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Detection of immunoglobulin isotypes from dried blood spots

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ABSTRACT

The study was designed to determine the sensitivity and reproducibility of recovering immunoglobulin (Ig) isotypes (IgG subclasses, IgA, IgE and IgM classes) from dried blood spots (DBS), a methodologic subcomponent of the Upstate KIDS Study. A multiplexed Luminex assay was used for IgG1/2/3/4, IgA and IgM analysis; an ELISA was used for IgE. Plasma samples from de-identified patients were used to compare the Luminex assay with nephelometry, which is routinely used to quantify IgA, IgG and IgM in clinical samples. The IgE ELISA was compared to an immunofluorescence assay. Prior to evaluation of punches from newborn dried blood spots (NDBSs), recoveries of Ig from punches of cord blood DBSs (CBDBSs) vs. plasma from the same cord bloods were compared. Although the recoveries of Ig from plasma and DBSs were not comparable, which could be due to cell lysates in the DBS samples, the analyses were reproducible. Additionally, the levels of IgA, IgG2, IgG4, and IgM recovered from CBDBSs positively correlated with those in plasma. The DBS data is a relative value since it is not equivalent to the plasma concentration. The majority of Ig concentrations recovered from 108 newborns of the Upstate KIDs Study were within the range of newborn plasma Ig levels with the exception of IgG3. The IgG4 values displayed the greatest variance with a wide range (0.01–319 mg/dl), whereas, IgG1 values had the narrowest range (85.2–960.4 mg/dl).
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1. Introduction

Eluted proteins from newborn dried blood spots (NDBS) have been used to assess the levels of cytokines and chemokines; however, to date, few studies have quantified the levels of immunoglobulins (Igs). Most likely, there have been few studies of newborn Ig levels, because most antibodies are of the IgG class, and maternal IgG antibodies are transported through the placenta. Previously, only the four IgG isotypes (IgG1/2/3/4) were suggested to be transported through the placenta. The exclusive transport of the IgG subclasses from mother to infant has recently been challenged with the investigation of immunodeficient babies with maternal IgA (Borte et al., 2012). The quantities of each of the Ig isotypes, including the IgG subclasses, regardless of whether a portion of the Ig is from the mother, could provide clues about developing disorders or altered processes.

The IgG subclasses are not equally transported through the placenta by the neonatal Fc receptor (FcRn) (Palmeira et al., 2012), in that usually IgG1 displays the greatest transport and IgG2 the lowest (Costa-Carvalho et al., 1996). IgG transfer to the fetus increases in the third trimester with the greatest amounts occurring in the last 4 weeks of gestation (Saji et al.,

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1999). Interestingly, it has been suggested that all IgG subclasses are transferred with reduced efficiency into at term neonates with a low birth weight, but IgG1 and IgG2 subclasses are transferred with significantly less efficiency than IgG3 and IgG4 (Palmeira et al., 2012).

All of the Ig isotypes have different biological activities (Schur, 1987; Ravetch and Kinet, 1991; Hamilton, 2001), including activation of complement and binding to specific Fc receptors on different cell types via their C-terminal (Fc) domain. IgG isotypes bind to different FcγRs (FcγRI, CD64; FcγRII, CD32; and FcγRIII, CD16). The FcRs of leukocytes have different affinities for the Ig isotypes; IgG1 and IgG3 bind with higher affinities than IgG2 and IgG4. Neutrophils bind IgA better than the other types of leukocytes (Lawrence et al., 1975). Human basophils, eosinophils, mast cells, neutrophils, monocytes, macrophages, dendritic cells, Langerhans cells, and platelets have a high-affinity receptor for IgE (FcεRI) (Kinet, 1999); whereas, low-affinity IgE receptors (FcεRII or CD23) are on lymphocytes (Lawrence et al., 1975; Conrad, 1990; Acharya et al., 2010). Both FcεRI (Untersmayr et al., 2010) and FcεRII (Kaiserlian et al., 1993) are expressed by intestinal epithelial cells and have been implicated in enteropathies. High levels of IgE usually reflect atopy or infections with parasites, such as *Schistosoma*. Although in utero *Schistosoma* infection leads to elevated cord blood IgE levels (Seydel et al., 2012), high levels of cord blood IgE as a predictor of later allergies or asthma has been controversial. Prenatal stress has been reported to increase the amount of IgE in cord blood (Peters et al., 2012). Environmental exposure to the immunotoxicant lead also has been linked to elevated levels of cord blood Pb levels (Annesi-Maesano et al., 2003). Lead preferentially enhances the activity of Th2 cells, which promotes IL-4 and IgE production (Heo et al., 1996); prenatal lead exposure of mice enhanced neonatal serum IgE levels (Snyder et al., 2000).

