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The challenge of assessing infant vaccine responses in resource-poor settings

Katie L Flanagan[1,*], **Sarah Burl**[1,*], **Barbara L Lohman-Payne**[2], and **Magdalena Plebanski**[3,†]

[1] Medical Research Council (UK) The Gambia, Fajara, The Gambia

[2] Departments of Medicine and Global Health, University of Washington, WA, USA

[3] Department of Immunology, Monash University, Alfred Medical Research and Education Precinct, Melbourne, Australia

Abstract

Newborns and infants are highly susceptible to infectious diseases, resulting in high mortality and morbidity, particularly in resource-poor settings. Many vaccines require several booster doses, resulting in an extensive vaccine schedule, and yet there is still inadequate protection from some of these diseases. This is partly due to the immaturity of the neonate and infant immune system. Little is known about the specific modifications to immunological assessment protocols in early life but increasing knowledge of infant immunology has helped provide better recommendations for assessing these responses. Since most new vaccines will eventually be deployed in low-income settings such as Africa, the logistics and resources of assessing immunity in such settings also need to be understood. In this article, we will review immunity to vaccines in early life, discuss the many challenges associated with assessing immunogenicity and provide practical tips.

Keywords

antibody; B cell; cord blood; developing country; immune response; infant; innate immunity; neonate; nonspecific effect; T cell; vaccine

Early in life, children have little immunological memory and a developing immune system, which increases their vulnerability to infectious diseases, particularly in neonates. This is, therefore, a crucial age group for vaccination programs. Indeed, in 2008 it is estimated that an unprecedented 106 million children received routine vaccinations throughout the world [1], saving 6 million lives [2]. It is not unusual for an 18-month-old child to have received up to 20 vaccines under the recommended Expanded Program on Immunization (EPI) (Table 1). This is also the ideal age at which to introduce new or improved potentially life-saving vaccines, including those for rotavirus, dengue, malaria, HIV and TB, preferably within the EPI framework.

†Author for correspondence: Tel.: +61 39 905 430, Fax: +61 399 054 302, magdalena.plebanski@med.monash.edu.au.
*These authors contributed equally to the manuscript

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Innate immunity in neonates & infants

Little is known about the neonatal innate immune response; however, a newborn infant has limited adaptive memory (apart from that primed by exposure to antigens *in utero*) and innate responses are likely to be a critical component of the immune response to vaccines in early life. Furthermore, the innate response strongly influences the type and magnitude of the adaptive response (reviewed in [3]). It is likely that many childhood vaccines stimulate an innate response via pattern-recognition receptors expressed by immune cells. Indeed, bacillus Calmette–Guérin (BCG) activates Toll-like receptor (TLR)2, TLR4 and possibly TLR8 [4,5]. Recent studies in South Africa showed that *in vitro* IL-12, IL-10 and IFN-γ production in response to BCG was predominantly from innate cells at birth, including monocytes and natural killer cells, but was T-cell-derived by 13 weeks after BCG vaccination [6]. Neonatal dendritic cells do not respond well to TLR agonists, with defective production of IL-12 [7,8], and defects in the innate signaling pathway through IL-1 receptor-associated kinase 4 and myeloid differentiation primary response gene 88 [9–11]. Overall TLR agonist studies suggest greater or similar IL-1β, IL-6, IL-23 and IL-10, but lower TNF-α and IL-12, and less polyfunctional responses in neonates compared with adults, even though basal TLR expression levels are often similar, suggesting a functional rather than quantitative deficit [3,7,12–17]. However agonists to TLR8 and the combination of TLR7/8 can induce adult-like responses in the cord blood and have potential as adjuvants for infant vaccines [7,14]. There is a lack of information concerning infant innate responses during the first year of life; however, the immune system is rapidly evolving in response to repeated immune challenges and responses are likely to be quite different to those observed at birth. Other pattern-recognition receptors of the innate immune system that are likely to be affected by vaccination include retinoic acid-inducible gene-like receptors such as RIG-1 and MDA-5; nucleotide oligomerization domain-like receptors; C-lectin receptors such as mannose-binding lectin; and dendritic cell (DC)-specific intercellular adhesion molecule-3-grabbing non-integrin [18,19]. Little is known regarding the developmental expression of most of these receptors in humans.

