Article

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Abstract

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Optimization of vaccine responses in early life: The role of delivery systems and immunomodulators

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Summary Infant immunization is a particularly important field with multiple challenges for vaccine research and development. There is, together with a high susceptibility to infections, a lower efficacy of most vaccinations in newborns and young infants, compared to those performed later in life. In the present review, the authors focus on problems arising from the attempt to vaccinate against pathogens very early in life, and on the role of selective adjuvants (i.e. antigen delivery systems or immunomodulators) that could be used to: (i) rapidly induce strong antibody responses of the appropriate isotypes; (ii) elicit sustained antibody responses extending beyond infancy; (iii) induce efficient Th1 and CTL responses in spite of the preferential Th2 polarization of early life responses; (iv) escape from maternal antibody mediated inhibition of vaccine responses; (v) show acceptable reactogenicity in early life; and (vi) allow incorporation of several vaccine antigens into a single formulation so as to reduce the number of required injections. How such objectives might be achieved by several of the vaccine formulations currently in development is illustrated by reviewing data from experimental models and clinical studies, when available.

Key words: adjuvants, delivery systems, immunization, immunomodulators, maternal immunity, newborn infants, Th1/Th2, vaccines.

Current strategies for infant immunization

Infant immunization has been identified as an essential determinant allowing the achievement of high vaccine coverage in populations where access to medical care strikingly decreases after the first year of life. Early immunization is, however, also required to protect from disease caused by pathogens to which exposure occurs in early life and which result in enhanced morbidity and mortality. Infant immunization programmes currently in most countries include: (i) vaccines against pathogens for which exposure is not expected to occur prior to several months or years; and (ii) vaccines that rely on the early induction of protective responses in order to be effective (Table 1).

With some exceptions such as the bacille Calmette-Guérin (BCG) vaccine, currently available live vaccines are difficult to use in very early life because of insufficient infant immunogenicity, excess reactogenicity or the presence of antibodies of maternal origin that persist for a prolonged period of time at levels capable of interfering with infant responses (see following). The use of live vaccines, such as measles or yellow fever vaccines, is thus best postponed until after the age of 6–9 months. Most infant vaccines therefore consist of subunit vaccines based on purified proteins, carbohydrates or on antigens generated by recombinant and biosynthetic technologies, which offer several advantages such as increased purity and safety. Again, with notable exceptions (i.e. hepatitis B vaccine), subunit vaccines are weakly immunogenic until several weeks after birth and their use in infants requires the administration of multiple vaccine doses during the first year of life. As an example, a single immunization with a conjugate vaccine against *Haemophilus influenzae B* (Hib) is sufficient to protect a 15-months-old toddler, whereas three injections followed by an early booster dose are required when immunization is initiated at 2–3 months of age.

Following the rapid development of vaccines, a number of additional immunization targets are being considered for inclusion into infant vaccines and immunization programmes. Pathogens responsible for significant morbidity in early life constitute a long list including bacteria (group B *Streptococcus, S. pneumoniae*, *Listeria monocytogenes*, *Escherichia coli*), viruses (respiratory syncytial virus [RSV], influenza and parainfluenza, herpes simplex virus, cytomegalovirus, enteroviruses, rotavirus) or other infectious agents (*Candida albicans*, *Toxoplasma gondii*, *Chlamydia trachomatis*). The rapid development of vaccines for potential use in early life emphasizes the need for antigen delivery systems capable of incorporating multiple antigens into a single vaccine to reduce the number of injections required, and of potent specific immunomodulators capable of overcoming the limitations of immune responses observed in early life.

Limitations of immune responses in early life

Early life antibody responses

Antibody mediated protection plays a crucial role in defence against extracellular bacteria by accelerating their

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Table 1 Current infant vaccines and formulations

Antigens	Expected age of exposure	Number of infant doses	Type of formulation
BCG	Early/delayed	1	Live bovine vaccine
Diphtheria	Delayed	4	Toxoid/alum
Tetanus	Delayed*	4	Toxoid/alum
Pertussis	Early	4	Whole cell//subunit/alum
Haemophilus influenzae B	Early	4	PS-protein conjugate
Poliomyelitis	Early	3–4	Live//inactivated viruses
Hepatitis B	Delayed†	3	Recombinant protein/alum
Measles	Early	1	Live attenuated virus
Rubella	Delayed	1	Live attenuated virus
Mumps	Delayed	1	Live attenuated virus

BCG, bacille Calmette-Guérin; PS polysaccharide.

*Apart from neonatal tetanus, prevented by maternal immunization.

†Except in infants born to infected mothers.

clearance through opsonization, complement fixation and/or antibody mediated cellular cytotoxicity (Hib, *S. pneumoniae*, *Neisseria meningitidis*), or by blocking the active sites of their toxins (tetanus, diphtheria). Antibodies also play an important role in the neutralization of viruses, as exemplified by the protection conferred during a few weeks or months by maternally transferred antibodies (measles) or parenterally administered specific antibodies (RSV).

Although newborn infants are able to produce IgM, IgG and IgA antibodies upon antigen exposure, important differences distinguish infant B cell responses from those generated later in life. Acquisition of full antigen responsiveness proceeds with the diversification of the antibody repertoire, which does not reach adult-like patterns before several months of age.[1] A progressive immune maturation is responsible for the stepwise increase in antibody responses to subunit vaccines observed in infancy, where a single month of delay in the age of first immunization (i.e. 3 *vs* 2 months) or in the intervals between vaccine doses (i.e. 2 *vs* 1 month) are sufficient to significantly enhance the magnitude of antibody responses to infant vaccines.[2] Furthermore, age-related differences in V region expression of antibody light chain in response to Hib polysaccharide-conjugate vaccine were demonstrated previously.[3] As a result, achieving high and specific antibody responses within the first weeks of life, as required for protection against early occurring infectious diseases such as RSV, remains a challenge. Infant antibody responses are, however, not only slower and weaker than later in life, they are also of shorter duration: even when good initial responses have been elicited by primary immunization, vaccine antibodies rapidly decline during the following months, reaching threshold levels requiring the administration of a vaccine booster by the second year of life.