Fetal B cells and T cells exist by the second trimester, suggesting humoral immunity may begin early in development if antigens or immunomodulators, such as lead, are presented, and fetal B cell clones may have skewed synthesis of IgG1, 2, 3, and 4 or the other isotypes with prenatal exposures. Thus, aberrant proportions of the isotypes could be predictors of unusual prenatal conditions that could increase the prevalence of disorders/diseases as the child develops. The early levels of the Ig isotypes may also shed light on other aspects of the fetomaternal relationship. Elevated levels of IgA or IgM could indicate that the protective germ-free environment has been compromised. Since IgM does not cross the placenta, neonatal IgM levels could be used to determine prenatal and early postnatal infections. IgM from DBSs has been used to detect antibodies to *Toxoplasma gondii* (Mei et al., 2011) and measles (Uzicanin et al., 2011). Neonatal IgG4 levels usually are low, because placenta transport is not efficient and maternal IgG4 levels are usually lower than the other IgG isotypes. Recently, IgG4 has been associated with sclerosing disease and other diseases (Stone et al., 2012); elevated levels of IgG4 could suggest unusual early immune responses. An abnormal increase in any isotype could relate to a lymphoma and aberrant immune activation.

This report describes the methods used to quantify the Ig levels from NDBSs and how these levels compare to those in cord blood. For the analysis of cord blood, the Ig concentrations of cord blood plasma and those eluted from dried cord blood of the same baby were compared. The rationale for undertaking this work was to determine if NDBSs could be used to characterize infants' immunologic status, and also for assessing in utero infection as a potential confounder of the relation between infertility treatment and infant's growth and development through three years of age. This latter purpose is the central research goal of the Upstate KIDS Study [www.albany.edu/sph/upstatekids.php]. The quantitative differences of measured Ig obtained from the various samples are discussed with regard to protein recovery and free vs. bound proteins.

2. Material and methods

2.1. Patients and samples

De-identified plasma or anonymized cord blood was provided by Dr. Mark Preissler or Dr. Brian Freed, respectively. Anonymized punches (3.2 mm) from NDBSs were obtained from the Newborn Screening Program of Wadsworth Center, NYS Department of Health under an exempt protocol (NYS IRB # 07-070); a 3.2 mm punch, which is the standard size for all Newborn Screening Programs, allows the most efficient number of punches/DBS (9 punches/DBS). De-identified punches from 108 participants of the Upstate Kids Project were obtained with written informed consents of a non-exempt protocol (NYS IRB # 07-097). The amount of blood contained in a 3.2 mm punch was calculated to be 3.275 μl (Fig. 1). Estimating that 50% of the blood is plasma, we calculated that each punch would therefore contain 1.6375 μl of plasma.

2.2. Sample preparation methods

The different preparations of each collected cord blood (CB) were processed and shipped overnight. The CB was first collected with a 16 gauge ultra thin wall needle into a 250 ml sterile Blood Pack Unit containing 35 ml of the anticoagulant Citrate Phosphate Dextrose Solution USP (CPD; Fenwal, Cat # 4R0837MC). For the collection of plasma, a portion of each CB was centrifuged (655 ×g for 10 min at 4 °C), and the plasma was then immediately frozen. For the preparation of the cord blood dried blood spots (CBDBS), 50 μl of CB was spotted five times onto a Guthrie card, then dried and sealed in a plastic

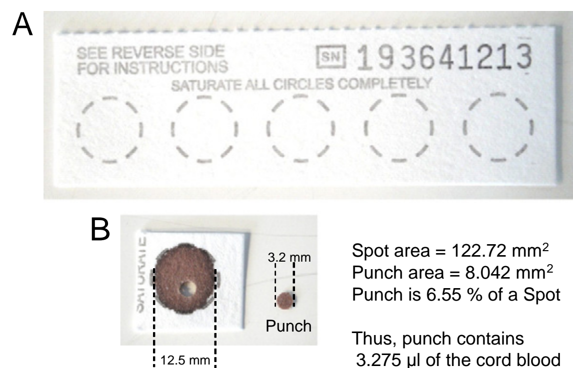

Fig. 1. The Ig was extracted from punches taken from a dried blood spot on a Guthrie Card (A). A hanging drop of 50 μl of cord blood was absorbed onto an encircled area and a 3.2 mm punch is taken (B).

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