p < .0001$] significantly higher levels were observed for smokers compared to non-smokers in a dose-response pattern and a high correlation was found between maternal and cord blood cotinine serum, $r = .87$, $p < .0001$, indicating a good method to confirm the presence of maternal smoking. No association was observed between cord blood IL-4 and maternal smoking, maternal serum cotinine, or cord blood cotinine, respectively. An association was observed between maternal IL-4 and cord blood IL-4, $r = .37$, $p < .01$, suggesting a maternal-fetal cytokine interaction. Newborn birth weight significantly decreased as a function of daily maternal smoking, and maternal and cord blood cotinine, in a dose-response manner. Data on maternal smoking effects on selected perinatal variables are reported. Discussion of the pertinence of additional studies required to elucidate mechanisms involved in prenatal sensitization in relation to maternal smoking are discussed.

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Introduction

Trends in the development and severity of allergic disease are increasing at an alarming rate resulting in overwhelming public concern on a global scale. In particular, we have witnessed soaring prevalence rates of asthma and allergy in children within the past two decades (Burr, Butland, King, and Vaughn-Williams, 1989; Haahtela, Lindholm, Bjorksten, Koskenvuo, and Laitinen, 1990; Hsieh & Shen, 1988; Robertson, Heycock, Bishop, Nolan, Olinsky, and Phelan, 1991; Shaw, Crane, O'Donnel, Porteous, and Coleman, 1990). Effective control of allergic disease is still elusive partly due to the paucity of knowledge in the underlying pathogenic mechanisms.

Sensitization is the most critical event in the development of allergic disease. At least four separate components can be identified as being responsible for the development of sensitization to environmental antigens: genetic susceptibility, dose, timing, and duration of exposure. Genetic susceptibility can be explained as the predisposition for generating inappropriate amounts of immunoglobulin E antibody (IgE) against common environmental allergens, expressed clinically as asthma, eczema, and rhino-conjunctivitis. This predisposition is referred to as 'atopy'. Dose and duration are involved with the ability of the immune system to become sensitized to the allergen in question. At critical doses, less duration would be required for sensitization to occur and vice versa. This relationship of course would be a function of the degree of genetic susceptibility expressed by the individual, lending evidence to a dynamic interplay of factors in the development of sensitization. Timing has recently proven to be an important variable in this context. It has become increasingly apparent that the first 12

months of life are more likely to induce IgE-mediated hypersensitivity than antigens encountered later (Arshad & Hide, 1992).

Of particular interest are allergens derived from domestic dust mites, proteins from domestic pets, cockroaches, fungal antigens and pollens (Holgate, 1997). In addition, environmental tobacco smoke and maternal smoking has been shown to contribute to the onset and exacerbation of allergic disease (Jedrychowski & Flak, 1997; Martinez, Cline and Burrows, 1992).

The sensitization mechanism is initiated by the participation of cells, which recognize, process, and, in turn present allergens to the T (thymus)-cell effector arm of the immune system. Antigen presenting cells (APCs) including dendritic cells and activated B-(bursal) cells and more commonly, although not as effective, macrophages, act to engulf antigen, initiating a cascade of membrane and intracellular events involving interactions with MHC (major histocompatiblity complex) class II molecules, ultimately ending in the surface expression of small peptide fragments (epitopes) coding for appropriate T-cell activity. With respect to allergic disease, the allergen tends to code for the CD4+ (cluster of differentiation) T helper (Th) cell population.

T helper cells possess a functional heterogeneity about them, including cytokine secretion and expression of various surface receptors and can, thus, be divided into subpopulations. The generation of interferon-gamma (IFN-γ), tumor necrosis factor-beta (TNF-β), and interleukin-2 (IL-2) are unique to Th1 cells, whereas Th2 cells produce predominately interleukin(s) -4, -5, -6, -10, and -13 (Mosmann, Cherwinski, Bond, Geidi, and Coffman, 1986; Mosmann & Coffman, 1989). These effector cells develop from the T helper progenitor (Thp) cells, which differentiate as a result of the microenvironment's

influence via signals delivered by secreted cytokines and co-stimulatory molecules. Factors responsible for the direction of differentiation at the Thp stage include; IFN-γ, promoting Th1 cell production, both *in vitro* (Gajewski et al., 1989; as cited in Maggi, Parronchi, Manetti, Simonelli, Piccinni, Rugio, Carli, Ricci and Romagnani, 1992) and in the *Leishmania* model *in vivo* (Locksley, 1991; as cited in Maggi et al., 1992). Reciprocally, IL-4 induces Th2 production, again both *in vitro* (Demeure, Yang, Byun, Ishihara, Vezzio, and Delespesse, 1995) and *in vivo* (Coffman, 1991; as cited in Maggi et al., 1992). Th1 and Th2 subsets are hypothesized to cross regulate phenotypic expression of each other.

In a recent *in vivo* study, infection with *Bacillus Calmette-Guérin* (BCG) resulted in Th1 responding, and subsequent aeroallergen challenge being met with an attenuated response (Erb, Holloway, Sobeck, Moll, and Le Gros, 1998). Conversely, IL-10 production by the Th2 subset attenuated the effects of IFN-γ (D'Andrea. Aste-Amezaga, Valiante, Ma, Kubin, and Trinchieri, 1993). Further evidence has addressed this issue following a review of epidemiological studies assessing the global incidence of allergic disease in children (Doull & Holgate, 1997). It was found that children of increasing birth order and those from third world countries had lower prevalence rates of developing allergic disease. The hypothesis presented to account for this seemingly idiosyncratic response was that exposure to viral infections in early life occurs more often in these populations, offering protection against allergy evolvement. Thus, reactive Th1 responding to infection prevents sensitization to provoking allergens by sufficiently suppressing Th2 participation.

In focusing on the role of Th2 responding, it is important to review the utility of relevant cytokines. IL-4 has proven to be the most influential cytokine in the induction of IgE synthesis in reaction to allergen/APC influence on T-cells. IgE synthesis takes place after virgin B-cells arrive at a secondary lymphoid organ, where they undergo antigen-dependant selection. Once mature, the B-cells can become memory cells or be prodded by IL-4 to mature into plasma cells. These plasma cells, in the presence of IL-4, will undergo a unique iso-type class switch from production of immunoglobulin M (IgM) to IgE. Antibodies of the IgE class have a central role in allergic disease, with elevated levels of both total and allergen-specific IgE in serum of atopic patients (Barbee, Halonen, Kaltenborn, and Burrows, 1991). IL-5 (Pène, Rousset, Brière, Chrétien, Wideman, Bonnefy and de Vries, 1988) and IL-6 (Vercelli, Jabara, Arai, Yokota, and Geha, 1989) have been demonstrated to play an obligatory role in the IL-4 dependent induction of IgE. In addition, asthma inspired CD4+ T-lymphocyte activation is accompanied by increased serum concentrations of IL-5 (Corrigan, Hamid, North, Barkans, Moqbel, Durham, Gemou-Engesaeth and Kay, 1993). IL-10 appears to act antagonistically to IFN-γ, which is responsible for attenuation of IL-4 induced IgE synthesis (D'Andrea et al., 1993), thus furthering the actions of Th2 effector cells.

IgE antibodies, coded for the antigen which began this myriad of immune events, attach themselves to surface receptors on mast cells. This process can last weeks and even months in anticipation of the second encounter with allergen. This 'priming' mechanism is the basis of sensitization, and interestingly, results in no overt symptomatology. In later encounters between the allergen and the body, allergen molecules promptly bind to IgE antibodies on mast cells. When one such molecule

connects with two IgE molecules in a bridge-like fashion, it draws together the attached IgE receptors, and the mast cell springs to life. Activation involves cascades of enzymatic reactions resulting in calcium-mediated expulsion of chemical-laden granules (Bissonnette & Befus, 1998). Various chemicals released by mast cells are accountable for expressed allergic symptoms.