In addition to these limitations in antibody responses to protein vaccines, infants and toddlers show a marked age-dependent limitation in the T cell-independent antibody response to most bacterial capsule polysaccharides (PS). Although certain PS may elicit antibody responses as early as age 3 months (i.e. *S. pneumoniae* serotype 3, *N. meningitidis* group A), most PS are poorly immunogenic before the age of 18–24 months. This failure creates an important 'window' of susceptibility to infections by encapsulated bacteria at the time of disappearance of protective levels of residual maternal antigen-specific IgG, that is, between 4 and 12 months of age. This is considered to be related to low complement activity, limiting the deposit of complement component C3d on PS, and reduced levels of CD21 (the CR2 complement receptor) on infant B lymphocytes, which result in lack of CD21/mIg synergy and defective B cell activation by PS.[4] Additional parameters, including a role for amplifier and suppressive T cells on B cell responses to PS are, however, considered likely to contribute to poor infant responses to most PS. These limitations can be overcome by coupling PS to carrier proteins, transforming the B cell response to a T cell-dependent type. Whereas such modified Hib vaccines have been shown highly effective for infant immunization, it appears that similar approaches may not be easily applicable for prevention against all serotypes of *S. pneumoniae* or against *N. meningitidis* infections.

Last, vaccine antibody isotypes induced in infancy may differ from those elicited later in life. Infant responses are characterized by a strong predominance of IgM, IgA1 and IgG1 responses, whereas the generation of IgG2 antibodies remains weak during the first 12–18 months of life, irrespective of the antigen or delivery system used. This physiological IgG2 isotype deficiency could reflect difficulties in using the Cγ2 gene located downstream of other heavy chain constant genes during the recombination process of immunoglobulin, and/or the preferential induction of Th2 versus Th1 responses[5] (see following). Whatever the reason, a reduced IgG2/IgG1 ratio could limit protection against infectious agents that require optimal complement binding for bacterial clearance.

In order to better identify the factors associated with impaired infant vaccine responses and strategies capable of overcoming such limitations, animal models of neonatal and early life immunization have been developed. The maturity of animals and humans at birth and in early life is difficult to compare: mice are considered as more immature than humans at birth in view of the relative ease with which immune tolerance can be induced during their first 24 h of life. However, infant mice 'catch up' more rapidly thereaf-

ter, and adult patterns of antibody responses to subunit or PS vaccines can generally be elicited in mice at the age of 3–4 weeks, compared to the several months of maturation required in humans. Thus, the maturation occurring in infant mice between 1 and 3 weeks of age somehow reflects the first year of human infant immune maturation. When neonatal and early life murine responses to candidate vaccine antigens such as tetanus, measles or RSV proteins given as subunit, inactivated, live or recombinant vectors were assessed, significant antibody responses could be raised if certain conditions were met.[6,7] However, a slower increase in IgG antibody titres, suggesting a neonatal restriction of antigen presentation or clonal expansion of B cells,[1] reduced IgG2a/IgG1 ratio,[6] and lower avidity of antibodies (C-A Siegrist *et al*. unpubl. data) were observed in neonatal compared to adult primed mice. These characteristics of early life vaccine responses are similar to those observed in human infants, indicating the potential usefulness of such animal models for the preclinical evaluation of novel delivery systems and immunomodulators.

Early life T cell responses

In 1953, Billingham, Brent and Medawar experimentally showed that exposure to an antigen (allogeneic spleen cells) either *in utero* or immediately after birth generated antigen-specific lymphocytes considered as self-reactive and that were eliminated in order to prevent the body from potential self-destruction.[8] This early alloantigenic exposure resulted in lifelong tolerance, as demonstrated by the acceptance of donor type skin grafts later in life. Based on Burnet's theory of clonal selection,[9] neonatal tolerance has subsequently been made responsible for the obvious difficulties in mounting effective and protective immune responses in newborns after immunization with foreign antigens such as lymphocytic choriomeningitis virus (LCMV) and staphylococcal enterotoxin B.[10,11] However, several independent research groups demonstrated that by modification of the experimental settings such as the type of antigen-presenting cells (APC)/costimulation,[12] the dose of a live leukaemia virus[13] or the use of adjuvant,[14] newborn mice were able to mount significant T cell responses to foreign antigens. These observations were extended to other peptides[15] and to several vaccine antigens.[6] However, qualitative and quantitative differences remained when comparing neonatal T cell responses with those elicited in adults. The qualitative differences included secretion of significantly higher IL-5 and lower IFN-γ levels by antigen-specific T cells, interpreted as reflecting a preferential polarization of neonatal T cells towards the Th2 rather than the Th1 phenotype. Impaired induction of cytotoxic T cell precursors was also observed upon immunization with live vaccines during the first weeks of life.[6]

Although relevant clinical studies are not yet available, the patterns of certain viral infections in early life strongly suggest that impaired human CD4 and CD8 responses limit the *in vivo* clearance of intracellular agents. When assessed *in vitro*, the majority of newborn human T cells present a 'naive' phenotype, characterized by the CD45RA+ marker, and absent activation markers such as CD40L. Neonatal CD4 T cells apparently follow preferential development into IL-4 and IL-5 producing effector cells[16, 17] and respond to specific cytokines such as IL-12 not only by IFN-γ but also by IL-4 production.[18] However, responses to alloantigens are normal and proliferative responses and cytokine production in response to T cell receptor ligation can be improved by CD28 cosignalling.[19] This suggests that neonatal T cells have normal intrinsic capacities, but altered thresholds of responsiveness requiring enhanced APC costimulation. With regard to cytotoxic T cells, although they are induced by viral infection early in life, as demonstrated for RSV or HIV-1, they are observed at a significantly reduced frequency that could well follow the impaired induction of Th1 responses.

This Th2 polarization of neonatal responses is thought to be linked in part to the persistence of maternal hormonal influences (such as progesterone and prostaglandins) that protect the foetus against the toxic effects of Th1 inflammatory cytokines during pregnancy. In addition to the progressive dilution of maternal hormone influence, the induction of Th1 and CTL responses after birth thus requires increased exposure to antigens, microbial costimulation and progressive maturation of the cellular immune network. It is now becoming more widely accepted that neonatal T cells are intrinsically immunocompetent in both humans and mice, but that their differentiation is biased towards a Th2 pattern by suboptimal interactions between APC, natural killer (NK) and T cells. Significant amounts of IL-4 are readily produced in humans by naive CD4+T cells, $\gamma\delta$-T-cells, NK1.1+T cells and basophils, which could be sufficient to inhibit the IL-12Rβ2 chain expression on naive T cells and therefore direct towards Th2 responses even in the presence of IL-12.[20] Thus, preventing the neonatal burst of Th2 driving cytokines in order to allow the induction of strong Th1 and cytotoxic responses probably requires enhanced APC–T cell interactions with optimal costimulation that can only be elicited by certain antigen delivery systems or immunomodulators. A summary of limitations of B and T cell responses in early life is shown in Table 2.