Adjuvants & innate immunity

Recent evidence suggests that aluminum adjuvants, first discovered empirically in 1921 and present in many of the inactivated EPI vaccines, including diphtheria–tetanus–whole-cell pertussis, hepatitis B vaccine (HepB) and pentavalent vaccine (diphtheria–tetanus–whole-cell pertussis–HepB–*Haemophilus influenzae* type b), stimulate innate responses via the intracellular inflammasome pathway leading to a Th2 adaptive response [20]. It is still debated if this is in addition to, or in contrast to, the ‘depot effect’ that was originally thought to be the function of the aluminum (reviewed in [21]). Many of the new-generation vaccines currently being tested in humans utilize novel adjuvants, for example, the malaria vaccine RTS,S that is currently in Phase IV studies (reviewed in [22]). Some of these contain intrinsic innate agonists and the effect of these on coadministered vaccines needs to be considered when measuring vaccine immunogenicity in clinical trials.

Practical considerations for assessment of infant innate immune responses

Measures of innate responses may provide critical information in vaccine studies in neonates and infants. Innate reactivity will vary according to age and possibly geographical location, and this will determine the choice of innate responses to be tested. Assessment of *in vitro* cytokine release to TLR agonists in whole-blood assays provides a simple method to gain a picture of innate reactivity before and after vaccination, but more detailed approaches might include analysis for innate receptors on immune cells and microarray analyses for innate signaling pathway molecules.

Adaptive immunity in infants

Humoral immunity

Maternal antibody—Traditionally, studies of vaccine efficacy have relied upon measurements of vaccine-specific antibodies. However, maternally derived antibodies, transferred trans-placentally during gestation and in breast milk, may influence vaccine antibody responses in early life. Thus, the presence of maternal antibodies can inhibit the development of the infant humoral response to both live and killed vaccines, dependent on maternal-derived antibody levels at the time of vaccination [23]. Inhibition of measles vaccine by high-titer maternal antibody has been well documented and can last until the infant is up to 12 months of age, and this is why measles vaccination is generally deferred until 9–12 months of age (reviewed in [24]). It is thought that maternal antibodies neutralize live vaccines, thus limiting immune priming. However, these antibody–antigen complexes are still presented by DCs and induce T-cell-mediated responses which, in some cases, are enhanced [23]. Furthermore, it is not possible to distinguish maternal from infant IgG, limiting the usefulness of antibody assays in the assessment of vaccine-specific immunity in this age group. Performing IgM measurements can overcome this problem, but does not give a measure of humoral memory in infants.

Antibodies & B cells—Neonatal antibody responses are delayed in onset, reach lower distribution peak levels, are of shorter duration, differ in IgG_2 and are of lower affinity with reduced heterogeneity than adult responses [23]. These effects are not thought to be associated with intrinsic B-cell defects, but rather a lack of plasma cell induction and limited development of B cells [25,26]. Twin studies in The Gambia have shown that genetic determinants control the early phase of the vaccine antibody response, whereas environmental determinants (e.g., total levels of IgG) predominantly influence antibody persistence and avidity maturation [27]. The earlier in life that infants are vaccinated, the poorer and more short-lived their antibody responses are, irrespective of maternal antibody levels. For instance, the group B meningococcal conjugate vaccine provides protection in 74% of adults but only 47% of children, and fails to elicit any protection in young infants [28]. Strong primary IgG responses to vaccines can be induced from 2 months of age, but persistence and adult-like levels cannot normally be achieved before 12 months of age, particularly to polysaccharide vaccines such as the pneumococcal vaccine [29], or the meningococcus C conjugate vaccine, which when administered at 2, 3 and 4 months of age produces an antibody peak at 5 months of age, but a sharp decline thereafter [30]. Similarly, three doses of diphtheria-containing vaccines within the first 6 months of life is followed by a rapid decline in antibody levels in the 6–10 months postvaccination in Swedish children [31]. As most pathogens enter the body via the mucosal system, induction of both mucosal and systemic immunity may be an advantage, especially as the mucosal system matures earlier. Many new vaccines are being developed to be administered through the mucosal system, which would avoid interference from maternal antibody. However, there are conflicting reports regarding aerosol effectiveness in young infants. The effectiveness of aerosol vaccines appears to be low in infants less than 10 months of age, possibly due to the congested noses frequently observed in early life, which inhibit the uptake of intranasal aerosol. After 10 months of age there appears to be equal or enhanced immunogenicity with aerosol measles vaccine, suggesting that it may be necessary to use adjuvants to induce effective immunity in younger children (reviewed in [32]). More work is needed to optimize the effectiveness of these vaccines in children and to develop the best ways of detecting mucosal immunity (reviewed in [33]).