Tissue damage occurs later as frequency of allergen exposure is maintained. Of considerable concern is the recruitment of eosinophils to the tissue area (Desreumaux & Capron, 1996; Gleich, 1996) by which IL-5 plays a central role (Egan, Umland, Cuss, and Chapman, 1996). Proteins secreted from these cells, including major basic protein (MBP) and eosinophil cationic protein (ECP), appear to be detrimental to epithelial integrity, thus contributing significantly to airway pathology exhibited by asthmatics. Evidence of this process includes correlations of eosinophil numbers and the pathogenesis of allergic disease (Walker, Kaegi, Braun, and Blaser, 1991).

Smoking has been shown to promote Th2 activity in the immune system. Sherill, Halonen, and Burrows (1994) reported on a twenty-year follow-up analysis describing the relationships between IgE levels, atopy and smoking. The findings revealed that smoking and atopy were both related to significant elevations in total serum IgE, in a dose-response fashion, suggestive of a causal association. Zetterström, Vega, Huyghe and Zurwaski (1981) examined the risks of smoking and occupational allergy. They found that IgE was increased in smokers concomitant with sensitization against occupational allergens. These data were further supported by Taylor, Gross, Joyce, Holland, and Pride (1985) where IgE was greater in smokers and ex-smokers versus non-smokers. In addition, they reported smokers displaying higher eosinophil counts. Byron,

Varigos, and Wooton (1994) demonstrated that phytohaemaglutinin (PHA)-induced IL-4 production by peripheral blood mononuclear cells (PBMCs) was significantly higher in smokers as compared to non-smokers. In a study questioning the effect of passive smoking on children, it was found that children of smoking parents had a higher incidence of respiratory illnesses per year, significantly higher total leukocytic and eosinophil counts, higher percentage of eosinophils and increased concentrations of serum IL-4 and IgE in a dose-response manner to parental smoking (el-Nawawy, Soliman, el-Azzouni, Amer, Demian, and el-Sayed, 1996). Given the evidence from smokers and their children, increased IL-4 production may be part of the mechanism responsible for the above increases in serum IgE and allergic-like symptoms associated with cigarette smoking.

Prenatal Origins

Recently it has been suggested that sensitization to allergens may occur as early as *in utero*. Following T-cell development during gestation, the first lymphocytes are detectable in the thymus at eight to nine weeks of pregnancy. It has been shown that the more mature T-cells can demonstrate proliferative responses at this time (Toivanen, Uksila, Leino, Lassila, Hirvonen, and Ruuskanen, 1981). More notably, T-cells migrate into fetal circulation at about 15-16 weeks of gestation. Warner (1997) reported proliferative responses to PHA in PBMCs starting at 17 weeks, which continued to increase, being significantly higher in the second and third trimester. Furthermore, a similar increase in proliferative responses to specific inhalant and ingestant allergens has been determined (Jones, Miles, Warner, Colwell, Bryant and Warner, 1996), supporting the theory of prenatal programming.

Prenatal programming is supported further by positive proliferative responses of fetal T-cells to a range of allergen sources including, house dust mite, cat fur, birch tree pollen, β-lactoglubulin, and ovalbumin from as early as the 22nd week of gestation (Warner, 1997). These data effectively support that maternal exposure to allergens is sufficient in priming of fetal T-cells such that, when confronted with environmental allergens antenatal, responding becomes consequential.

A growing body of research suggests that maternal tobacco smoking is associated with decreased lung development and function in offspring. For example, Taylor & Wadsworth (1987) conducted a national study in the United Kingdom on 12, 743 children and confirmed that maternal smoking had a significant influence on the reported incidence of bronchitis and admission to hospital for lower respiratory tract infections (LRTIs) during the first five years of life. In Sweden, Carlsen, Jaakkola, Nafstad, and Carlsen (1997) assessed a birth cohort of 3, 754 newborns for breathing parameters and respiratory mechanics. They found that maternal smoking affected tidal breathing parameters in newborns, and the decline in lung function was directly proportional to the number of cigarettes smoked per day by the mother. Chen, Kimizuka, and Wang (1987) in Quebec, Canada, studied lungs from fetuses (postmortem) of smoking mothers compared to those of nonsmoking mothers and showed that there were significant differences between the groups morphologically. To compare lung function between infants of smoking mothers to infants with a family history of asthma, Sheikh, Goldsmith, Howell, Parry, and Eid (1999) conducted a cross-sectional study. Findings included evidence that the infants showed no statistically significant difference on a variety of respiratory measures, however in response to bronchodilators the asthmatic history group

responded positively. Jedrychowski & Flak (1997) studied a sample of 1,129 grade two students from Poland and found that the relative risk of developing respiratory infection became significant after prenatal exposure to smoking was included in addition to postnatal environmental tobacco smoke (ETS). Martinez et al. (1992) from Arizona, USA, revealed that in a group of 786 children, those who where exposed to maternal tobacco smoke *in utero* (10+ cigs/day) were 2.5 times more likely to develop asthma and had a 15.7% lower maximal midexpiratory flow. In an epidemiological study assessing whether reported deficits in lung function in newborns persist in later childhood, it was found that maternal smoking and/or ETS exposure in the first few years of life was attributable to sustained altered pulmonary function throughout the child's life (Cunningham, Dockery, Gold, and Speizer, 1994). Arshad & Hide (1992) followed a group of 1,167 infants for 1 year in a population-based prospective study to assess the effect of environmental factors on the development of allergic disorders. They found that maternal smoking produced odds ratio of 2.30 ($p < 0.003$) as being a risk factor for the development of asthma. In a similar study by Infante-Rivard, Gautrin, Malo and Suissa (1999) a group of 457 children were followed in Montreal, Canada after meeting experimental criteria, most notably to have been diagnosed for the first time with asthma at an emergency room at the designated hospital. The findings included maternal smoking as a risk factor for childhood asthma. In particular smoking less than 20 cigarettes per day produced an odds ratio of 1.22 and maternal smoking of greater than 20 produced an overwhelming odds ratio of 3.84.

Taken together, these studies indicate that maternal smoking has the potential to alter lung development and increase susceptibility to LRTIs and/or asthma thereafter. It

has been hypothesized that the increase in prevalence rates of childhood asthma may be partially due to increased prevalence of smoking among women of childbearing age (Martinez, 1997). With respect to Canada, the prevalence of female smokers of childbearing age (20-44) is 34%. Ontario was estimated at 33% and Thunder Bay, Ontario was reported at 44% (Ontario Health Survey, 1990). These statistics make Thunder Bay an excellent candidate for research in this growing field.

Less attention has been directed towards elucidating the process by which maternal smoking leads to increased potential for respiratory complications in offspring. Magnusson (1986) reported increased levels of IgE in umbilical cord blood of smoking mothers after controlling for parental allergic history. Contrary to these findings, Ownby, Johnson and Peterson (1991) and Oryszczyn, Godin, Annesi, Hellier, and Kauffman (1991) both found that, after increasing the sample size and controlling for several more confounding variables, IgE in cord blood was not affected by maternal smoking. Neutrophils are shown to be decreased in cord blood of smoking mothers (Mercelina-Roumans, Breukers, Ubachs, and van Wersch, 1996; Harrison, 1979), as well, IgG and IgA are elevated in cord blood (Cederqvist, Eddey, Nagwa, and Liwin, 1984), suggesting an explanation for the enhanced incidence of postnatal infection seen in children of smoking mothers. What has yet to be investigated is fetal cytokine production in cord blood of smoking mothers. One possible explanation for the disparate IgE findings is that the child is not yet producing IgE since the child has not yet been exposed to ETS or the allergen directly. However, the fetal immune system may have been primed, *in utero*, by excessive Th2 responding by the smoking mother. Such priming could influence fetal

immune functioning in the Th2 direction, so that when exposed to ETS antenatally, rapid responding ensues, leading to IgE-mediated sensitization.

