IMMUNE RESPONSE OF NEONATES ELICITED BY SOMATIC TRANSGENE VACCINATION WITH NAKED DNA

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1. ABSTRACT

Neonates display lower immune responsiveness and higher susceptibility for high-dose tolerance. Quantitative as well as functional differences between the neonatal and adult lymphocytes or antigen presenting cells (APC) respectively, explain the particular immune responsiveness during the early stages of the postnatal development. Reduced numbers of lymphocytes and APCs as well as a modified responsiveness of T cells in neonates, are the main factors that account for the low threshold of tolerance in newborns. Taking into account these particularities, the design of effective vaccines for neonates poses significant difficulties. We hypothesized that a continuous exposure to low doses of antigens may avoid high-zone tolerance and may lead instead, to effective expansion of effector and memory cells. Indeed, inoculation of newborn mice with plasmids encoding nucleoprotein (NP) or hemagglutinin (HA) of influenza virus, led to the priming of specific cytotoxic (CTL), helper (Th) and B cells, rather than induction of unresponsiveness. Mice immunized as neonates with naked DNA and challenged later with lethal doses of influenza virus, displayed significant protection. Thus, DNA immunization may be a promising strategy for vaccination against serious infectious diseases of infants and children.

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2. INTRODUCTION

Early studies demonstrated that the immune responsiveness varies with the stage of the development. Countless observations showed that during the early stages of postnatal development or during the fetal period, exposure to antigens was followed by unresponsiveness. Billingham *et al.* (1) noted that newborns injected with allogeneic hematopoietic cells failed to reject subsequent skin grafts. Later, Owen (2) showed that genetically non-identical bovine twins which shared placenta and were exposed to each other's blood, failed to reject allogeneic cells as adults. Based on these data and theoretical considerations, Burnet (3) proposed that the ability to distinguish self from non-self results from the deletion of self reactive clones during the early stages of the immune system development. This concept was supported by experiments that showed an impaired ability to produce specific antibodies following neonatal injection of large amounts of foreign antigens (4,5) or antibodies specific for idiotypic, allotypic or isotypic determinants of Ig receptors (6-8). Furthermore, Etlinger and Chiller (9) showed that aggregated human gamma-globulin, a highly immunogenic T-dependent antigen in euthymic mice, was able to induce immune specific unresponsiveness when injected into newborn mice. More recent data in transgenic animals also showed that thymic exposure to soluble or cellular antigens (10) as well as exposure of immature B cells to membrane-bound antigens (11) caused deletion of the antigen-specific clones. Thus, it was indirectly assumed that neonatal susceptibility to tolerance was a consequence of the predominance of immature lymphocytes during this developmental stage.

Based on these assumptions regarding the neonatal susceptibility to tolerance, several groups

succeeded in preventing or delaying the occurrence of spontaneous or experimental autoimmune diseases by injection of self-antigens into newborns. Prevention of experimental allergic encephalitis (EAE) by treatment of neonates with myelin basic protein (MBP) (12) or an MBP immunodominant peptide (13) provided a good example of anergizing the autoreactive T cell clones during the neonatal stage of life. In this particular case, the autoreactive T cell clones are not deleted during the thymic stage of development because of the lack of MBP self antigen. It is known that compacted myelin is produced in substantial amounts only after the first postnatal week and it is essentially sequestred in the nervous system. Thus, neonatal exposure to exogenous MBP prevented the subsequent responsiveness of the MBP-specific T cells. In the same manner, neonatal injection with glutamic acid decarboxylase (GAD) was also capable of delaying the spontaneous occurrence of diabetes in NOD female mice (14). Noteworthy, while the animals injected with MBP or GAD antigens as neonates developed specific T cell unresponsiveness, they were able to produce anti-MBP or anti-GAD antibodies (12,14). The phenomenon of 'split tolerance' was further investigated by Singh *et al.* (15) who demonstrated that neonatal inoculation of the 106-116 hen egg lysozyme (HEL) peptide in tolerogenic doses induced a mixed Th1/Th2 response that evolved into a strongly biased Th2 response after subsequent antigen exposure. Furthermore, following *in vitro* treatment with anti-IL-4 or anti-IL-10 monoclonal antibodies (mAbs), the Th2 response was reversed to a Th1 response associated with increased T cell proliferation (15). Mice were able to mount, at all stages, HEL 106-116 specific antibodies, the isotype pattern being largely determined by the Th profile (15). In some cases, neonatal injection of self peptides may rather facilitate or trigger autoimmune diseases. For example, treatment of neonatal NZB/NZW F1 mice with a self-autoantigenic peptide from an anti-DNA mAb that was followed by Th2 response and decreased T cell proliferation, led to the induction of pathogenic IgG anti-dsDNA auto-antibodies associated with signs of glomerulonephritis (15). Thus, it appears that injection of neonates with antigens may have various outcomes regarding the susceptibility for autoimmune diseases, depending on the particular pathogenic mechanism that leads to the disease.