Influence of maternal antibodies on infant vaccine responses

Maternal antibodies are transferred across the placental layer of the syncytiotrophoblast by an active and specific transfer process.[21] They play an important role in infant protection against a number of viral and bacterial pathogens during a period of several weeks to months, until their catabolism leads to their decline below protective levels. Unfortunately, the presence of maternal antibodies at the time of immunization has been responsible for the inhibition of responses to infant vaccines such as measles[22] and other live (poliomyelitis[23]) and non-live (tetanus, diphtheria, pertussis[24]) vaccines. This phenomenon results in a window of susceptibility to infectious disease in the case of early exposure.

This passive antibody mediated inhibition is a trans-disease phenomenon observed with most infant vaccines, albeit at highly variable levels of clinical significance. The first step in maternal antibody mediated inhibition of im-

Table 2 Limitations of vaccine responses in early life

Effector mechanisms	Affected parameters	Expected consequences
Antibodies	Slower kinetics of induction	Delay before protection
	Lower magnitude (antibody titres)	Lower reaching of protective thresholds
	Shorter persistence	Shorter duration of protection
	Lower avidity	Correlates with protection (?)
	Different isotype distribution	Reduced complement fixation capacity
	Weak/no response to most PS	Failure of inducing protection
CD4 T cells	Lower magnitude (proliferative responses)	Reduced cytokine production
	Reduced Th1 cytokine production	Impaired clearance of intracellular micro-organisms
	Preferential Th2 cytokine production	Enhanced IgGl, IgG4, IgE responses
CD8 T cells	Lower magnitude (frequency of CTLp)	Impaired elimination of infected cells

CTLp, cytotoxic T cell precursors; PS, polysaccharides.

mune responses lies in the specific interaction of antibody molecules with their corresponding antigenic determinants, as demonstrated early by epitope-specific effects of monoclonal antibodies against sheep erythrocytes.[25] Multiple hypotheses have been suggested to explain the immunological events following this initial antigen–antibody interaction, partly contradictory results preventing a clear understanding of the underlying mechanisms. A recent reappraisal in several murine models of early life immunization indicates that vaccine-specific Th and CTL responses may not be inhibited even when high titres of maternal antibodies completely inhibit infant antibody responses (C-A Siegrist *et al*., unpubl. data).

In order to overcome maternal antibody inhibition of infant responses to vaccines, strategies up until now have consisted of either the administration of repeated vaccine doses (i.e. tetanus, diphtheria, pertussis, poliomyelitis), taking advantage of the progressive decline in maternally derived antibodies, or in the delay of age at first immunization (measles). Neither approach obviously allows early ($<$ 3–6 months) induction of protection, and novel vaccine formulations/strategies capable of doing so in the presence of high levels of maternal antibodies are needed for protection against certain early life infections.

Future vaccine strategies and formulations for infant immunization

Several of the factors that interfere with or limit the induction of vaccine responses in the neonatal period or early infancy could be positively influenced by novel vaccine formulations and/or immunization strategies. Thus, the concept of 'vaccine adjuvanticity' has been broadened to include (in the present review) a variety of factors that may ensure and enhance the protective capacity of newly designed infant vaccines. For clarification, one will follow the classification of adjuvants into 'antigen delivery systems' and 'immunomodulators'. Antigen delivery systems incorporate the antigen(s) and determine its uptake, processing, and presentation by APC to B and T lymphocytes, whereas immunomodulators directly activate APC or lymphocytes to specifically enhance or modulate immune responses. Obviously, selected antigen-presentation systems and immunomodulators can and are being combined to act simultaneously at both levels of antigen presentation and lymphocyte recognition/differentiation.

Objectives for new vaccine formulations/strategies capable of improving infant vaccine responses in order to allow neonatal immunization and rapid induction of protective mechanisms have been summarized in Table 3. In addition to the already mentioned specific immunological requirements, vaccine formulations for infant use must fulfil stringent safety requirements in order to: (i) take into account the relative immaturity of this period of life (live vaccines); and (ii) avoid local or systemic inflammatory reactions potentially deleterious to a young individual. As an example, the mere induction of vaccine-related fever in the first 4 weeks of life would lead in most industrialized countries to a full medical work-up in order to rule out underlying bacterial infection!

Clinical vaccine studies involving neonates and young infants are difficult for both logistic and ethical reasons. Assessment of the capacity of novel vaccine formulations to optimize neonatal immune responses has only recently been initiated in experimental (animal) models. In the absence of a true understanding of the mode of action by which adjuvants mediate their effect on vaccine responses, their influence on vaccine-driven antibody responses is being analysed quantitatively in terms of their respective influence on antibody titres, kinetics and persistence, and qualita-

Table 3 Objectives for future infant vaccine formulations

Objective
1. Rapid induction of strong antibody responses of the appropriate isotypes
2. Induction of antibody responses extending beyond infancy
3. Rapid induction of efficient Th1 and CTL responses
4. Escape from maternal antibody mediated inhibition of vaccine responses
5. Acceptable reactogenicity in early life
6. Compatibility with several vaccine antigens for simultaneous administration

tively in terms of functional efficacy, specificity, avidity, isotypes and localization. The influence of vaccine formulations on T cell responses are evaluated in terms of modulation of the Th1/Th2 differentiation of vaccine CD4 responses, reflected by their antigen-specific cytokine production, and of induction of CD8 cytotoxic T cells.

In the next sections, antigen delivery systems and immunomodulators will be reviewed for their capacity to meet the defined objectives of infant immunization. This will be based on reported studies of early life immunization, when available, or on the characteristics observed upon adult immunization with such formulations. Because of space limitations, vaccine formulations for use in mucosal vaccines will not be addressed here.