The focus of this study is to examine tobacco smoking effects on immune functioning in relation to allergic asthma by comparing maternal and cord blood samples of smoking and non-smoking mothers. Consequently, a comparison of perinatal variables between these groups is also intended. Particularly, the fetal immune response and perinatal outcome to intrauterine tobacco exposure via maternal smoking deserves to be investigated.

Sensitization is critical when considering what might be responsible for smoker's childrens' greater susceptibility to asthma. As explained earlier, any allergic disease becomes phenotypic only after sensitization occurs. The question then arises, what mechanisms are in place to allow or prevent sensitization from occurring? The above discussion clarifies the important role T-cells and cytokines play in orchestrating the asthmatic or allergic response and most notably the role of IL-4 and IFN-γ. Researchers agree that excessive Th2 responding is primarily responsible for initiating IgE synthesis and subsequent mast cell sensitization to allergens. One could envisage an immune system favouring Th2 and deficient in Th1 responding, which would be conducive to sensitization, just as, conversely, exposure to infections in third world countries has been speculated to be protective since it encourages Th1 responding.

Despite the intimate contact between fetal-derived allogenic tissue and maternal-derived decidual tissue, generally the immunocompetent mother does not reject the fetus. Several mechanisms have been proposed, which create an intrauterine milieu propitious to the continuation of pregnancy. The regulatory role cytokines play to this avail has

been recently elucidated. Successful pregnancy has been shown to be associated with a skew in T-cell operation towards Th2 expression (Wegmann, Lin, Guilbert and Mossmann, 1993). This shift in T-cell population of the fetoplacental unit to produce Th2 cytokines inhibits maternal Th1 cytokine production. Thus, whether the mother is allergic or not, the fetus is exposed to conditions that would favour the development of Th2-like responses (Warner, Jones, Jones, and Warner, 2000). It is believed that, towards the end of gestation, IFN-γ (Th1) levels elevate to guard against fetal allergic sensitization. However, the interaction between pregnancy and smoking may inhibit this mechanism due to excessive Th2 suppression of IFN-γ. Supporting this argument is evidence of low IFN-γ levels in cord blood being predictive of atopic eczema (Warner, Miles, Jones, Quint, Colwell and Warner, 1994). Hence, it would appear probable that interactions between Th1 and Th2 cytokines during pregnancy are influential in determining the type of immune response that develops *in utero* and carries on into early life. Taken together, pregnancy already coupled with Th2 activity with the addition of further Th2 responding from maternal smoking, may partially be responsible for sensitization or securing a Th2 immuno-environment at birth.

Maternal smoking is hypothesized to play an integral role in influencing the fetal immune system at critical periods of immuno-development. Because of the degree of Th2 responding to smoking and the susceptibility of the naïve fetal immune system, fetal T-cell repertoires may be significantly shifted in the Th2 direction, away from Th1. A cord blood measure of IL-4 would verify this assumption and a measure of IFN-γ would verify a deficiency of Th1 activity.

The proposed study attempts to provide evidence of intrauterine priming of Th2 expression resulting from maternal smoking in relation to allergic asthma.

Methods

Participants

Participants were recruited in Thunder Bay, Ontario, Canada, from prenatal clinics and classes. To be eligible mothers must have planned to deliver at Thunder Bay Regional Hospital within the testing window, be expecting a live singleton fetus and have completed a questionnaire and provided informed consent (Appendix A).

In total, 99 mothers were recruited from the above-mentioned sources. Within the total, 21 participants were excluded from the analysis; 10 were missed for reasons of the investigator not being called to the delivery; 4 did not provide viable blood samples (hemolyzed or lack of volume); and 7 delivered after the study was completed. Thus, included in the study were 78 mothers and their respective newborns.

Test Materials and Procedure

Questionnaire. Development of the "Lifestyles in Pregnancy" questionnaire (Appendix B) was intended to assess a select group of variables, with respect to maternal lifestyles during pregnancy, and to explore how these variables influence chosen immune system parameters. Special attention was given to maternal smoking rates in comparison to maternal non-smoking.

Dissemination of research results was provided via participants filling out the "request for results" form (Appendix C).

Delivery and Postpartum Data Collection of Perinatal Variables. Information was collected surrounding the time of each delivery including the duration of gestation, birth order of the newborn (parity), APGAR scores taken at 1 and 5 minutes of life, newborn

weight, method of delivery, presence or absence of gestational diabetes, and the presence or absence of meconium stain. Collectively these data are referred to as 'perinatal variables'.

Sample Collection and Analyses. Upon admission to the hospital for delivery, participants having routine admission blood work performed had an additional 10 ml tube drawn for the purpose of this study. Once the fetus had been delivered umbilical cord blood was collected by following this protocol when possible: the clamped cord attached to placenta was washed using saline solution intending to clean away maternal blood; the cord was unclamped and allowed to drain into a sterile cup by elevating the attached placenta; cord blood was then collected by pouring into serum separator tubes. The removal of cord blood and pouring into tubes technique was developed in order to eliminate the possibility of hemolysis caused by use of a syringe, and to ensure purity of the samples, washing was performed.

Blood samples were allowed to sit for 30 minutes after collection to allow clotting followed by centrifugation to separate the serum. Serum was aliquoted into 4 tubes, marked and frozen at -20 °C until assay was performed.

Maternal contamination of the umbilical cord blood is certainly a confounding variable. This issue was dealt with by screening the cord blood for the presence of immunoglobulin-A (IgA) (Piastra, Stabile, Fioravanti, Castagnola, Pani, and Ria, 1994). IgA does not cross the placental barrier and is elevated in the pregnant mother, thus its existence in cord blood would be a marker of maternal contamination. Each cord sample collected fell below the sensitivity of the IgA assay employed (<0.07 g/L). The IgA reference range for an adult is 0.639-3.82 g/L.

Total IL-4 and IFN-γ was quantified by employing a sandwich enzyme immunoassay technique (ELISA), using a commercial immunoassay (R&D Systems, Minn, USA.). Only 60 of the 78 participant's blood and corresponding cord blood samples were selected to be subjected to the ELISA. The selection was based on choosing the 30 highest smokers and 30 non-smokers. Standards and serum samples were added individually in duplicate to microplate wells coated with a monoclonal antibody specific for IL-4 or IFN-γ cytokines. The immobilized antibody would bind any cytokine present. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for the cytokine was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to induce a colour reaction in proportion to the amount of cytokine bound in the initial step. A stop solution was applied at a specific time and the intensity of the colour was measured using an ELISA plate reader. Techniques for the assay precisely followed those provided for in the kits. Controls were assayed in duplicate by the investigator to ensure the reliability of the ELISA's and consistency of techniques. The sensitivity of the standard ELISA's used ranged from 31.2 pg/ml to 2000 pg/ml for the IL-4 assay and from 15.6 pg/ml to 1000 pg/ml for the IFN-γ assay. High sensitivity IL-4 ELISA was performed producing results sensitive from 0.25 pg/ml to 16 pg/ml.

Calculation of the results were obtained by creating a standard curve and plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and drawing a best fit curve through the points on the graph.

To assess the validity of self-reported smoking histories from the mothers, cotinine was measured in both maternal and cord samples by using a commercial

immunoassay (Serex Inc., New Jersey, USA) validated for the semi-quantification of cotinine in human saliva and urine. The immunoassay was validated for use with human serum by the investigator by means of spiking negative serum samples with known levels of cotinine to create a standard curve for which to compare unknowns. The sensitivity of the assay ranged from 8 to 250 ng/ml. In addition, this information was used to assess placental transfer of cotinine and to explore relationships between cotinine levels and the selected perinatal variables. Of the major metabolites of nicotine, cotinine is a suitable candidate for a marker of smoking as it has a relatively long half-life of 10-40hrs and is a proven marker of maternal smoking (Oryszczy n et al., 1991; Ownby et al., 1991; Mochizuki, Maruo, and Masuko, 1984).