In a distinct experimental system, neonatal B6AF1 female mice injected with a self ZP3 ovarian-specific peptide, developed autoimmune ovarian disease (AOD) associated with a significant T cell proliferative ability and Th1/Th2 mixed profile (16). In contrast, males that were identically treated developed strong Th2 responses associated with low T cell proliferation and resistence to induction of AOD (16). This study suggested that Th1 pathogenic cells induced by injection of neonates with ZP3 peptide, were rescued by continuous exposure to the endogenous self-antigen in the female mice. In another study, it was shown that the continuous

exposure to low doses of antigen following injection of neonates with plasmid, led to the priming of specific CTLs rather than unresponsiveness (17). Furthermore, Th1 and CTL induction were noted subsequent to neonatal injection of antigens at low doses or in complete Freund's adjuvant (CFA) (18,19). CTLs specific for H-Y antigens were induced by transfer of dendritic cells from adult males to newborn females, indicating that 'professional' presentation may lead to effective cellular responses in neonates (20). Thus, whereas low doses of antigens presented by professional APC (pAPC) are associated with Th1 and CTL induction upon immunization of neonates, moderate or high-doses of antigens lead to generation of Th2 cells that have a poor proliferative potential. Consequently, neonates exposed to antigens display the tendency to develop an immunodeviated response rather than a true immunologic tolerance.

During the past years, studies conducted in our laboratory regarding the effect of DNA immunization of neonates, showed that sustained exposure to low doses of antigens during the early stages of postnatal development was followed by significant priming of protective humoral and cellular responses rather than induction of tolerance. Thus, this review is aimed at analyzing information, sometimes contradictory, regarding the immune responsiveness of neonatal B and T cells.

3. IMMUNE RESPONSIVENESS OF THE NEONATAL B CELLS

Susceptibility of the neonatal B cells to high-dose tolerance was previously documented in various experimental systems. Neonatal injection with antibodies specific for immunoglobulin (Ig) antigenic determinants caused a long lasting suppression of the B cells that express the corresponding determinant (6-8). Furthermore, injection of large amounts of bacterial polysaccharides (4,5) or T-dependent antigens (9) impaired the subsequent synthesis of specific antibodies.

However, Howard and Hale were among the first to show that in certain conditions, adult animals injected as newborns with lower amounts of bacterial polysaccharides can mount an antibody response (21). Studies carried out later demonstrated that some antibody responses and the expression of their corresponding idiotypes can be elicited in 1-14 day-old mice subsequent to immunization with various antigens (Table 1).

Notably, recent studies showed that some immunization protocols that led to T cell unresponsiveness in terms of proliferation, were, in fact, followed by humoral responses (15, 16). Due to the fact that low antigen doses are associated with Th1 responses while higher doses are followed by poor proliferative Th2 responses (18, 19), one should expect different isotype

Table 1. Antibody responses elicited by neonatal immunization

Age of immunization	Antigen	Idiotypes associated with antibodies	Reference
Day 1	alfa1-3 Dextran	J 558	(22)
	beta 2-6 Fructosan	A48 IdX	(5)
	Salmonella tranaroa	MOPC 348Id	(23)
	Galactan	X24 Id1 and IdX	(24)
	TNP-LPS	460 Id	(23)
Day 7	Phosphocoline	T15 IdX	(25)
	Phenylarsonate	CRI	(26)
Day 14	Flagellin	467 Id	(23)
	Pneumococal polysaccharide	4.65 III Id	(27)

profiles in T-dependent humoral responses, depending on the antigen dose inoculated in newborn organisms. It becomes apparent that the mechanisms of neonatal B and T cell tolerance are different. Whereas the induction of neonatal B cell tolerance may require higher antigen doses that can directly turn off the antigen-specific B lymphocytes, the neonatal T cell tolerance is a modified response rather than a true unresponsiveness.