Practical considerations when measuring B-cell & antibody responses to vaccines in infants—Many EPI vaccine antibody assays are standardized and performed by accredited reference laboratories. There are specific ‘cut-off’ protective levels for a number

of vaccines, although there are only a few epidemiological studies determining the actual level of protective immunity. Measures of IgG subclass and antibody avidity may give a better indication of the quality of the humoral response. Not all measured antibodies are functional, and the development of 'in-house' functional assays can provide additional information, such as neutralization assays used to assess polio and measles antibody responses. However, while superior, these are laborious and difficult to standardize, limiting their widespread use. Maternal antibody levels must be taken into consideration in the first year of life, and cord blood levels provide a good measure of the maternal-derived levels at birth. If possible, cellular measures of vaccine immunogenicity should be measured to complement antibody measurement. However, they are even more difficult to standardize and assess in terms of protection.

Cell-mediated immunity

Many of the commonly administered EPI vaccines were developed empirically, with little understanding of their effect on cellular immune responses. Furthermore, there is a surprising paucity of information on human infant cellular immunity in general. Maternal lymphocytes do not normally cross the placental barrier and only low levels are present in breast milk; therefore, infants are susceptible to infections requiring T-cell-mediated immunity. It is increasingly evident that cellular responses are an important component of the immune response to natural infections. A mature $CD8^+$ T-cell response is induced in response to congenital human cytomegalovirus (CMV) infection, which is common in developing countries, whereas the $CD8^+$ T-cell responses to HIV infection are low and often not associated with good clinical outcomes as observed in adults [34–36]. In addition, $CD4^+$ T-cell responses are of lower magnitude in response to both of these infections. Cell-mediated responses are required for efficient vaccine responses against intracellular organisms such as *Mycobacterium tuberculosis* [37] and measles [38], and in the case of BCG vaccination at birth, an adult-like IFN-γ response is induced [39]. It is worth considering that antigens introduced early in life can activate specific T cells that are highly prone to tolerance, and are mainly dependent on antigen route and dose [40]. Furthermore, the infant immune system actively eliminates self-reactive T cells, thus, vaccine-specific T cells may be deleted without a deposit of memory. Human newborns are intrinsically Th2 biased, with defective Th1 inducing IL-12 production by antigen-presenting cells. BCG at birth induces quantitatively and qualitatively similar Th1 responses as vaccination in adulthood, while many other vaccines (HepB and diphtheria–tetanus–pertussis) induce Th2 responses when given early in life [36]. However, the proinflammatory Th1 response to BCG may be short-lived as shown in Gambian infants vaccinated at birth. The initial increase in IFN-γ response to purified protein derivative observed at 4.5 months of age was reduced by 9 months of age [Burl S, Cox M, Flanagan KL *et al*., Manuscript Submitted].

There has been a tendency to focus on vaccine-induced IFN-γ production in human studies, but there is increasing evidence that other cytokines (proinflammatory and anti-inflammatory) and polyfunctional T cells (those producing more than one cytokine) provide more meaningful information [41–43]. Despite this, good correlates of protective cellular immunity for most vaccine-preventable diseases have not been defined for adults or infants, and without these it is difficult to be certain which cytokines will be the most informative. For example, in TB, IFN-γ although essential for protection, is not sufficient [44]. Newborns have very high levels of the Th2-polarizing cytokine IL-6 [15], and antigen-specific CD4 T cells expand more vigorously *in vivo* when IL-6 is present during immunization due to reduced apoptosis [45]. In the presence of IL-23 and/or TGF-β, high levels of IL-6 induce a pro-inflammatory Th17 response, whereas an absence of IL-6 biases towards $FOXP3^+$ Tregs [46]. Despite this, cord blood IL-17 production to the bacterial superantigen staphylococcal enterotoxin B is negligible, suggesting an inability of a newborn to mount an IL-17 response [47]. By 4.5 months of age,