Results

Descriptive characteristics of the participants are presented in Table 1 (page 44).

Both IL-4 and IFN-γ were undetectable in both maternal and cord blood using the standard ELISA kit sensitive to 31.2 and 15.6 pg/ml respectively. A high sensitivity IL-4 ELISA (sensitivity range: 0.25 – 16 pg/ml) was performed on a subset of 59 matched maternal/cord blood samples able to detect presence of cytokine. No significant difference in IL-4 levels was found between smoking and non-smoking groups in both maternal and cord blood samples. ANOVA methods designed to test differences between non-smoking, light smoking (1-8 cigarettes/day), moderate smoking (9-15) and heavy smoking (>15) as a function of IL-4 levels produced no significant findings for both maternal and cord blood samples. No association was observed between maternal allergic and asthmatic history and maternal and cord blood IL-4. Maternal and cord blood cotinine levels were separated into 7 groups (0, 8, 16, 31, 62, 125, and 250 ng/ml) to search for differences as a function of IL-4 concentration. No main effect was found in either maternal or cord blood cotinine groups. Contrasts evaluation revealed significant differences between grouping lower cotinine concentrations (8, 16, and 31 ng/ml) against higher (62, 125 and 250 ng/ml) in maternal blood samples, $t(18.84) = 2.82$, $p < .011$. Parallel analysis for cord blood approached significance, $t(5.67) = 2.47$, $p < .051$. Correlational analysis presented a positive relationship between maternal and cord blood IL-4 concentrations, $r = .37$, $p < .004$ (Figure 1).

Maternal smoking was separated into subgroups as a function of cigarettes smoked per day: non-smoker = 0, light smoker = 1-8, moderate = 9-15, and heavy = > 15 for ANOVA. Maternal cotinine concentrations were found to increase with maternal

smoking, $F(3, 74) = 26.43$, $p < .0001$. The concentration of cord blood cotinine was found to increase with the degree of maternal smoking, $F(3, 74) = 25.20$, $p < .0001$. Daily caffeine consumption by the mother increased with the degree of maternal smoking, $F(3, 74) = 11.43$, $p < .0001$. Newborn birth weight followed a downward trend as maternal smoking, maternal cotinine and cord blood cotinine increased (Figure 2), $F(3, 74) = 4.36$, $p < .007$; $F(6, 71) = 3.33$, $p < .006$; and $F(6, 71)$, $p < .017$ respectively. No significant findings were revealed to indicate a relationship between the degree of maternal smoking and newborn APGAR scores (1 or 5 min.) as a function of maternal cotinine, cord blood cotinine or reported daily maternal smoking habits.

The cotinine assay employed allowed for the discrimination between 7 concentrations measured in ng/ml; 0, 8, 16, 31, 62, 125 and 250. These concentrations were used in ANOVA for the following results. The concentration of maternal serum cotinine increased with the number of cigarettes smoked per day, $F(6, 71) = 25.21$, $p < .0001$, creating a dose-response relationship (Figure 3). Maternal weekly exercise is associated with increasing maternal cotinine, with the highest frequency taking place in the highest concentration and the lowest frequency in the zero level, $F(6, 71) = 25.21$, $p < .013$. Simple effects reveal that the differences largely lie between grouping the lower (8, 16, and 31ng/ml) against the higher (62, 125, and 250ng/ml) cotinine groups, $t(3.73) = 4.66$, $p < .015$. Differences in average daily caffeine consumption by the mother was found to produce differences in the concentrations of maternal serum cotinine, $F(6, 71) = 2.95$, $p < .013$. Further analysis on this variable via contrasts revealed that the differences largely lie between zero level serum cotinine and 62ng/ml, 125ng/ml, and 250ng/ml respectively, at $t(3.24) = 12.61$, $p < .007$; $t(4.79) = 13.14$, $p < .0001$, and $t(3.90) = 7.11$, p

< 0.006. Maternal age of the smoking and non-smoking groups were significantly different, $t(76) = 2.08$, $p < .05$.

Cord blood cotinine levels were found to be significantly associated with maternal cotinine in a dose response fashion, $r = .865$, $p < .0001$ and have significant differences between concentration groups, $F(6, 71) = 21.64$, $p < .0001$ (Figure 4). Maternal cigarettes smoked per day indicates an increase in cord blood cotinine, $r = 0.66$, $p < .0001$, and a difference between cotinine groups, $F(6, 71) = 21.64$, $p < .0001$. Maternal daily caffeine consumption is associated with an increase in cord blood cotinine, $F(6, 71) = 4.3$, $p < .001$.

Table 2 (page 46) summarizes significant correlations (Pearson) found between select variables. In particular the findings associated with birth weight indicate a consistent inverse relationship. From daily maternal smoking, maternal cotinine down to cord blood cotinine levels, correlations remain stable, $r =$-.37, $p < .001$; $r =$ -.36, $p <$ .001and $r =$ -.37, $p < .001$ respectively. Maternal caffeine consumption on a daily basis follows a comparable trend with respect to birth weight, $r =$ -.33, $p < .01$, as it correlates with maternal smoking, $r = .49$, $p < .0001$; maternal cotinine, $r = .38$, $p < 0.001$ and cord blood cotinine, $r = .37$, $p < .001$. A positive relationship between maternal serum IL-4 and cord serum IL-4 was also demonstrated, $r = .37$, $p < .004$.

Mean daily smoking habits were divided by the mean maternal cotinine concentration to produce a maternal cotinine per cigarette value. Parallel calculations were performed to produce cord blood cotinine per maternal cigarette and birth weight decrease per unit of cord blood cotinine. The following results were generated. Each cigarette represented 8.34 ng/ml and 7.3 ng/ml maternal and cord blood cotinine

respectively. For every 1 ng/ml maternal and cord blood cotinine, birth weight decreased by 4.32 and 4.93 g, respectively. In other words, each cigarette smoked produced a decrease of 36 g of birth weight as indicated by the maternal and cord blood cotinine levels.

Discussion

No association was observed in this study conducted on 78 Canadian mothers and their respective newborns between cord blood IL-4 and maternal smoking or between maternal smoking and maternal IL-4. A positive association was found however between maternal and cord blood IL-4 levels. No association was observed in IL-4 levels as a function of maternal allergic or asthmatic history.

Smoking validation

Validation of smoking habits was verified by serum cotinine measurements. Cotinine levels are more relevant to consider than nicotine levels, since cotinine has a larger serum half-life (about 18 hours in adults) than nicotine (about 2 hours) and is exclusively a product of *in vivo* metabolism, thus avoiding contamination problems. Smoking data assessed by questionnaire correlated well with both cord and maternal serum cotinine levels. Levels observed are comparable to those found in the literature (Oryszczyn et al., 1991). Our results demonstrate that cord blood may be used to assess maternal smoking in varying degrees as determined by the strong correlation between maternal and cord blood cotinine (Figure 4), which supports findings in the literature (Jauniaux, Gulbis, Acharya, Thiry, and Rodeck, 1999; Hayde, Bernaschek, Stevenson, Knight, Haddow and Widness, 1999). Our results indicate that for every cigarette smoked by the mother there is an increase of 8.34 ng/ml and 7.3 ng/ml in maternal and cord serum cotinine, respectively. These data can be compared to that in the literature for cord serum increase per maternal cigarette at 4.4 ng/ml cotinine (Nafstad, Kongerud, Botten, Urdal, Silsand, Pederson, and Jaakkola, 1996). Questionnaires however should

not be ignored but rather used in conjunction, as cotinine levels reflect short-term exposure only, thus not accurately depicting the occasional smoker.