This hypothesis is supported by the predominance of immature B cells in the neonatal repertoire. Indeed, neonatal B cells differ phenotypically from their mature counterparts since they are B220^{hi}, sIgM^{hi}, sIgD$^-$, whereas most adult B cells are B220^{hi}, sIgM^{lo} and sIgD^{hi} (28). The predominance of immature B cells (sIgM$^+$IgD$^-$) was considered the major cause to explain why the neonatal B cells are more susceptible to tolerance induction by various T-independent antigens (9, 29). An early study showed that *in vitro* tolerance induction required 10^3 times more antigen in the case of adult B cells as compared to neonatal B cells (30).

Several hypotheses were entertained to explain the unresponsiveness of the immature B cells, which are dominant in the periphery of newborns. The most prevailing hypothesis considers that the differences in isotype expression of the Ig receptors are responsible for the susceptibility of the immature B cells to tolerance. Thus, while the binding of antigens to sIgM only causes anergy or deletion, the interaction with sIgD induces a positive signal (31). Negative signaling through sIgM may be related to the fact that sIgM is not coupled downstream to the inositol-phospholipid signaling pathway (32). Furthermore, a recent study showed that 3 day-old mice have marked deficiencies in *lyn*, *fgr* and *src*-family tyrosine kinase expression (33). This model is supported by the observation that mature B cells stripped of IgD become more sensitive to the induction of tolerance (31). However, this model cannot easily account for the fact that both sIgM and sIgD can mediate negative selection in the transgenic mice (34). A more detailed analysis carried out in B cell receptor transgenic mice by Carsetti *et al.* (35) demonstrated that the subpopulation directly affected by tolerance is the late-immature (sIgM^{hi}, sIgD$^-$, HSA^{bright}, B220^{low}, CD44^{hi}) subpopulation rather than the dominant (sIgM^{hi}, sIgD$^-$, B220^{hi}) subpopulation of immature B cells.

Another hypothesis aimed at explaining the poor humoral immune responsiveness of newborns considers that neonatal B cells are unable to receive second signals from T cells, independent on their stage of differentiation. This idea is supported by recent studies which showed that human neonatal T cells have decreased expression of CD40-L and the cord-blood B cells displayed an impaired ability of isotype switch following CD40 binding (36). It is well known that the interaction between these two molecules is important for the activation and differentiation of both B and T cells (37, 38). Thus, both the abilities of B cells to undergo

isotype switching and of T cells to differentiate to Th1 cells could be affected. Furthermore, the lack of CD40 - CD40-L interaction may lead to a predominant nonprofessional presentation of antigens to T cells because the up-regulation of B7 molecules on APCs is consequently impaired (39).

The discrepancy between the *in vivo* data (Table 1) demonstrating the ability of neonates to mount humoral responses in certain instances and the *in vitro* observations indicating that signaling through IgM leads to apoptosis of the immature B cells, can be explained by the presence of a few mature IgM^+IgD^+ B cells in the periphery of newborns. Thus, injection of antigens in neonates is followed by deletion or anergy of the immature, antigen-specific B cells and priming of few differentiated B cells. Lower doses of antigens may be unable to delete most of the specific precursors but may still be able to prime some mature B cells, leading to a humoral response. If the antigen dose is above a certain threshold, most of the specific B cell precursors would be deleted and the mature B cells anergized, leading to a state of unresponsiveness with a variable duration. It is noteworthy to mention that exposure to lipopolysaccharide (LPS) which is a polyclonal stimulator for B cells, can trigger a more rapid recovery from the state of B cell unresponsiveness induced by injection of antigens into neonates (9).

3.1. Humoral responses of mice immunized as neonates with naked DNA

There are numerous studies demonstrating that immunization of adult mice (40), chickens (41), ferrets and monkeys (42, 43) with plasmids bearing HA or NP genes of various strains of type A influenza virus, can induce protective immune responses.

In order to study the neonatal humoral response to mammalian expression vectors, we immunized newborn mice with a plasmid bearing the HA gene cDNA from A/WSN/33 virus (pHA), driven by the initial-early cytomegalovirus (CMV) promoter (Fig.1A). Newborn and adult BALB/c mice were immunized three times with 100 µg of plasmid and the immune response was studied at various intervals (Fig.1B).