we found high levels of mycobacterial-specific IL-6 and IL-17 in infants vaccinated with BCG at birth, although these levels waned by 9 months of age [Burl S, Cox S, Flanagan KL *et al.*, Manuscript Submitted]. Thus, Th17 proinflammatory responses might also be an important component of vaccine-induced memory in addition to the classic Th1/Th2 responses.

Regulatory T cells—Tregs play a key role in suppressing immune responses to foreign antigens and pathogens, suggesting that responses to vaccination could be affected by Tregs. High levels of functional naturally occurring Tregs (nTregs; $CD4^+FOXP3^+CD25^{high}$) are present *in utero* [48–55] and decline to adult levels by birth [52]. Cord blood Tregs are of a more naive phenotype ($CD45RA^+$) compared with adults [52] and are phenotypically distinct from maternal Tregs [47]. Cord blood Tregs ($CD4^+CD25^+FOXP3^+$) have higher expression of the $CD127^-$ and $CD27^+$ phenotype but a reduced expression of $CD95^+$, $CCR6^+$, $CD45RO^+CD62L^+$ and $CD28^+$ compared with adult Tregs [47]. When and how they mature into adult-type Tregs is not known.

Murine studies suggest that Tregs can attenuate vaccine immunogenicity [56,57] and immunopathology [58]. Depletion of nTregs using anti-CD25 antibody prior to vaccination with modified vaccinia Ankara–ciscumsporozoite protein malaria vaccine in mice enhanced IFN-γ responses, increased parasite control and durable immunity (at least for up to 100 days when the effects of anti-CD25 would be eliminated) compared with vaccine alone [59]. We and others have shown an upregulation of nTregs after BCG vaccination in human infants at birth [60] [Burl S, Cox M, Flanagan KL *et al.*, Manuscript Submitted]. Novel vaccine strategies have been designed to suppress the Treg induction and improve immunogenicity (reviewed in [61]), but overall the data on vaccines and Tregs from human infant studies are limited.

Measuring T-cell responses—The standard T-cell assays can be used to measure T-cell responses to vaccines in early life, including ELISpot assays, flow cytometry for intracellular cytokines and functional substances such as perforin and granzyme, cytotoxity assays, proliferation assays (carboxy-fluorescein diacetate succinimidyl ester, Ki67, thymidine incorporation and bromodeoxyuridine), and assessment of cytokines by ELISA or multiplex assays. It should be borne in mind that infant cells are highly susceptible to apoptosis and are less robust than adult peripheral blood mononuclear cells (PBMCs). Several cord blood studies have shown increased apoptosis along with increased proliferation of lymphocytes compared with adults [62,63]. Thus, fresh rather than cryopreserved PBMCs may be more suitable for infant immune studies. Furthermore, the blood volumes available are generally smaller, thus limiting the assays that can be performed.

Practical troubleshooting & advice for optimized measurement of T-cell responses to vaccines in infants—Memory T-cell induction is an important component of vaccine immunogenicity and should be assessed in vaccine studies, particularly in trials of novel vaccines. The standard T-cell assays used in adult studies can be used, although infant cells are less robust in culture and, therefore, assays need to be optimized to suit local conditions. In addition, methods to increase the culture sensitivity of the assay should be considered, such as increasing the number of cells per well, using serum-free media or human serum. Defrosted PBMCs from infants often require a shorter rest period prior to setting up *in vitro* functional assays than might be used in adults, for example, a 4-h rest is preferable to longer rest periods. Whole-blood assays are useful since they can be set up on small volumes of unmanipulated fresh blood. Methods to freeze infant whole blood for flow cytometry analysis that is collected in the field have been developed in South Africa [64] but the stability of particular phenotypic markers after freezing needs to be considered when applying these techniques supporting the use of fresh samples where possible. The cells may differ phenotypically from adult cells and, therefore, markers to be assessed by flow cytometry should be optimized in preliminary assays. For example, almost all newborn T cells express the