Maternal smoking and perinatal variables

Maternal daily smoking habits and maternal and cord blood cotinine all produced a significant decrease in newborn birth weight (Figure 2) when compared against their non-smoking counterparts, which is in accordance with much previous research (i.e. Bai, Wong, Gyaneshwar and Stewart, 2000; Cooke, 1998; Schellscheidt, Jorch, and Menke, 1998; Cornelius, Taylor, and Geva, 1995). Evidence of decreased birth weight in mothers who smoked by Haug, Irgens, Skjaerven, Markestad, Baste and Schreuder (2000), indicated a 232 g mean reduction in comparison to non-smokers. The current study's data shows a further average reduction in birth weight in the order of 415 g. Further analysis of the data in this study revealed that for every 1 ng/ml of cord blood cotinine, birth weight decreased by 4.93 g, $r = -.37$, $p < .001$. Low birth weight is a significant risk factor in subsequent occurrence of sudden infant death (Haustein, 1999). Literature reports (Windham, Hopkins, Fenster and Swan, 2000) of increased incidence of pre-term birth (< 245 days) in relation to maternal smoking were not confirmed in this study. The mean gestational length in the non-smoking group (n = 42) was 281.6 days and 277.72 days in the smoking group (n = 36).

An interesting finding was revealed when comparing maternal cotinine levels to corresponding cord blood cotinine. In 19% of the smoking group (7 of 36), cord blood cotinine levels exceeded those of the matched maternal levels. Higher cotinine levels were reported in fetal serum compared to maternal serum in a study conducted by Jauniaux et al. (1999). Reported levels were in a population of mothers and their aborted

fetuses. This finding in conjunction with our own suggests that the fetus may not be as metabolically equipped as the adult mother in dealing with nicotine and cotinine degradation both in early pregnancy and at term, another point of interest when considering teratogenic effects of nicotine on prenatal development – sustained concentration for increased duration.

Counter to common thought was a finding that maternal exercise increased significantly with maternal smoking. Considering exercise to be a healthy activity requiring assiduousness, one would assume that this would carry over into other 'healthy lifestyles', notably, non-smoking behaviour. It should be noted that exercise frequency was self-reported via questionnaire and may have been inflated to compensate for other consciously unhealthy pregnancy practices, such as smoking. If indeed the correlation is accurate further research may be warranted to explore this relationship.

Average daily caffeine consumption measured by questionnaire had a strong association with maternal smoking indicating, as daily maternal smoking frequency increased, caffeine habits followed suit. This finding supports evidence in the literature suggesting that caffeine has the ability to potentiate the reinforcing properties of nicotine, thus highlighting the importance of environmental factors in shaping and maintaining tobacco smoking (Shoaib, Swanner, Yasar, and Goldberg, 1999). It seems logical then to request of smoking pregnant mothers who refuse to quit to deny themselves caffeine in lieu of abstaining from smoking and perhaps, as research suggests, smoking behaviour should decline having less rewarding properties attached to it (not considering the behavioural component of addiction).

Iinterleukin-4 Findings

Interleukin-4's involvement with the progression to sensitization can be considered a critical one. Without its presence, iso-type class switching would fail to occur in B-cells, disallowing production of IgE antibody essential for mast cell degranulation. In the past IgE has been measured in cord blood in relation to maternal smoking only to produce discrepant results. Magnusson (1986) found that in 186 newborn infants, for which 41 were exposed to maternal smoking, the association between maternal smoking and IgE was significant. In opposition are results generated from a sample of 325 newborns, 62 of whom had smoking mothers, (Johnson, McCullough, Blocki, Strauchman, Jacobsen and Ownby, 1989) no association between maternal smoking and cord blood IgE was found.

It was believed that IgE might not be the marker of choice in the maternal smoking - fetal sensitization paradigm since the fetus had not yet been directly exposed to tobacco smoke and IgE is unable to cross the placental barrier. Among adults, the relationship between IgE and smoking has been determined in most studies, however the mechanisms involved are unclear. IL-4 levels have been demonstrated to be elevated in adult smokers as well (Byron et al., 1994). In their study, the method used to assess differences in IL-4 levels was *in vitro*, via prodding T cells to measure their potential to produce cytokines.

An *in vivo* measure of IL-4 and IFN-γ performed in the current study was chosen to test the theory of intrauterine priming through placental transfer of cytokine's influence on fetal T cell differentiation (Figure 5). IL-4 was chosen to represent Th2 expression and IFN-γ was selected to represent Th1 expression. The cytokines were

hypothesized to exhibit an inverse relationship. In the smoking group (maternal and cord serum) the direction was believed to reveal elevated IL-4 and decreased IFN-γ. By employing traditional *in vitro* methods of prodding cells to produce desired cytokines, the assessment of placental transfer of cytokines could not have been performed using the design chosen. By measuring cytokine levels, as they exist *in vivo* in the mother and the fetus (cord blood), an accurate depiction of the relationship between cytokine levels could be explored. Although the association between maternal smoking and cytokine levels was not established according to the hypothesis, a positive correlation was revealed between maternal (smoking and non-smoking) IL-4 and cord blood IL-4 (Figure 1), suggesting some cytokine activity being shared between mother and fetus. In order to confirm whether this finding is a result of maternal cytokine influence on fetal T cell differentiation favouring Th2 production, further research is warranted.

The Th1/Th2 paradigm proposed in the literature to represent immune operation during pregnancy and that of allergic responding is indeed evidence based. Following this model, in addition to adult smoking effects on the immune system, one would deduce that the outcome would be upregulated Th2 responding in both maternal and newborn units when taking measurements at birth. However, our findings do not bear this presumption. Considering our method accurately represents our intent, some other mechanism may be at play influencing this model or another variable within our population sample requires examination. A recent paper in the literature alludes to the proposition of abnormal Th2 responding in atopic positive women and their fetuses. Williams, Jones, Miles, Warner and Warner (2000), sought to examine IL-13 (possesses IL-4 like activity) expression by fetal and neonatal cells and the placenta via cell

culturing in the presence or absence of PHA. They found that cells stimulated to produce IL-13 in samples from atopic positive histories had lower counts compared to those without parental atopic histories. The authors concluded that this may be representative of an inherent immaturity in the development of T cell-cytokine responses in babies at genetic risk for atopy or could be a consequence of downregulation of responses by other factors.

It is possible that a subsystem not yet identified in the Th2 arm of the immune system is not influenced normally by changes in Th1 functioning during pregnancy. While the model has Th1 responding attenuated during pregnancy, allowing Th2 functioning to be elevated vis à vis disinhibition, this Th2 subsystem may not follow suit. We propose this alternate model following observational data and subsequent literature review. Throughout data collection the principal investigator had numerous opportunities to speak with the enrolled mothers and fathers on the labour and delivery ward. A recurring item of information discussed with the parents with asthmatic histories was levels of symptoms before and during the pregnancy. Most often the mother explained that asthma inhaler use before pregnancy was considerably increased as compared to during her pregnancy. According to evidence of Th2 control during pregnancy and the known Th2 supremacy concomitant with asthmatic symptomatology, it seems logical that allergic/asthmatic women who become pregnant should be at risk for elevated responding and thus increased inhaler use. Several investigators have examined this relationship between pregnancy and asthma. Schatz, Harden Forsythe, Chilingar, Hoffman, Sperling and Zeiger (1988), found that in 330 maternal asthma associated pregnancies, symptoms worsened in 35%, improved in 28%, and was unchanged in 33% of the women. Similar

findings were found by Stenius-Aarnaila, Piirila, and Teramo (1988) in a study conducted on 198 asthma associated pregnancies. They found that anti-asthma medication use during pregnancy increased in 42%, decreased in 18% and remained unchanged in 40% of the women.

Future studies should stratify the allergic disease population, in prenatal origins of allergic disease research, into groups representing levels of symptomatology before and during pregnancy. These groups would be a function of the level of asthma/allergic resistance and proneness to changes in pregnancy associated modifications of immune function. It may be that clinically similar allergic disease differ at the level of the immune system or genetically and based on these differences offspring are at a greater or lesser risk to develop allergic disease. This could be carried out by immuno profiling the groups via quantifying Th1/Th2 expression and by using cDNA micro-array technology to further classify differences genetically.