Antigen delivery systems

Particulate substances

Aluminium As for other particulate adjuvants, aluminium salts create a depot at the site of injection, from which antigen is slowly released and which activates macrophages and complement.[26] The adjuvanticity of aluminium salts essentially depends on the biochemical properties of the antigen and its resulting adsorption capacity. As an example, although aluminium hydroxide enhances the immunogenicity of tetanus toxoid, its use reduces antibody responses to Hib-tetanus conjugate vaccine.[27] Interestingly, this interference of aluminium hydroxide-containing formulations on Hib antibodies was not observed with aluminium phosphate-containing formulations, which could thus be of advantage for use in combined infant vaccines incorporating multiple antigens.

Aluminium salts have been demonstrated as influencing the differentiation of T lymphocyte responses towards the Th2 phenotype both in mice and humans,[28] which explains their enhancing effects on antibody responses, the generation of IgE-mediated allergic reactions and their inability to elicit cell-mediated Th1 and CTL responses. As an example, an immunodominant peptide of the tetanus toxin (TTP30) adsorbed onto alum elicited a much stronger Th2 pattern (high IgG1 and IL-5 levels) in young compared to adult mice.[29] Early immunization with alum-adsorbed measles virus Schwarz strain (MV-S) similarly led to preferential Th2 responses, whereas mixed Th1/Th2 responses were induced in adult animals.[6] The role of aluminium salts in enhancing Th2 neonatal responses was further exemplified by early immunization with a MV-S given without alum; although this formulation still preferentially induced IgG1 versus IgG2a antibodies and a higher production of IL-5 compared to adult primed mice, IFN-γ production was now detectable, and significant CTL responses were elicited in spite of the intrinsic neonatal Th2 polarization of vaccine responses (C-A Siegrist *et al*., unpubl. data). Although advantageous in inducing strong antibody responses; that is, to tetanus or diphtheria toxoid, the Th2 driving activity of aluminium salts is, therefore, a major disadvantage for infant vaccines aiming at the induction of Th1 and CTL responses to viral/bacterial agents. Such Th1 responses, which are difficult to elicit in early life, will be, furthermore, driven towards Th2 responses in the presence of aluminium salts. This could be of significant importance for certain new vaccines as the priming effect of this Th2-polarizing alum formulation could only be partially reverted even by boosting at adulthood with a strong Th1-driving adjuvant.[29]

However, aluminium salts compounds have been the sole adjuvant registered for use in childhood vaccines for many years, which renders them difficult to be substituted for by novel adjuvant preparations. They have, indeed, gained an extensive safety record, although local reactions do occasionally occur and increase with repeated exposure.[30] Thus, among the objectives for infant vaccine formulations, aluminium salts essentially fulfil the criteria of acceptable reactogenicity in early life and the induction of good antibody responses essentially of the IgG1 isotype. In contrast, they fail to induce Th1 and CTL responses, do not escape from the influence of maternal antibodies,[24] elicit transient responses that require early boosting and are not compatible with all vaccine antigens that one would like to include in paediatric vaccines. These limitations certainly warrant the efforts required to develop and characterize new adjuvant formulations for infant vaccines.

Emulsions Various mineral oils incorporated in numerous types of water-in-oil (w/o) and oil-in-water (o/w) emulsions stabilized by emulsifiers have been developed and evaluated for their adjuvanticity and reactogenicity profile.[30] They have generally little intrinsic immunogenic properties, but can, nevertheless, enhance antibody responses to hydrophilic antigens by constituting a short-term depot from which antigen is slowly released.[31] Water-in-oil emulsions with external antigenic peptides have also been described as capable of inducing CTL in clinical studies.[32] The main interest in emulsions is their capacity to accommodate various substances, and formulations currently in preclinical or clinical trials are complex formulations incorporating both antigen(s) and immunomodulatory substances (see following).

Liposomes and virosomes Liposome microspheres encapsulating either water-soluble or lipid-soluble molecules were shown to enhance both humoral and cell-mediated immune responses.[33,34] Whereas surface-linked antigens preferentially remain in the endosomal compartment and stimulate CD4 T cells, encapsulated antigens may escape into the cytosol and the MHC class I pathway and thus be presented to CD8 T cells.[35] The insertion of virus fusion proteins into liposomal bilayers has led to a specific type of liposomes referred to as virosomes.[36] These fusion proteins enhance cell binding, endocytic uptake and delivery of the virosome content into the cytosol through fusion to the endosomal membrane. However, CTL induction has not yet been reported, which could be because of preferential induction of Th2 rather than Th1 responses by virosomes. Clinical studies have demonstrated the safety of virosomes in adults.[37,38] However, antigenic competition limited their immunogenicity when virosomes were used as carriers for multiple antigens as a new type of combined vaccine.[39]

Studies assessing the immunogenicity of liposomes or virosomes in early life have not yet been reported either in clinical or in experimental models. Their characteristics in adults suggest that they may have to face limitations such as preferential induction of Th2 rather than Th1 responses, and antigenic competition if used for combined vaccines. Whether such formulations would be capable of inducing CTL or protecting incorporated antigens from maternal antibodies has not yet been reported.

Microspheres Microspheres of biodegradable polymers (polylactide-coglycolide, polyphosphazenes) can be used for sustained delivery of vaccine antigen.[40,41] Polymers with various sizes; that is, with various rates of *in vivo* degradation, can, in principle, be manufactured so as to achieve either progressive or pulsate antigen release over a prolonged period. A single injection of such formulations thus limits the number of required injections,[42] which would be an advantage for infant immunization. Although preliminary experiments with tetanus toxoid-containing microspheres failed to circumvent the inhibitory influence of high levels of maternal antibodies upon immunization of 2-week-old mice (C-A Siegrist *et al.*, unpubl. data), delivery formulations could potentially be adapted for use in the neonatal period, if technical difficulties still inherent in their manufacturing process can be solved.

Live vaccines and vectors

The main interest in live agents is their capacity to target specific antigen-presenting cells and to efficiently trigger both the innate and specific immune system, which could be of utmost importance in early life. The capacity of live replicating vectors to induce adult-like responses in early life appears superior to the one of many antigen presentation systems (C-A Siegrist *et al.*, unpubl. data). The main concerns for their use in early life relate to safety and to potential interference by pre-existing anti-vector immunity, including maternal antibodies.