Antibodies specific for WSN virus were detected by both hemagglutination-inhibition (HI) and radio-immunoassay analysis (RIA) (44). The data presented in Fig. 2 show the presence of WSN specific antibodies as detected by HI assay at 1 and 3 months after the completion of immunization, in mice inoculated with pHA as adults or as neonates

Serum HI titers dramatically declined between 3 and 6 months after the immunization of adult mice. Immunization of adult mice with live WSN virus resulted in HI titers that were 10-20 higher than those obtained following inoculation with pHA. In contrast, inoculation
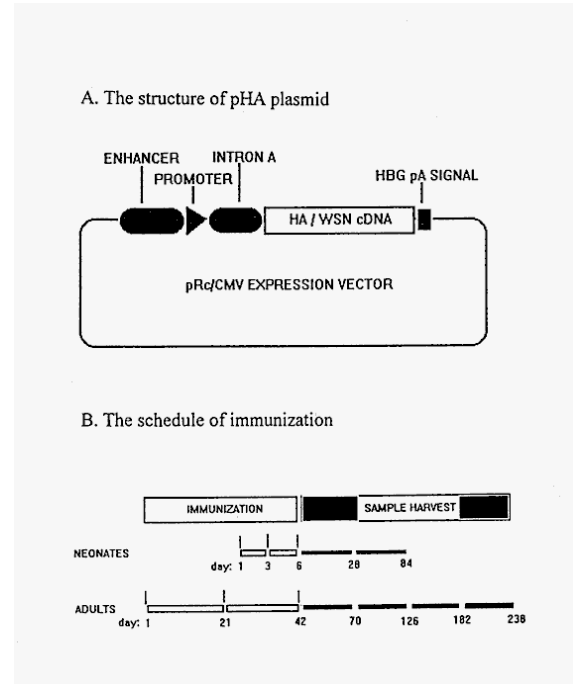

Figure 1. The structure of the pHA plasmid (A) and the immunization schedule with naked DNA of neonatal and adult mice (B). The cDNA of the HA was inserted into a plasmid bearing the regulatory elements (enhancer + promoter) of the initial-early genes of CMV and the polyadenylation signal of the bovine growth-hormone gene. Mice were immunized three times in the muscle with 100µg of plasmid/dose. Blood or tissue samples were harvested at various intervals after the completion of the immunization. Some of the mice were boosted with live-virus 7 days prior to the sample harvest.

of neonates or adults with a control plasmid that did not express HA was not followed by humoral response. The mice immunized with pHA were able to mount significant secondary humoral responses following the virus boost, indicating that the B cell repertoire was not affected by the prolongued exposure to the antigen (44). The plasmid could be detected even at 9 months after the completion of immunization, in approximately 25% of the immunized mice (Fig. 2 and data not shown).

Two major differences were noted between the humoral responses of mice immunized with pHA as adults and neonates: first, the isotype profile of the WSN specific antibodies was distinct in mice immunized as neonates versus adults, after the virus boost (ref. 44 and below). Whereas IgG_2 antibodies predominated in the mice immunized as adults, the newborn immunized mice displayed increased titers of IgG_1 and IgG_3 antibodies after the virus boost. Second, the reactivity analysis of clonotypes obtained from mice immunized as adults or newborns with pHA, showed that in contrast to mice
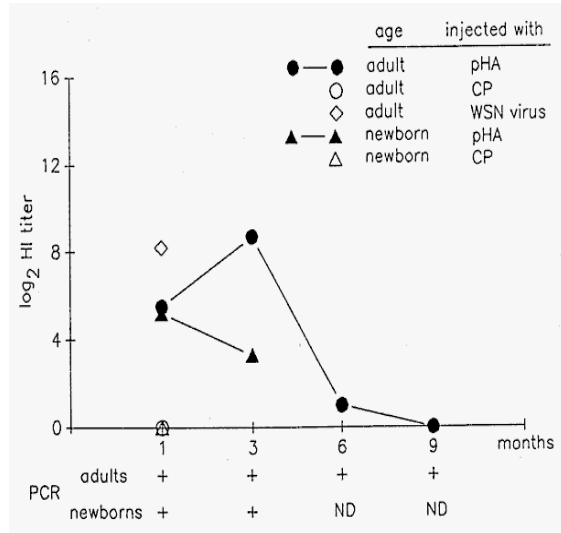
Figure 2. The HI titer of serum antibodies obtained from animals immunized with pHA as adults or newborns, at various intervals after the completion of immunization. HI titers of WSN specific antibodies, which are good correlates of the neutralization ability, were estimated in groups of 5-10 mice. The results were expressed as means of $\log_2$ of the individual HI titers at various intervals after the immunization. Control mice were injected with the plasmid lacking the HA open reading frame (CP) or with live WSN virus, 1 week previous to the blood harvest. PCR results showing the persistence of pHA plasmid at the site of injection were considered positive (+) if at least one mouse in a given group was positive. ND - not done.

immunized as neoantes, those inoculated as adults displayed more frequent cross-reactive clonotypes (data not shown). These differences can be accounted for by the particular T cell responsiveness of neonates and by the more restricted B cell repertoire of the young mice (45).