In relation to our findings it is possible that the lack of differences in cytokine production found as a function of smoking status and allergic/asthmatic history is due to factors not considered at the outset of this investigation. It would be interesting to run a retrospective pilot study involving re-questioning of participants on their allergic/asthmatic symptoms before, during, and after pregnancy and see how this classification influences Th1/Th2 cytokine production. If a trend is observed another study could be designed with larger numbers in each group.

Conclusion

The association between maternal smoking and the prevalence of allergic disease in offspring has long been determined (Arshad & Hide, 1992; Infante-Rivard et al., 1998;

Jedrychowski & Flak, 1997). The mechanism to account for this relationship has yet to be elucidated. This paradigm deserves further research into the biological and molecular underpinnings since it's a model free of important confounds found in similar studies relating to the prenatal origins of allergic disease, most notably, consistency of insult. When maternal smoking occurs it likely does so daily and in similar amounts. Other models suffer from seasonal variation of allergen, high variability in duration of exposure, and insufficient biological markers for maternal and fetal levels of the suspected insult. For these reasons the maternal smoking paradigm is fitting to provide a base from which to develop. To isolate possible targets in the chain of events leading to sensitization that is being exploited in the fetus during gestation, points of intervention could be explored and the birth of prenatal management of allergic disease may not be far to follow.

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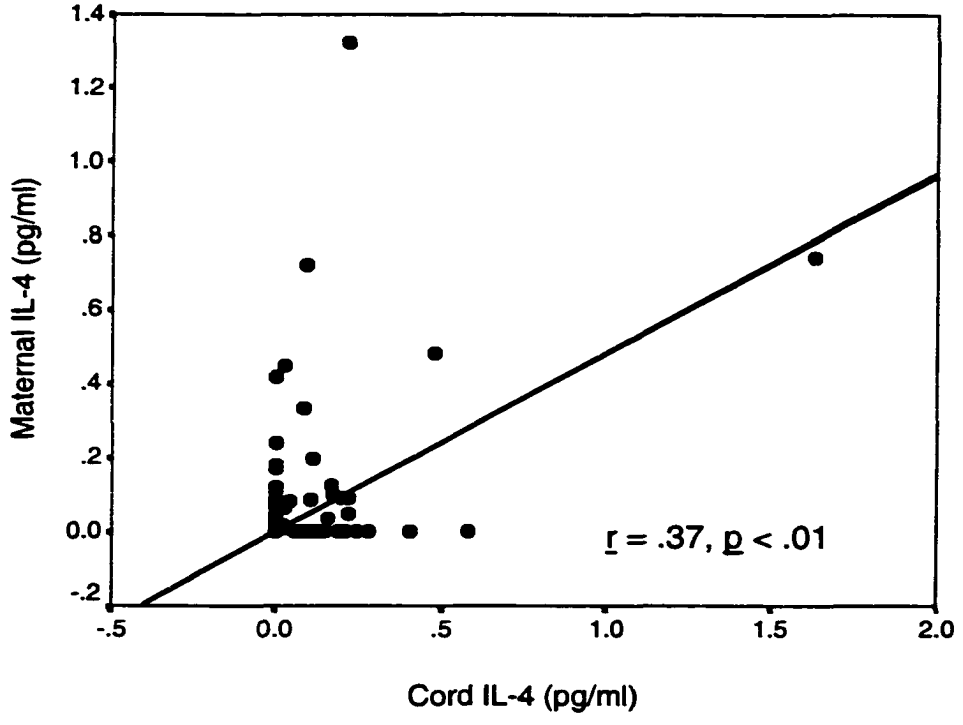

Figure 1. Relationship between maternal serum IL-4 (pg/ml) and umbilical cord serum IL-4 (pg/ml).

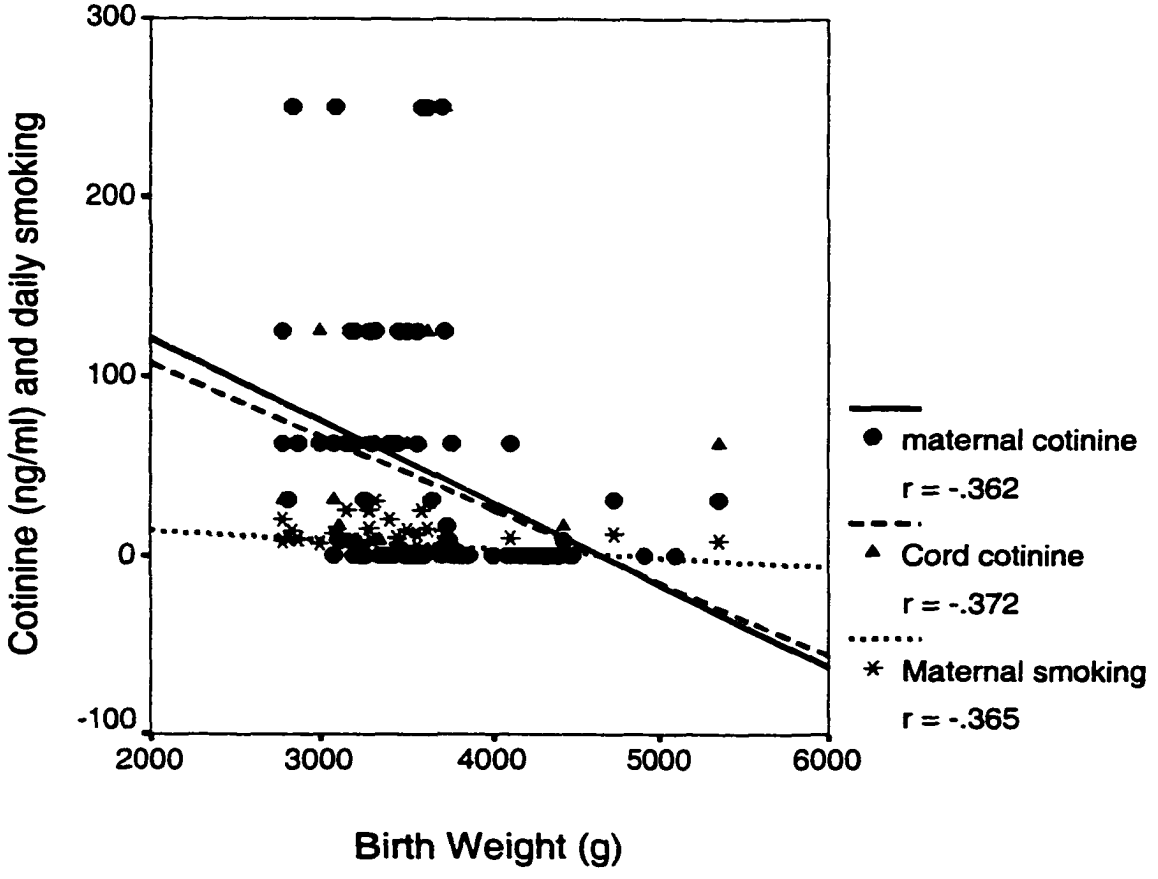

Fig. 2. Daily maternal smoking habits, maternal serum cotinine and cord serum cotinine according to newborn birth weight.