Bacterial vectors such as the BCG strains have a good safety record even in newborns, persist in tissues for weeks or months and can elicit strong cell-mediated immune responses. Although immunization with BCG, which induces very strong IFN-γ responses upon immunization of adult mice,[43] was not able to induce similar IFN-γ levels in newborn mice,[6] the absence of IL-5 in culture supernatants indicated the successful induction of preferential CD4+ Th1 responses. Similarly, although a higher rate of T cell responses to purified protein derivative (PPD) was observed when BCG immunization was delayed from the first week of life to 9–12 months,[44,45] protection from miliary or tuberculous meningitis in the first year of life can be conferred by neonatal immunization. In view of the capacity of mycobacterial vectors to induce strong CD4 responses, a BCG-measles recombinant strain was constructed for potential use in early life.[46] Whether it would be capable of inducing strong measles-specific CD4 responses upon neonatal priming, or escaping from the influence of measles-specific maternal antibodies remains to be addressed. Among bacterial vectors, avirulent species which constitute the normal flora (*Lactobacillus*, *Streptococcus*, *Enterococcus*, *Staphylococcus*) offer obvious safety advantages for use in early life. They have, however, only recently been shown to be able to induce antibody responses to foreign antigens[47] and require further characterization of their immunogenic potential prior to consideration for use in early life.

Live recombinant viral vaccines or vectors may be relatively unstable, present a risk of *in vivo* recombination (adenovirus, polio, influenza), and be limited by the enhanced risk of establishing latent infection (herpes).[48] However, recently developed non-replicating live viral vectors circumvent these safety issues. As an example, pox viruses genetically manipulated for enhanced safety (NYVAC, MVA strains) or derived from avipox viruses (e.g. canarypox, ALVAC); that is, unable to replicate in mammalian cells, have been generated.[49] They were demonstrated to induce antibody, CD4 and CTL responses to encoded foreign antigens in numerous animal and a few human studies. The canarypox vaccine ALVAC-RG conferred protection against rabies in both adult and young dogs,[50] as well as in humans,[51] but the same vectors were less immunogenic when expressing the measles-haemagglutinin (ALVAC-HA) or the gp160 of HIV-1.[52] Initial studies involving administration of ALVAC-gp160 to human infants are currently planned.

In newborn and young mice, ALVAC-HA resulted in significant lower antibody levels and in a reduction of IgG2a antibodies compared to adult mice.[6] Additionally, immunization below 2–3 weeks of age still generated high levels of IL-5 and low IFN-γ secretion by *in vitro* restimulated splenocytes, as well as poor CTL responses. In contrast, splenocytes from mice immunized at adulthood secreted large amounts of IFN-γ and no IL-5, and generated strong CTL responses. Thus, in spite of its capacity to generate strong Th1/CTL responses in adult mice, this non-replicating live vector was not able to overcome the Th2 bias of neonatal responses of BALB/c mice. Whether it will do so in human infants will need to be addressed carefully. Last, ALVAC-HA was not able to induce measles-specific antibodies when administered to pups born to immune mothers (C-A Siegrist *et al.,* unpubl. data). However, the induction of measles-specific CD4 responses by ALVAC-HA was remarkably similar whether immunization was performed in the presence or absence of maternal antibodies. Whether the neonatal immunogenicity of such vectors in humans would allow neonatal T cell priming against measles even in the presence of maternal antibodies is an interesting question in view of their good safety profile.

DNA vaccines

The basis for DNA vaccines was the finding that intramuscular and transdermic injection of plasmid DNA could lead to the *in vivo* expression of antigen by transfected cells.[53] Such newly synthesized antigen was shown to be taken up by APC and subsequently led to the efficient induction of specific antibody and T cell responses.[54,55] As an antigen-presentation system, DNA vaccines thus have the advantage of inducing direct *in vivo* synthesis of the antigen

in its native form, followed by authentic antigen processing and prolonged presentation by surface MHC class I molecules to CD8 + T cells. In adult animal models, immunization with plasmid DNA expression vectors can induce strong Th1 and CTL vaccine responses (reviewed by Ulmer *et al*.[56]), which are important for clearance of intracellular agents and could thus be beneficial in early life.

Importantly, adult-like Th1 and CTL responses were induced in newborn and young mice upon injection of DNA encoding either measles HA or nucleoprotein (NP) of measles, sendai, influenza or LCMV virus,[57–59] as well as retroviral antigens[60] or rabies virus glycoprotein.[61] Adult-like mixed Th1/Th2 responses were induced using a plasmid encoding tetanus toxoid in young mice.[57] In contrast, a persisting neonatal Th2-bias was observed after injection of the gB-gene of herpes simplex virus,[62] and one group even described the induction of tolerance with a plasmid DNA encoding for a malaria circumsporozoite protein.[63] It remains to be clarified if those differences in the generation of Th1/CTL, Th2 responses or tolerance in newborn versus adult mice are related to the use of different antigens, plasmids, amounts of DNA injected, injection sites or to other yet unknown factors. Protective immune responses after DNA vaccination in neonates seem to be equally well inducible in species other than mice, as shown with chimpanzees.[64]

Although antibody responses induced by DNA vaccines are, in general, lower than those elicited by other antigen presentation systems, DNA vaccines have been described in a few models as able to escape from the interference with residual maternal antibodies.[59,62] This was clearly not the case in other models[65] including measles-HA DNA (C-A Siegrist *et al*., unpubl. data). As for conventional vaccines, a number of factors could explain such discrepancies. In comparative analyses involving immunization with various formulations of measles-HA, the level of maternally derived antibodies at the time of immunization was found to exhibit a greater influence on vaccine responses than the nature of the antigen-presentation system itself, whether conventional or novel. The potential advantage of DNA vaccines over conventional vaccines is, therefore, likely to depend essentially on the titres of antigen-specific maternal antibodies present at immunization, and thus on their persistence during the few weeks during which DNA-driven antigen production occurs.

In addition, DNA plasmids can be designed to code for several epitopes of the same antigen or for multiple pathogens, and even costimulatory molecules and cytokines, such as IL-12 or granulocyte/macrophage colony stimulating factor (GM-CSF), can be integrated into the same vector in order to exert local rather than systemic effects.[66,67] Such single immunization approaches make DNA vaccines a very interesting tool for immunizations, including in early life. However, the risk of integration with chromosomal DNA and the induction of autoimmune diseases are clearly potential drawbacks of this approach.