immune activation marker CD38 without expression of $CD25^+$ and $HLA\text{-}DR^+$ [65], potentially limiting its usefulness as a marker of immune activation. In addition, recent thymic emigrants express higher levels of CD127, which may alter the definition of Tregs in neonates (reviewed in [66]). Since the DCs are less mature it can be useful to add costimulatory molecules into the cell cultures that drive their maturation, a commonly used example is the use of soluble recombinant CD49d, which acts to induce IL-2 secretion and IL-2R expression, enhancing proliferation of T cells [67]. Notably, cytokine levels elicited from T cells after antigen-specific activation are often lower than those produced by adults (reviewed in [29]), and, therefore, assays need to establish the range of cytokine concentrations that are relevant for measurement in target populations within different age groups. The initial primed response should be tested again later, since it may revert to a Th2-biased profile as seen for BCG [Burl S, Cox M, Flanagan KL *et al.*, Manuscript Submitted].

Other considerations in vaccine immunogenicity studies

Nonspecific effects & vaccine interactions

A host of studies particularly from Guinea-Bissau suggest that, aside from the vaccine-specific effects, vaccines can also have non-specific effects on childhood morbidity and all-cause mortality (reviewed by [68,69]). Broadly, live vaccines such as BCG [70–72] and measles vaccine seem to have nonspecific beneficial effects on child survival, whereas inactivated vaccines such as the diphtheria–tetanus–pertussis combined vaccine have a detrimental survival effect [73,74] and the sequence in which these vaccines are given also appears to be important [75]. The immunological mechanisms have not been elucidated and are becoming a research priority, and will also need to be considered for new vaccines entering clinical trials. Vaccines given alongside the standard EPI vaccines can potentially interact and interfere with the immunogenicity of these vaccines, and vice versa. For example, BCG at birth increases responses to the polio and HepB vaccines [76]. Interference can also occur when combining vaccines into multivalent formulations (reviewed in [77]). Although the efficacy of multivalent vaccines compared with monovalent vaccines is considered in clinical trials, there are little data in infants regarding the interactions between the new multivalent vaccines with other live and killed vaccines in the EPI schedule. It is, therefore, important to test any new vaccines to be used in early life for interference with other vaccines. It is also important that participants in vaccine studies in early life have their EPI vaccines administered on time and in the correct order to minimize the impact of this potentially confounding variable as observed in Guinea-Bissau [75].

Sex-specific differences

A number of studies show sex-specific differences in immunogenicity of vaccines. Although sex hormones might explain many differences in adults, a number of studies have shown differences in vaccine responses in infants who are prepubescent [74,78–82]. The explanation for these gender differences in early life is unclear, although levels of gonadotropins and sex hormones are differentially elevated in a males and females in the first few months of life, but generally decrease by 1 year of age [83]. Furthermore, a number of immune response genes are encoded on the X chromosome, leading to sex differences in immunity (reviewed in [84]). It would be helpful if researchers considered analysis by sex when performing vaccine studies, since it is becoming increasingly clear that the immune response profile can be quite different according to sex.

Confounders of vaccine responses in infants

Many studies have shown that nutrition early in life affects later immune competence, and nutrients such as vitamins A and D, zinc and iron have all been shown to have effects on innate and adaptive immunity and, thus, potentially affect vaccine immunogenicity (reviewed in

[85]). However, a recent meta-analysis of the literature surrounding this area suggest that, in general, malnutrition has little or no effect on vaccine responses (although severe malnutrition can reduce immunity). This analysis showed only weak evidence of adjuvant effects of micronutrients at time of vaccination, mostly due to the paucity, poor quality and heterogeneity of data (reviewed in [86]). In addition breast feeding can influence the intestinal microflora and enhance immune responses to vaccines [87], suggesting that similar feeding patterns should be considered in pediatric immunology studies.