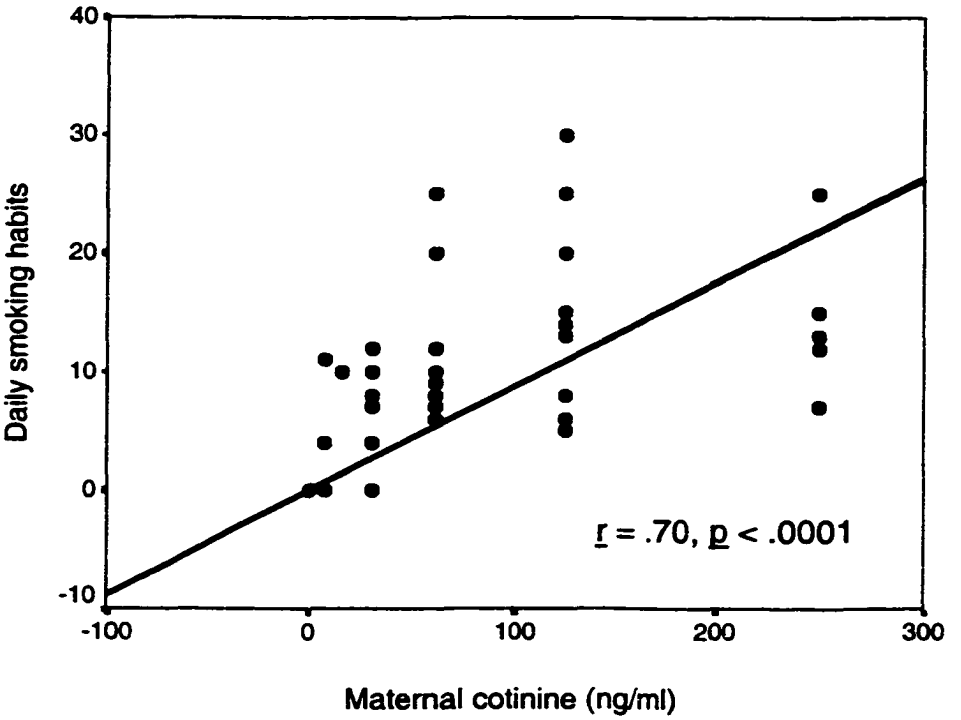

Fig. 3. Relationship between maternal daily smoking habits and corresponding maternal blood cotinine.

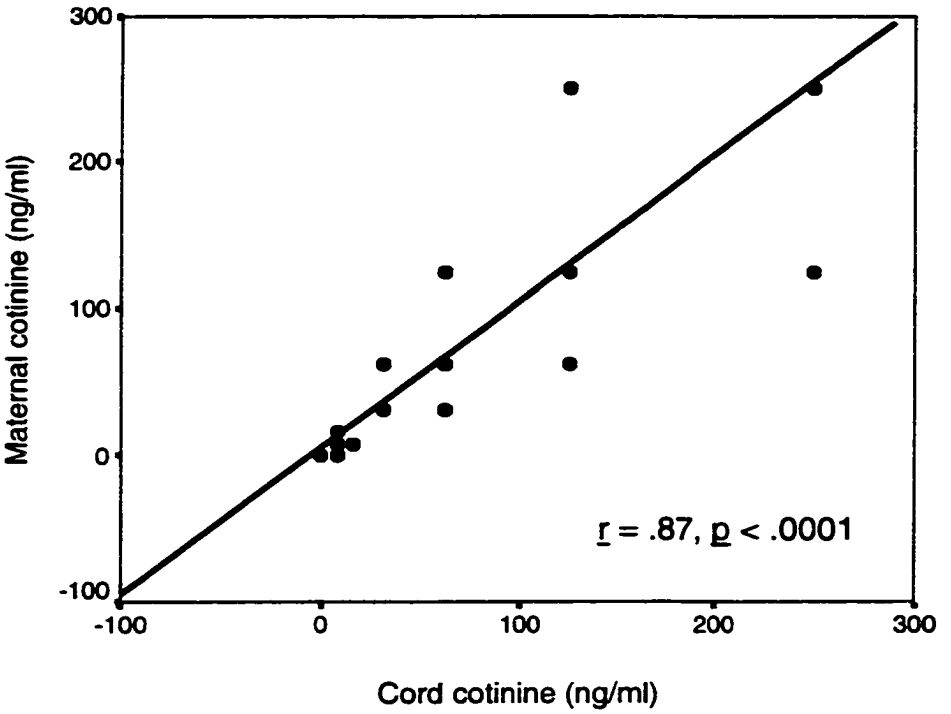

Figure 4. Relationship between maternal blood cotinine and newborn cord blood cotinine.

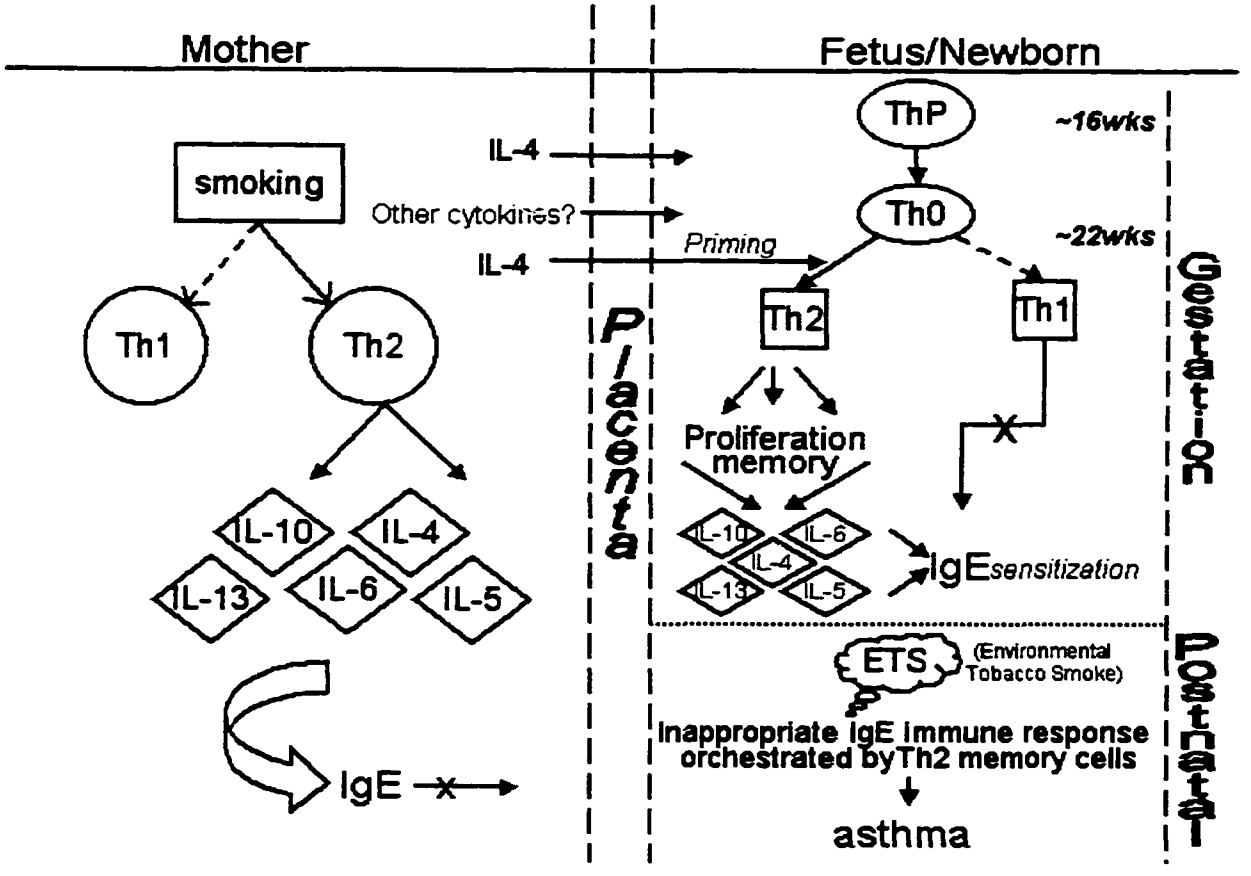

Figure 5. Intrauterine priming model. Maternal smoking generates increased production of Th2 type cytokines while suppressing expression of Th1 type cytokines. Cyokines cross the placental barrier priming Th0 cells to differentiate in the Th2 direction beginning at 22 weeks gestation. Sustained Th2 proliferation and Th2 cytokine expression in the fetus driven by cytokines derived from maternal circulation create an immuno environment favouring IgE sensitization. Postnatal exposure to ETS is met with vigorous responding in the newborn encouraging development of allergic disease.