Immunomodulators

MPL, QS21 and MDP derivatives

From the lipid A region of LPS, the less reactogenic derivative monophosphoryl lipid A (MPL) appears to be promising in enhancing immune responses.[68] The mode of action of MPL is primarily through activation of APC to produce increased levels of cytokines such as IFN-γ, TNF-α and IL-1β,[69] leading subsequently to a Th1 polarization of T cell responses.[70] This could represent a significant advantage for use in certain early life vaccines, if MPL administration can be shown to be as safe in infants as it appears in adults.[71]

A purified fraction of the bark-saponin Quil A (*Quillaja saponaria*; QS21), appears as a potent adjuvant,[72] which preferentially enhanced IgG2a antibody responses and strong Th1 and CTL responses when administered with subunit antigens in various animal species including mice, baboons and rhesus macaques.[73–75] Although initial human studies performed in adult melanoma patients mention an acceptable reactogenicity, the safety requirements for use in healthy infants may, however, differ from those accepted for adult therapeutic vaccines and could represent a limitation to the use of QS21 in infancy.

Attempts to purify components of *Mycobacteria* that would be deprived of the toxicity of Freund's adjuvant led to the identification of the minimal structure needed for adjuvanticity, *N*-acetyl muramyl-L-alanyl-D-isoglutamine (MDP).[76] The MDP-induced pyrogenicity led to further development of Murabutide and Romurtide, two derivatives that are less toxic and whose adjuvant effects are under evaluation.

Thus, several immunomodulatory substances are available for incorporation into antigen delivery systems, such as emulsions and liposomes, resulting in complex vaccine formulations whose most interesting properties for infant vaccines could be related to their capacity to induce strong Th1 and eventually CTL responses if their reactogenicity profile can be shown to be acceptable.

Cytokines and interferons

It seems likely that at least part of the specific effects of adjuvants on antibody isotype switching is mediated by the production of different cytokines. When *in situ* hybridization was performed on splenocytes of mice immunized with various adjuvants leading to either Th1 or Th2 preferential humoral responses, clear differences in the cytokine patterns were observed.[77] The direct incorporation of cytokines into antigen delivery systems, or their co-administration with vaccine antigens, allows their selection for a specific action. Given the characteristics of early life immune responses, administration of cytokines capable of: (i) enhancing the activation of neonatal dendritic cells; or (ii) supporting the differentiation of CD4 T cells towards the Th1 phenotype could be of interest.

The GM-CSF increases the recruitment and activation of dendritic cells and may thus enhance vaccine responses. Enhancement of antibody responses to hepatitis B vaccine was reported in clinical studies involving non-responsive haemodialysis patients,[78] and protective antibody titres were observed after a single vaccine dose in 11/18 healthy subjects receiving GM-CSF, compared to none of the controls.[79] Such a reduction in the number of vaccine doses required could have a significant impact on immunization programmes. The influence of GM-CSF on T cell responses

remains to be demonstrated. Results from clinical studies, including prematurely born infants, are expected within the next years.

To support the preferential polarization of vaccine T cells towards the Th1 phenotype, administration of interferons or of IL-12 appears to be the most rational approach. Co-administration of IFN-γ with antigen, indeed, resulted in enhanced DTH responses and helper T cell-mediated antibody production.[80] Interferon-α and IFN-γ have already been used in vaccine trials, enhancing the immunogenicity of hepatitis B vaccines in non-responders[81] and of a synthetic sporozoite vaccine for *Plasmodium falciparum* in healthy volunteers.[82] Other studies using interferons are currently in progress.[83]

In man and mouse, IL-12 is considered the most important Th1 driving cytokine, acting in antagonism with IL-4, the major promoter of Th2 responses.[84] Its major biological activity on Th1 differentiation is attributed to strong induction of IFN-γ secretion from NK and T cells[85,86] which, in turn, enhances the ability of phagocytic cells to produce IL-12 and other pro-inflammatory cytokines. Interleukin-12 was demonstrated capable of increasing the lytic activity of CTL[85,87] and to activate NK and lymphokine-activated killer (LAK) cells,[85,88] which are considered as limiting steps of neonatal cellular responses.

The production of human and murine recombinant (r)IL-12 has led to the testing of this potent cytokine in various experimental and clinical settings. Parenteral administration of IL-12 to mice together with protein antigens adsorbed to alum strongly enhanced their antibody responses, increasing the synthesis of Th1-associated antigen-specific antibodies of the IgG2a, IgG2b and IgG3 subclasses 10–1000-fold. The Th2-associated antibody subsets, IgG1 and IgE were only slightly enhanced (IgG1) or even suppressed in the case of IgE.[89] Accordingly, mice that were immunized with a hapten–protein conjugate while treated parenterally with IL-12 showed a marked inhibition of IL-4 but an increase of IFN-γ secreting cells, and suppression of anti-hapten IgG1 antibodies.[90] Therefore, apart from the expected effect of IL-12 on the cellular immune response, IL-12 clearly exhibits potent helper activity for B cell Ig production. This effect, which could also be beneficial in the neonatal period, was particularly obvious when alum was used in the antigen formulation,[91] and seems, furthermore, to be partly independent of IFN-γ production, as demonstrated in mice containing disruptions in the IFN-γ gene.[92] The rIL-12 was also successfully used as adjuvant in some clinical settings to increase Th1 and CTL responses in adult individuals, as reviewed by others.[93,94]

A number of limitations to the administration of cytokines, which could narrow their use as vaccine adjuvants, have already been recognized. First, the issue of their potential toxicity, either immediately at the time of immunization or later, following the induction of strongly polarized immune responses that could participate in delayed immunopathological manifestations, appears essential. Excessive cytokine toxicity was observed after IL-12 administration,[95] which was even synergized by viral LCMV infection.[96] The capacity of a newborn or young infant to tolerate either acute or long-term cytokine-triggered side effects will have to be carefully assessed. Ongoing experiments injecting IL-12 into young mice at the time of immunization, indeed, suggest that the balance between efficacy and toxicity might be very difficult to achieve for cytokines acting at multiple levels of the immune responses. Second, the effect of IL-12 in inducing Th1 responses seems to be only very transient,[97] requiring the administration of multiple doses of IL-12 that would render its clinical use very difficult. Some of the concerns related to the many potential limitations of cytokine use (toxicity, specificity, stability and cost) could potentially find elegant solution through the use of viral vectors or cytokine–encoding DNA plasmids delivering minute amounts of cytokine exclusively within the micro-environment where vaccine responses are being triggered.[98,99]