In developing countries, infants are exposed to many infections in early life, and respiratory infections, malaria and diarrhea remain the leading cause of death. Helminth and parasite infection rates are also high in many settings and can further skew the immune system towards a Th2 response (reviewed in [88]). The relatively poor efficacy of the BCG vaccine close to the equator has been attributed to attenuation by exposure to environmental mycobacteria [89,90]. A striking 85% of children in The Gambia are infected with CMV by 1 year of age, and approximately 4% are infected intrapartum or during delivery [91]. Immunological studies in The Gambia have shown that CMV infection in early life has a profound effect on the immune system, leading to very high frequencies of highly differentiated CD8 and CD4 T cells [91,92]. Thus, coinfection with CMV, and the somewhat less prevalent herpes virus Epstein–Barr virus, might be important confounding variables in vaccine studies in infants in the developing world. Indeed, measles-specific antibodies and CD4 IFN-γ responses were reduced in CMV-infected children following measles vaccination compared with their uninfected counterparts, suggesting that CMV can decrease immunological memory to vaccines [92]. In addition, CMV peak viral loads are associated with HIV-1 infection in infants, suggesting that CMV load can indicate global T-cell changes [93].

The highly polymorphic HLA system suggests that differences between individuals and populations in response to vaccines may be partially attributed to genetics. This has been shown to be the case alongside polymorphisms in other molecules involved in the immune signaling pathways (reviewed in [94]).

As discussed previously, age has profound effects on immunogenicity, and this is also the case for premature and low-birth-weight children. Those born earlier than 32 weeks often show defects in some vaccine responses; however, responses to tetanus, diphtheria, polio and pertussis antigens were sufficient. BCG vaccine seems most immunogenic in preterms if delayed to 34–35 weeks of postmenstrual age (reviewed by [95]).

Practical considerations regarding confounding factors in vaccine studies in infants—Consider the nutritional status and prevalence of infections in the study population as important potential confounders. Collect information on breast-feeding practices and interventions such as vitamin A and antihelminth campaigns. Collecting anthropometric data such as weight, height and mid-upper arm circumference in longitudinal vaccine studies can provide useful information about the uniformity and general health of the study population. Analyses must also consider age-related changes in longitudinal cohorts and include identification of preterm babies. In addition, genetic heterogeneity between populations (for example, western versus southern settings) or ethnicity should be considered when designing studies. Other factors that may be particularly important to consider in low-income settings include parental education, orphan status and distance from a health center, which have been associated with low or delayed vaccine uptake, and high mortality in many populations [96].

Logistic challenges to early-life vaccine studies

Clinical trials to assess immune responses to current or novel vaccines require strict regulations that include working under the International Committee of Harmonization Good Clinical

Practice guidelines designed to protect the participants, and ensure accurate and reproducible results [101]. For children younger than 18 years of age, parental consent is required to take blood, although children who are able to form an opinion must be involved in the decision making and give assent to the procedure or trial [97]. It is worth noting that in some populations there are social beliefs and superstitions regarding donating blood, and public awareness campaigns are essential to provide accurate information and details of studies to be carried out in these communities. Peripheral blood is the main source of immune cells in humans and it is relatively straightforward to obtain substantial volumes (up to 50 ml) from consenting adults. However, in young children ethical guidelines set up according to US Federal guidelines and/or Institute Review Boards limit the volume of blood that can be collected. Local guidelines may also state that no more than two attempts at venipuncture may be undertaken at one time from a child, and ideally the interval between venipuncture should not be too short, although this may not be practical for some vaccine trials. Although necessary, these guidelines can make it difficult to recruit and collect sufficient blood from young children for proposed studies, and limits intensive longitudinal studies to assess vaccine responses over time. The sample size of a proposed vaccine trial is also an important consideration given the large heterogeneity in immune responses in infants requiring large numbers of recruits to obtain accurate and significant results in infant vaccine studies [98]. Furthermore, the paucity of data on infant immunity makes it difficult to perform accurate sample-size calculations in the first place.