Mice immunized with pHA as newborns or adults and challenged with one 100% lethal dose (1LD$_{100}$) of WSN virus one month after the completion of immunization, displayed 50-80% long-time survivors (44). However, the cross-protection against influenza virus PR8 drift-variant and the pattern of virus lung-titer decrease between day 3 and 7 after the infection, suggested that T cell immunity is an important protective factor following the immunization with pHA (44).

It appears that plasmid immunization of newborn mice leads to protective humoral responses rather than unresponsiveness. Continuous exposure to small doses of antigen following plasmid immunization did not lead to deletion of the specific immature B cells or anergy of the more mature, virus-reactive B cells. In contrast, the mature B cells present during the early stages of the postnatal development were effectively primed to antibody-forming and memory B cells. Thus, whereas antibodies are continuously generated as long as the antigen persists in the organism, the animals are able to mount strong protective responses following the virus infection.
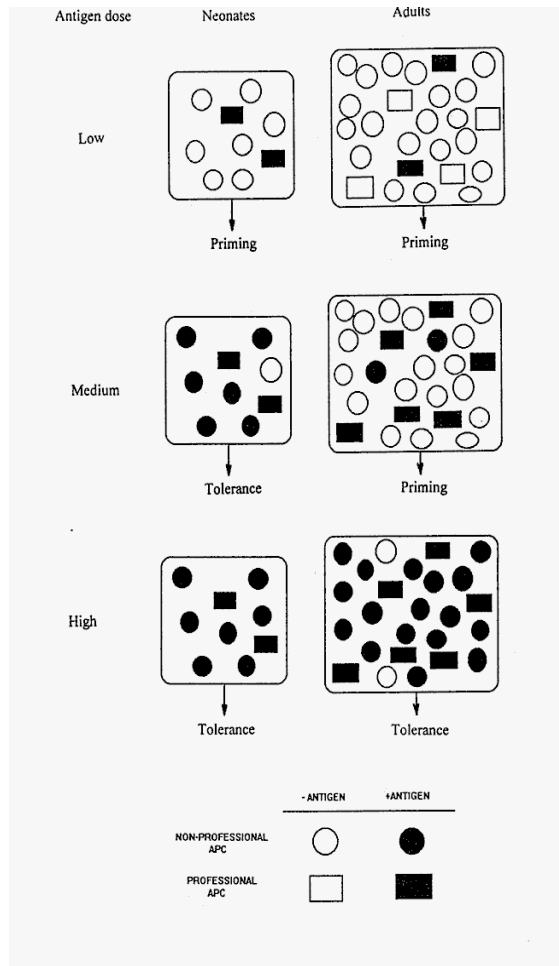
4. IMMUNE RESPONSIVENESS OF THE NEONATAL T CELLS

The classical experiment of neonatal tolerance induction following the injection of allogeneic cells (1) suggested that self-nonself discrimination is a consequence of the deletion of lymphocytes that encounter antigens during a certain neonatal time-window. However, only few things about the biology of the T cells were known at that time. More recent studies showed that rather than being unresponsive, the neonates mount immune responses that are qualitatively different as compared to adult animals (8,15). Furthermore, in certain circumstances, newborns display immune responses that resemble those of the adults (17,19). Two non-mutually exclusive models could explain the particular immune responsiveness of the neonatal T cells: the first one considers that differences in the total numbers of APC and T cells between adults and neonates account for the differences in the immune responsiveness (the quantitative model). The second, considers that the neonatal T cells respond in a functionally different manner to antigens (the qualitative model). Interestingly, there are numerous studies that support both hypotheses.

4.1. Quantitative differences between the immune systems of neonates and adults

This model is based on the following observations: (a) neonates are able to mount T cell responses to various types of antigens (8,17,19,20,46); (b) the T cell response of neonates to low doses of antigens is adult-like (8,19); (c) the T cell response of adults to high doses of antigens resembles the immune response of newborns to 'tolerogenic' doses (20) and (d) antigen presentation by professional APC (pAPC) during the neonatal period, is followed by adult-like responses (20).