CpG oligodeoxynucleotides

Part of the potency of DNA vaccines to induce Th1 and CD8 cytotoxic T cells could be caused by the 'immunomodulatory' property of bacterial DNA itself, which induces strong production of cytokines that support the differentiation of CD4 T cells towards Th1-like responses.[100] This was recently reported as being because of the presence within bacterial DNA of specific immunomodulatory motives (CpG motifs) capable of rapidly enhanced transcription of IFN-α, IFN-β and IL-12 in monocytes.[101–103] The leucocyte stimulatory activity of oligodeoxynucleotides (ODN) was first discovered in 'anti-sense' approaches where particularly CpG-rich ODN showed a strong capacity to stimulate B cell proliferation.[104] Although CpG-rich ODN induced cell cycling in over 95% of all B cells (resting or activated) at high concentrations, a synergy with signals through antigen-specific activation pathways could be detected by lowering their concentration. On this basis, CpG-rich ODN were shown to possess adjuvant activity in several preliminary studies.[105–108] Using CpG-rich ODN, a Th2 immune response induced by hen egg lysozyme (HEL) antigen administered in IFA in adult mice could even be completely reverted to a Th1-type response with high levels of IFN-γ and IgG2a antibodies.[109]

The exact molecular mechanism by which CpG-ODN mediate their effect is not yet known. It is suggested that single-stranded CpG DNA binds to endosomal or nuclear protein and directly or indirectly transduces stimulatory signals. Induction of NFκB in monocytes by CpG-DNA was recently reported,[110] and triggering of neonatal APC to full activation is presumably the mechanism by which DNA vaccines are able to induce Th1 and CTL responses even in the early period of life. Co-administration of CpG containing oligonucleotides with subunit vaccines is currently being evaluated for the modulation of neonatal immune responses to various vaccine antigens.

In comparison to DNA, the use of ODN, which could be incorporated together with vaccine antigens in various types of emulsions, would eliminate the risk of chromosomal integration. However, other safety concerns remain. When ODN were administered to mice in very high doses, the excessive immune stimulation resulted in toxicity and death, presumably by releasing high quantities of TNF-α[111] and priming for the Shwartzman reaction.[112] Their strong direct stimulatory activity on B cells could also potentially

lead to the induction of auto-antibody production and autoimmunity, and CpG-rich ODN were even shown to rescue WEHI-231 cells from anti-IgM-induced apoptosis.[113] Although more studies are obviously needed to assess the safety of CpG-rich ODN administration with regard to possible autoimmune disease exacerbation,[114,115] their potential benefits appear to warrant such efforts.

Combined antigen presentation systems and immunomodulators

Immune stimulating complexes

Immune stimulating complexes (ISCOM) form cage-like lipid structures into which antigens can be multimerized, and where a saponin derivative (Quil-A) is included as a built-in immunomodulator.[116,117] Immune stimulating complexes activate macrophages[118] and, furthermore, enhance uptake and processing of antigen by several other types of APC,[119] partly through binding of the saponin carbohydrates with the DEC-205 receptor of dendritic cells.[120] Immune stimulating complexes were also shown to fuse with the endosomal membranes and deliver antigen into the cytosol, allowing presentation to CD8 cytotoxic T cells.[121]

In 3-week-old BALB/c mice, vaccination with ISCOM containing either the measles virus fusion (F) or HA glycoprotein was reported 10 years ago as protecting from subsequent viral challenge,[122] reflecting the induction of efficient antibody and T cell responses. More recently, ISCOM were shown to overcome the interference of passively administered IgG antibodies on measles antibody responses,[123] which could be of obvious interest for use in early life. Although protection against challenge has been demonstrated in numerous species including primates, clinical trials await safety studies of Quil-A, which interestingly appears devoid of significant toxicity when incorporated into ISCOM.[124] Another important limitation to the use of ISCOM appears related to their delicate manufacturing process.

Formulations based w/o emulsions

In spite of the limitation of w/o emulsions, which only form short-term antigen depot and do not readily support the generation of CTL responses, w/o emulsions with built-in immunomodulators could, nevertheless, be suitable for use with hydrophilic immunogens. The potency of CFA is regarded as reflecting a combination of the immunomodulatory properties of mycobacterial extracts along with the short-term depot effect of a w/o emulsion.[125] In adult animals, CFA-containing formulations change the immune response to peptide antigens from Th2 to Th1.[126] This was also observed in newborn mice, where CFA (but not formulations without mycobacteria [IFA]) elicited adult-like Th1 responses to hen egg white lysozyme.[14] Both CFA and IFA have, however, largely been excluded from animal experimentation, because of their potential for irritations,[127] and are unacceptable for use in vaccine trials.

Incorporation of non-ionic block copolymers in w/o or o/w emulsions enhances antibody responses to a variety of viral, parasite or bacterial antigens.[128,129] When assessed in early life, Hunter's (CRL8941) w/o adjuvant (TiterMax®, CytRx Corporation, Norocross, GA, USA) partially corrected the neonatal Th2 bias induced by immunization of a tetanus peptide in BALB/c mice.[29] The adjuvanticity of CRL8941 significantly increased IgG2a, IgG2b and IgG3 antigen-specific responses in 1-week-old mice compared to the alum-treated group, shifting their titres to adult-like levels after two vaccine doses. However, the kinetics of antibody responses remained slower in young mice, and CRL8941 was only able to reduce the neonatal IL-5 burst, but not to significantly increase IFN-γ levels in the cytokine assay. Inflammation at the injection site was significantly more important in young compared to adult mice, and confirmed the reactogenicity of this product, which was considered too high for use in humans and is currently leading to the development of novel formulations.

Formulations based on o/w emulsions

Amphipathic immunogens are best incorporated into o/w emulsions, and several formulations with interesting properties have been developed, some of which have reached the stage of clinical trials. Of these, some appear able to induce the Th1 and/or CTL responses desirable for use in early life.