Standardization of assays

In September 2008, a workshop entitled ‘CMI Standardization Techniques in Evaluation of Vaccine Response’ organized by the Fondation Merieux took place in Annecy, France [102]. The results of a questionnaire were presented about methods used to measure cell-mediated immune responses in young children. It was completed by academic groups and companies throughout Europe, North and South America, Asia, Australia and Africa, and demonstrated wide variability is assay conditions and methodology used. A third of researchers routinely used whole-blood assays, while the remaining two thirds tended to work with frozen PBMCs. Freezing cells facilitates study logistics but several T-cell markers and functional assays are susceptible to freezing [99] [Burl S, Cox M, Flanagan KL *et al.*, Pers. Comm.]. Flow cytometric intracellular cytokine staining was the predominant assay used to study cell-mediated immunity (74.4%), followed by ELISA and multiplex assays to examine cytokines (47.6%), with IFN-γ ELISpot assays used by 33% of the groups. The majority had standard operating procedures in place, but only 52.5% validated their assays and 38% had a quality control on their samples. In particular, assays to measure cell viability and positive controls were not consistent. It was recognized that there is a need to standardize assays in order to compare studies in infants and that a working group should be established to advise investigators and industry on how best to measure immune responses in infants.

Rapidly advancing molecular techniques, alongside decreasing costs, provide a novel way to investigate the immune response to vaccination. PCR assays to analyze for factors correlated with vaccine efficacy can be carried out easily and cheaply, including quantitative assessment. The whole human transcriptome approach is expensive and impractical in most situations, but has provided new insights into gene expression profiles following yellow fever vaccination [100]. By applying a systems biology approach and looking for immune response pathways to vaccination it has provided new insights into how vaccines work. Taking RNA samples either *ex vivo* or from *in vitro* cell cultures can provide a powerful tool for looking at signaling pathways and the effects on vaccines. The whole human transcriptome can be analyzed from RNA extracted from as little as 300 μl of whole blood collected into PAXgene tubes and, thus, extensive analyses can be performed from using very small volumes of blood. Standardizing the analysis of these large gene-based studies is currently under debate but the expanding role of bioinformatics has been valuable in processing this type of data.

Statistical analysis can differ widely between publications, making it difficult to compare studies. It would be beneficial if hypothesis-driven analysis strategies were predetermined and appropriate for large human cohort studies prior to commencing the study. The aid of both epidemiologists and biological statisticians would be an advantage in designing these proposals.

Conclusion

The study of infant immune responses to vaccines presents with some specific problems, particularly in resource-poor settings. Our lack of understanding of how the infant immune system develops, and how the innate and adaptive arms of the immune system interface in early life presents a major hurdle. Ethical issues and confounding variables such as intercurrent infection are also of particular relevance to the developing world. Infants are the target age group for many of the vaccines being developed at the present time, including TB, malaria and HIV vaccines, and administration within the EPI framework makes the most sense logistically. It is, therefore, critical that we begin to understand more about the development of immunity and the interactions of vaccines in this age group. Newer technology, including multicolor flow cytometry, multiplex cytokine analysis and whole human genome screening with very small volumes of blood are now available, opening the potential to study this vulnerable age group in more detail. This should become a research priority if we are to reduce mortality in early life.

Expert commentary

It is apparent from this review that infant immune responses differ in many ways to adults. Assumptions from animal and adult studies have often proved inappropriate, illustrating the importance of human infant studies. Cord blood studies have demonstrated the immaturity of the immune system at birth but the difficulties and acceptability of venous blood collection make it more difficult to study infant responses. The confounders that affect responses to vaccines include prior infection, sex and nutritional status, and are particularly important in low-income settings. Understanding the mechanisms for these interactions is still limited but is of great importance to vaccine development. Indeed, it is now possible to vaccinate against many life-threatening infectious diseases. However, the downside of this is that in particular settings the number of vaccines delivered to an infant population is extensive and include many multivalent formulations and complicated sequences of vaccines. Thus, more evidence regarding these potential interactions, and their effects on specific vaccine responses, and morbidity and mortality in general is needed. This has been a controversial area of research but is becoming more widely studied within high-infant-mortality settings.