The central assumption of this model is that T cells are turned-on by the pAPC, whereas non-professional APC (npAPC) that do not express enough co-stimulatory molecules, turn-off the naive T cells. The 'Danger Model', that attributes the ability of initiating the immune response to the pAPC (47), regards the DCs, the activated monocytes and B cells as pAPC, whereas the resting B cells are npAPC (20,48). Thus, the ratio between the pAPC and npAPC that present the antigen to naive specific T cells would determine the outcome of the response, i.e. priming versus tolerance. This model explains why whereas low doses of antigens produce adult-like immune responses in neonates, very high doses of antigens are tolerogenic even in adults (Fig. 3).

Figure 3. The quantitative model that addresses the differences of immune responsiveness between the neonates and adults. The number of professional (rectangles) and nonprofessional (circles) APC is proportionally decreased in neonates. The outcome of the immune response is regulated by the ratio between professional and nonprofessional APC that present the antigen. Thus, the inoculation of antigen in doses that are immunogenic for adults ('medium') is followed by predominant non-professional presentation and subsequent tolerance in the neonates. It can be inferred that immunization with low doses of antigens may have priming effects in newborns, whereas injection of large doses of antigen may lead to tolerance in adults as well as neonates.

Simply, moderate doses of antigens are sufficient in neonates to shift the overall balance toward nonprofessional presentation, because the number of APC is lower. In contrast, low doses of antigens would lead to an exclusive loading of pAPC. Thus, this quantitative model explains the observations that neonatal tolerance can be avoided by immunization with low amounts of

antigens (8,15,19,45,49) or with pAPC bearing the antigen (20).

The quantitative model has to take into consideration the data regarding the decreased number of class II$^+$ macrophages in the peripheral lymphoid organs of neonatal organisms (50-52) and the low efficacy of the neonatal B cells as APC (53). Thus, besides the lower number of APC, the ratio between pAPC and npAPC seems to be decreased in the neonatal as compared with the adults.

A consequence of the quantitative model is that the *in vitro* responsiveness of the neonatal and adult T cells are the same. Numerous observations argue against this fact, although they do not discard the validity of the quantitative model.

4.2. Functional differences between the lymphocytes of neonates and adults

The model based on qualitative differences between neonatal and adult peripheral T cells is based on the following observations: (a) the susceptibility of the neonates to high zone tolerance (1); (b) the strong tendency of the neonatal T cells to produce Th2 cytokines and the decreased ability to produce Th1 cytokines following *in vitro* stimulation with polyclonal activators (54-56); (c) the strong tendency of the neonates to mount Th2 biased immune responses that are refractory to a subsequent switch toward Th1 responses (15,16,57).

This qualitative model is strongly supported by studies that show phenotypic differences between neonatal and adult T cells. For example, most of the mouse neonatal CD4$^+$CD8$^-$ T cells lack Qa-2 membrane antigen and display low responsiveness to polyclonal stimulators (58). Furthermore, developmentally immature T cell subsets are present in the periphery of young rodents and they may undergo post-thymic maturation in the peripheral lymphoid organs (59,60). At present, little is known about the expression of co-receptors (i.e. CTLA-4, CD28, CD27, CD50, Fas) or cytokine receptors (i.e. TNFalfa-R) on the neonatal T cells. A functional study indicated a high tendency for the neonatal T cells to undergo apoptosis following *in vitro* polyclonal stimulation (56).

A recent study carried out with human neonatal T cells demonstrated that polyclonal activation by anti-CD3 mAb in the presence of anti-CD28 mAb led to Th1 or Th2 differentiation, depending on the addition of IL-2 or IL-4 (61). Interestingly, neonatal T cells were able to produce both IL-2 and IFNgamma (61), suggesting that strong co-stimulation circumvented the reduced ability of the neonatal T cells to differentiate to adult-like Th1 cells. Because the human neonatal T cells do not express detectable CD40-L (36), a direct CD28 engagement would bypass the requirements for B7.2 expression and IL-12 secretion by pAPC, that are otherwise dependent on

Table 2. Summary of the results showing different responsiveness profiles of naive antigen-specific T cells from neonatal and adult TCR transgenic mice, following *in vitro* priming with HA 110-120 peptide in the presence of adult accessory cells