A squalene oil emulsified with Tween 80 and Span 85 (MF-59®, Chiron Vaccines, Siena, Italy) was shown to be a safe and potent stimulator of animal and human cellular and humoral responses to subunit antigens such as herpes simplex virus, HIV, hepatitis B and influenza.[130,131] Its immunogenicity and safety in children was demonstrated in a large vaccination study involving toddlers.[132] Recently, the adjuvant effect of MF-59 was shown to be superior to an alum preparation with regard to the antibody response to Hib and *N. meningitidis* conjugate vaccines when administered to infant baboons.[133]

Several formulations including various combinations of immunomodulators (alum, MPL, QS21) incorporated in various w/o and o/w emulsions have been developed and are progressively reaching the stage of clinical trials. As an example, inclusion of a recombinant circumsporozoite protein (RTS,S) into the SB (SmithKline Beecham Biologicals, Rixensart, Belgium)-AS2 formulation, an o/w emulsion containing both MPL and QS21, was reported to protect against *Plasmodium falciparum* malaria.[134] In contrast, formulations containing either alum and deacylated MPL (SB-AS4) or without additional immunostimulants (SB-AS3) failed to induce protection. Inflammation and pain at the site of injection, which increased with number of vaccine doses were, however, commonly reported and are currently considered as preventing infant clinical trials using formulations such as SB-AS2. However, efforts to design rational novel vaccine formulations have generated formulations with improved immunogenicity/reactogenicity ratio, which are currently being evaluated for early life immunization in mice and monkeys. They could significantly enhance the immunological properties of existing vaccines (i.e. acellular pertussis vaccines), and allow the

Table 4 Potential advantages and limitations of antigen delivery systems for infant immunization

Delivery system	Potential advantages	Potential limitations
Particulate substances		
Aluminium salts	Enhancement of IgG1 responses	Transient Ab responses
	Enhancement of Th2 responses	Lack of Th1/CTL induction
	Acceptable reactogenicity	Susceptibility to maternal Ab
		Incompatibility with certain vaccine antigens
Emulsions	Capacity to incorporate multiple antigens/immunomodulators	
Liposomes/virosomes	Enhancement of Ab responses (?)	Antigenic competition
	Acceptable reactogenicity (?)	Lack of Th1/CTL induction
Polymer microspheres	Prolongation of Ab responses	Complex manufacturing process
	Escape from maternal Ab?	
Live vectors		
Bacterial vectors		
BCG derived	Enhancement of Th1 responses (?)	
	Acceptable reactogenicity (?)	
Viral vectors		
Pox derived	Enhancement of Ab responses (?)	Susceptibility to maternal Ab
	Enhancement of Th1 responses ?	
	Induction of CTL responses?	
	Good safety profile	
DNA vaccines	Prolongation of Ab responses (?)	Susceptibility to maternal Ab?
	Enhancement of Th1 responses	Safety issues
	Induction of CTL responses	

Ab, antibody; (?), remains to be evaluated in early life; ?, may not occur in early life; BCG, bacille Calmette-Guérin.

Table 5 Potential advantages and limitations of immunomodulators for infant immunization

Formulation	Potential advantages	Potential limitations
Immunomodulators		
MPL	Enhancement of Ab responses (?)	Reactogenicity (?)
	Enhancement of Th1 responses (?)	
QS21	Enhancement of Ab responses (?)	Reactogenicity
	Enhancement of Th1 responses (?)	
	Induction of CTL responses (?)	
MDP derivatives	Enhancement of Ab responses (?)	Reactogenicity (?)
	Enhancement of Th1 responses (?)	
Cytokines		
GM-CSF	Enhancement of Ab responses (?)	Safety issues
Interferons	Enhancement of Ab responses (?)	Safety issues
IL-12	Enhancement of IgG2 responses (?)	Safety issues
	Suppression of IgE responses (?)	Transient effect
	Enhancement of Th1 responses (?)	
CpG oligonucleotides	Enhancement of Ab responses (?)	High-dose toxicity (?)
	Enhancement of Th1 responses (?)	Autoimmunity (?)
Antigen delivery systems with built-in immunomodulators		
ISCOM	Enhancement of Ab responses	Reactogenicity (?)
	Enhancement of Th1 responses	Manufacturing process
	Induction of CTL responses	
	Escape from maternal Ab	
Block copolymers	Enhancement of Ab responses	Reactogenicity
	Induction of Th1 responses	
MF-59	Enhancement of Ab responses	
	Enhancement of Th1 responses (?)	
	Induction of CTL responses (?)	
	Acceptable reactogenicity (?)	
SB-AS2	Enhancement of Ab responses (?)	Reactogenicity (?)
	Enhancement of Th1 responses (?)	
	Induction of CTL responses (?)	

Ab antibody; (?), remains to be evaluated in early life; MPL, monophosphoryl lipid; QS21, *Quillaja saponaria*; MDP, *N*-acetyl muramyl-L-alanyl-D-isoglutamine; GM-CSF, granulocyte/macrophage colony stimulating factor; CpG, ISCOM, immune-stimulating complexes.

induction of early life immune responses against weak immunogens such as the RSV glycoproteins.

Conclusion

Numerous different antigen delivery systems and immunomodulators are currently being developed (Tables 4,5), several of which could be useful to enhance antibody and T cell vaccine responses in infants. Based on the identified limitations of infant immune responses, antigen delivery systems/immunomodulators of special interest for infant vaccines include those capable of: (i) rapid induction of long-lasting B cell responses, ideally even in the presence of maternal antibodies; (ii) induction of strong Th1 and CTL responses against antigens from intracellular micro-organisms; and (iii) stable formulations that would allow the inclusion of multiple vaccine antigens so as to lower the number of required injections. Evaluation of all these objectives, together with extended safety studies, will be required for assessment of novel compounds in several preclinical models of early life immunization, which should be selected to optimally mimic the conditions considered to prevail in human infants. Legitimate safety concerns should certainly postpone the clinical evaluation of novel formulations in human infants until wide demonstration of its acceptable reactogenicity and long-term safety, not only in human adults or older children but also in young animals, taking into consideration some of the unique features of this period of life.

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