Five-year view

Although there are many areas of infant vaccine research that are still unknown, the next 5 years is likely to bring many advances in this field. As we understand more about the specific cells involved in immunity both as part of the innate and adaptive immune systems and the regulatory mechanisms, it becomes easier to characterize vaccine responses. In addition, the advance of more sophisticated flow cytometers that can analyze up to 17 parameters and microarrray technology allows smaller volumes of blood to be utilized for a vast array of assays, therefore allowing detailed analysis on very few cells.

It is necessary to consider many of the confounding issues described in this paper and in particular with respect to vaccine interactions and sex. Many infant immunologists are in the process of setting up randomized trials to address these issues. Although it is known that live vaccines induce the best responses in infants, there have been safety issues that have driven

vaccine research to develop more suitable recombinant vaccines. Much of the research in the next few years aims to develop better vaccine adjuvants. TLR agonists and other molecules of the innate immune system appear to show promise. In addition, mucosal immunity has become of additional interest recently with increasing interest in the development of aerosol vaccines. Infant gut immunity is of particular interest in low-income settings where exposure to many microbes occurs via the gut. This is an area that will develop in the future and may provide valuable information about how dietary supplements could be modified to enhance vaccine responses.

Incredible progress has been made in the last few years towards saving children's lives but funding still remains a problem. As reducing child mortality is a Millennium Goal, increased investment has been made in this area and the recent commitment by the Gates Foundation of US$10 billion towards vaccine research is very promising.

Key issues

- Infant mortality has been greatly reduced by the development of vaccines against infectious diseases.
- The immaturity of the infant immune system can reduce antibody and cell-mediated memory responses to vaccines. Innate immunity can also be defective in infants, leading to the reduced adaptive response observed.
- Transplacentally transferred maternal antibodies can protect against infections that mothers have been exposed to but can inhibit protective antibody production against vaccines administered to the infant. The presence of these maternal antibodies also makes it difficult to distinguish between mother and child antibody responses.
- Lymphocytes are not transferred across the placenta so infants are vulnerable to pathogens that require a cell-mediated response. In addition, induction of long-term T-cell immunity in infants is often of different quality to that of adults (e.g., Th2 rather than Th1 response).
- It is important to assess both quantity and quality of infant immune responses as the presence of a response does not always indicate functional capacity, necessitating assays such as neutralization assays for detection of polio antibodies.
- Immune correlates of protection are not well defined for many diseases, but it is likely that these correlates will not be the same for adults and infants especially cell-mediated parameters.
- The optimization of many assays to measure infant responses need to be standardized in infants and consideration needs to be given to differences in cytokine profiles in early life, in cell characteristics during culture at different ages and volumes of blood that can be collected from infants.
- Vaccine adjuvants, many based on inducing stronger innate responses (e.g., Toll-like receptor agonists) are being developed to enhance responses to recombinant and conjugate vaccines. Different Toll-like receptor agonists are stimulatory in adults and children and, therefore, this needs to be considered when developing vaccines for infants.
- Confounding factors such as vaccine interactions, sequence of vaccines, sex, intercurrent infection, nutritional status, genetics and other nonspecific effects should all be considered when assessing vaccine responses, particularly in infants.

- There is a great need for studies in infants to assess the mechanisms of immunity for both old and new vaccines in order to optimize vaccine schedules for future use in infants and children.

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Table 1

Current Expanded Program on Immunization vaccine schedule in The Gambia (2010).

At birth	2 months	3 months	4 months	9 months	>12 months
BCG					
HepB					
OPV	OPV	OPV	OPV	OPV	OPV (18 months)
	Pentavalent	Pentavalent	Pentavalent		DTwP booster (16 months)
	PCV-7	PCV-7	PCV-7		
				MV	
				YF	

BCG: Bacillus Calmette–Guérin; DTwP: Diphtheria–tetanus–whole-cell pertussis combined vaccine; HepB: Hepatitis B vaccine; MV: Measles vaccine; OPV: Oral polio vaccine; PCV-7: Seven-valent pneumococcal conjugate vaccine; Pentavalent: Diphtheria, tetanus, whole-cell pertussis–*Haemophilus influenzae* type b–HepB; YF: Yellow fever vaccine.