Accessory cells	Assay	Responder cells	
		Neonatal T cells	Adult T cells
Adult splenocytes	Proliferation[a]	+	+++
	Cytotoxic activity[b]	++	++
	IFNgamma production[c]	-	++
	IL-4 production[c]	++	-
Enriched DCs[d]	IFNgamma production[c]	-	+++
	IL-4 production[c]	+++	-

[a] Proliferation index at least 10 times higher for adult compared to neonatal T cells;
[b] Following *in vitro* priming with HA 110-120 peptide, effector cells displayed specific lysis values between 50-70% in both cases;
[c] (-) represents values below the sensitivity of the ELISA assays (5 pg/ml); (++) corresponds to 150-300 pg/ml secreted by $1x10^5$ T cells after 4 days in culture with HA 110-120 peptide; (+++) corresponds to higher concentrations of cytokines (above 350 pg/ml);
[d] Enriched dendritic cells (DCs) were obtained from adult BALB/c splenocytes by overnight incubation of plastic-adherent cells in presence of GM-CSF (10 U/ml), followed by the recovery of the cells detached into the medium.

the CD40/CD40-L interaction (39,62). This hypothesis is indirectly supported by studies which showed that production of IL-2 by polyclonally stimulated neonatal T cells of mouse origin, can be enhanced by co-stimulation with anti-CD28 or IL-6 (63). Furthermore, strong co-stimulatory factors like IL-2, IL-6 or anti-CD28 mAb partially rescued the neonatal T cells from anti-CD3 mAb induced apoptosis (56). Thus, the overall view is that the neonatal T cells are impaired in their ability to receive and transduce co-stimulatory signals, leading to predominant differentiation to Th2 cells or apoptosis.

The above mentioned studies were carried out using polyclonal stimulation of the T cells. In order to circumvent this caveat, we addressed the question regarding the *in vitro* responsiveness of neonatal T cells

in a TCRalfa/beta transgenic system. The transgenic receptor is specific for HA 110-120 peptide in the context of I-E^d molecules and the transgenic mice express the

receptor (TCR-HA) on both CD4$^+$ and CD8$^+$ T cells (64). If quantitative differences were solely responsible for the particular immune responsiveness of neonates, we would expect an identical behavior of neonatal and adult T cells *in vitro* stimulated by antigen and pAPC. Interestingly, the responsiveness profiles of neonatal and adult T cells

from TCR-HA transgenic mice are distinct: (a) whereas neonatal T cells secreted large amounts of IL-4 and no IFNgamma, adult T cells secreted large amounts of IFNgamma and no IL-4; (b) proliferation ability of the neonatal T cells was very low compared to that of the adult T cells (Table 2).

The overall frequency of the TCR-HA$^+$ T cells in the spleens of adults and neonates were comparable, so that this quantitative factor alone cannot explain the above-mentioned differences (see below and Fig. 4). Furthermore, stimulation of the T cells in presence of enriched adult pAPC (Table 2) or various doses of HA 110-120 peptide (not shown) did not change the responsiveness profiles. Surprisingly, high CTL activities could be generated by *in vitro* peptide priming of both neonatal and adult TCR-HA$^+$ T cells (Table 2). This indicates that high CTL activity can be generated during an exclusive Th2 response, although it is not clear if this result is valid only in the case of MHC-II restricted CTLs.

The study of presence and phenotype of TCR-HA$^+$ T cells in the spleen or blood of transgenic mice (Figure 4) showed that: (a) the overall frequencies of TCR-HA$^+$ T cells in newborns and adults were comparable, around 5-15% of the total T cell population; (b) the frequency of CD4$^+$ CD8$^-$ TCR-HA$^+$ T cells was

significantly lower in the neonates; (c) in contrast to adult mice, most of the neonatal TCR-HA$^+$ T cells were CD4$^-$ CD8$^-$. Thus, although the relevance of this model for the wild-type organisms is still unclear, it appears that there are important functional and phenotype differences between the peripheral T cells of newborns versus adults. Furthermore, a recent study regarding the TCR-HA transgenic mice, suggested the age-dependent accumulation of T cells with activated phenotype, in the absence of intentional antigenic stimulation (65).

Taking into account all the data mentioned above, it becomes clear that a better model has to combine both quantitative and qualitative differences between APC and T cells respectively, from adults and neonates. The quantitative aspect is still very important and can explain some peculiarities of the neonatal immune system as a whole: (a) the low magnitude of the 'adult-like' immune response obtained in certain conditions, due to the presence of a few functional lymphocytes in the periphery of neonates and (b) the susceptibility of neonates to high-zone tolerance simply because the number of specific T cells to be turned-off is significantly lower. Indeed, an analysis of the results regarding the phenotype of TCR-HA$^+$ T cells, suggested that the total number of specific T cells in neonates is at least 100 times lower than in adult organisms (Fig.4 and data not shown).