Table 1

Descriptive characteristics of participants; combined, smoking and non-smoking groups

	Complete Sample	Range	Standard Deviation	Non-Smoking Sample	Range	Standard Deviation	Smoking	Range	Standard Deviation
Newborns									
Sex ratio M/F	38/40			22/20			16/20		
Birth weight (grams), mean	3670.13	2780-5360	543.77	3861.67	3080-5100*	477.57	3446.67	2780-5360*	536.61
APGAR at 1 min., mean	7.24	2-9	1.68	7.23	2-9	1.75	7.25	3-9	1.61
APGAR at 5 min., mean	8.67	3-10	0.94	8.62	3-10	1.17	8.72	7-9	0.57
Cord blood cotinine (ng/ml), mean	39.06	0-250	59.57	0.38	0-8	1.72	84.19	8-250	62.55
Cord blood IL-4 (pg/ml), mean	0.12	0-1.63	0.24	0.139	0-1.63	0.32	0.103	0-0.41	0.10
Delivery, frequency									
Spontaneous cephalic	44			27			17		
Forceps	10			3			7		
Cesarean section	20			10			10		
Breech	1			0			1		
Vacuum	2			2			0		
Gestational length (days), mean	279.81	253-296	9.02	281.60	259-296	7.9	277.72	253-294	9.88
Meconium stain, (%)	9			7.1			11.1		
Parity, mean	1.78	1-6	0.97	1.67	1-4	0.82	1.92	1-6	1.13
Gestational diabetes, (%)	6.3			7.1			5.6		

Table 1. Continued.

	Complete Sample	Range	Standard Deviation	Non-Smoking Sample	Range	Standard Deviation	Smoking	Range	Standard Deviation
Mothers									
N	78			42			36		
Age (years)	27.6	18-44	4.99	28.67			26.36	18-36	4.64
Asthma (%)	16.7			21.4			11.1		
Allergy (%)	29.5			35.7			22.2		
Smoking (cigs/day), mean	5.32	0-30	7.35	0	0	0	11.53	4-30	6.73
Cotinine (ng/ml), mean	44.49	0-250	69.01	0.19	0-8	1.23	96.17	8-250	73.31
IL-4 (pg/ml), mean	0.111	0-1.32	0.23	0.104	0-0.74	0.20	0.117	0-1.32	0.26
20 minutes of exercise per week, mean	3	0-12	2.38	2.21	0-7	1.62	3.92	0-12	2.79
Ethnic origin, (%)									
Caucasian	92.3			92.9			91.7		
First Nation	6.4			4.8			8.3		
Latin	1.3			2.4			0		
Caffeine products per day, mean	2.46	0-10	2.04	1.52	0-6	1.38	3.56	0-10	2.14
ETS exposure, (%)	61.5			38.1			88.9		
Fathers (mothers questionnaire)									
Asthma, (%)	16.7			14.3			11.1		
Allergies, (%)	19.2			14.3			25		

*These cases have diagnosed gestational diabetes

Table 2.

Relationships among chosen variables

Variables	*r*	*Variables*	*r*
smoking / M. cotinine	.70††	C. cotinine / caffeine	.37†
smoking / C. cotinine	.66††	caffeine / exercise	.40††
M. cotinine / C. cotinine	.87††	↑caffeine / ↓birth weight	-.33**
↑smoking / ↓birth weight	-.37†	smoking / exercise	.41††
↑M. cotinine / ↓birth weight	-.36†	APGAR / APGAR (1 min) (5 min)	.62††
↑C. cotinine / ↓birth weight	-.37†	M. age / parity	.24*
smoking / caffeine	.49††	gestational length / birth weight	.36†
M. cotinine / caffeine	.38†	ETS / M. cotinine	.24*
M. IL-4 / C. IL-4	.37**		

* $p < .05$
** $p < .01$
† $p < .001$
†† $p < .0001$

M - maternal, C - cord blood
↑,↓ - description of correlation
ETS - environmental tobacco smoke

APPENDIX A

Dear Participant,

Hello, my name is James Koprich and I invite you to be a participant in a very exciting study. I am a graduate student attending Lakehead University, completing a research project as part of my Master's degree. The basis of this research is to explore the possibility that during pregnancy the fetus may develop the potential to become allergic or asthmatic. This study will attempt to better understand immune system functioning during pregnancy in hopes of developing an intervention to reduce childhood allergic disorders.

First you will be asked to fill out a confidential questionnaire (attached). It is important that you read the instructions carefully and be completely honest when answering. Completed questionnaires will provide me with information about you and your pregnancy. I will attempt to determine how items on the questionnaire influence immune functioning.

As part of routine delivery, blood samples from you and the umbilical cord are collected for hospital lab analysis. In order to investigate the immune system I require your permission to examine these already collected blood samples. I will be looking at parts of the immune system related to asthma and allergy development. By participating in this research absolutely no part of the birthing routine is altered. All information gathered will remain CONFIDENTIAL and will be stored at Lakehead University for a period of seven years, after which time will be destroyed or remain confidential in my hands.

Community participation in any research is extremely valuable. It is an opportunity to be a part of science and provide information that may lead to improving quality of life. By volunteering consent to participate in the research at hand, you will be credited with improving the quality of our children's lives.

Ethics committees from Lakehead University and Thunder Bay Regional Hospital have approved this research project. Feel free to call James Koprich (344-9010) if you have any questions or concerns. If you decide at any time to withdrawal from this study your medical care will not be affected. By consenting below, you have agreed to all of the requirements outlined above and volunteer to participate in this research. This study is supported by the Asthma Society of Canada.

Date _____ Name _____ _____

Participant signature

_____

James B. Koprich, HBSc
Principal Investigator

APPENDIX B

Code M-_____

Pregnancy Lifestyles Questionnaire

Many thanks for participating in this research. Your contribution will be absolutely confidential and more importantly will facilitate our knowledge of pregnancy and science!

Instructions: Please provide answers to all questions which relate to personal lifestyles during your pregnancy. When a number is required please provide a **single number**, not a range (i.e. 10, not 5-10) in the **boxes**. This number can represent an average. Other questions simply require a '✓' in the **circles**.

Age ☐ Ethnic Origin: Asian ◯ African ◯ Caucasian ◯ First Nations ◯ East Indian ◯

Latin ◯ Other _____

1. On average, how many caffeine products (coffee, tea, cola, etc.) do you consume daily? (i.e. 2 Colas + 3 coffees + 1 tea = 6 caffeine products) ☐
2. How often, in one week, do you engage in at least 20 minutes of activity intended to be exercise? ☐
3. Have you participated in any prenatal classes? (i.e. Lamaze) Yes ◯ No ◯

4. How many cigarettes, on average, do you smoke in one day?
(smoking history will be evaluated additionally by chemically analyzing the blood samples)

   1 pack of 25 ◯ Less than a pack (state number of cigs) ☐ Other ☐

   2 packs of 25 ◯ Less than 2 packs (state number of cigs) ☐

   Are you exposed to second hand smoke? Yes ◯ No ◯ If Yes, where? At home ◯ At work ◯

5. Do you have a history of allergies? Yes ◯ No ◯ Asthma? Yes ◯ No ◯

   Does the father have allergies? Yes ◯ No ◯ Asthma? Yes ◯ No ◯

   Have your allergies been diagnosed by a doctor? Yes ◯ No ◯ Asthma? Yes ◯ No ◯

   Are you taking medication for your allergies and/or asthma? Yes ◯ No ◯

6. Have you been taking any vitamins or supplements during your pregnancy? Yes ◯ No ◯

Thank you kindly, and best wishes to you and your baby

APPENDIX C

Request for Results

Dear Participant,

If you wish, please write in your name and address on the label attached to this sheet and a copy of the results and conclusions will be mailed to you.

Thank you,

James Koprich