4.3. Cellular immune responses of mice immunized as newborns with naked DNA

In influenza virus infection, the cell mediated immunity plays an important role in the recovery from disease (66). First, virus-specific CTL mediate the clearance of the virus from the infected lungs (67). Secondly, in the absence of CD8$^+$ T cells and MHC-II expression on the lung epithelial cells, CD4$^+$ virus-specific T cells are able to clear type A influenza viruses from the infected lungs (68). Whereas Th1 cells are able to mediate a protective effect, Th2 cells display rather detrimental effects on the evolution of influenza pneumonia (69). Thus, multiple arms of the cellular immunity participate to the defense reaction in influenza virus infection.

Previous studies showed that plasmid immunization of adult animals led to protective cellular responses in the influenza virus system (40,43). We hypothesized that small amounts of antigen would be inefficient in inducing high-zone tolerance even in very young animals, so that the few mature antigen specific T cells could be primed and subsequently expanded because of the persistence of antigenic stimulation. We immunized newborn and adult BALB/c mice with a plasmid encoding NP of the PR8 strain of influenza virus (NPV1) (70) according to the protocol described in Fig.1. We studied the CTL immune response against target cells infected with influenza viruses or coated with NP 147-155 peptide, that is the
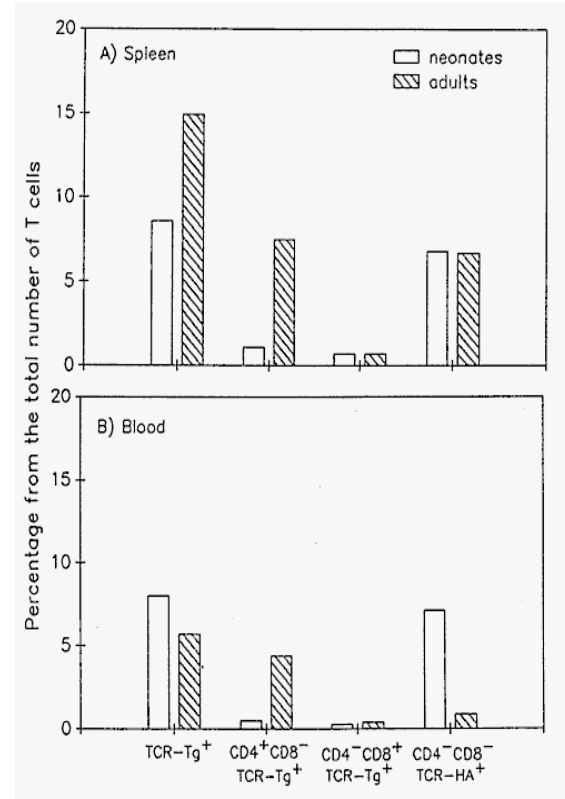

Figure 4. Frequency and immunophenotype of TCR-HA$^+$ T cells in neonatal and adult spleen (A) or peripheral blood (B) of transgenic mice. Samples were pooled from 3-10 mice in each group, T cells were separated and triple-staining FACS analysis was carried out using reagents specific for the TCR-HA receptor, CD4 and CD8 surface molecules. Results were expressed as percentage of cells in a given phenotypic group, relative to the total number of T cells.

immunodominant K^d epitope. Interestingly, immunization of newborn mice with NPV1 primed a strong CTL response rather than inducing unresponsiveness (17,71). CTL priming by neonatal NPV1 inoculation was demonstrated by: (a) increased CTL precursor (pCTL) frequency in the spleens of animals immunized with NPV1 and boosted with PR8 virus, compared to animals inoculated with virus or plasmid only (Fig.5); (b) significant cytotoxic activity specific for various type A influenza viruses as well as for the NP 147-155 immunodominant epitope of splenocytes from mice immunized as neonates or adults with NPV1 (17,71); (c) CD8$^+$ T cells from mice immunized as neonates with NPV1 secreted IFNgamma but no IL-4 when restimulated with NP 147-155 peptide, like those from adult animals immunized with live-virus (71); (d) T cells from animals immunized as newborns with NPV1 and boosted with live-virus displayed significantly enhanced proliferative abilities following antigenic stimulation, compared to
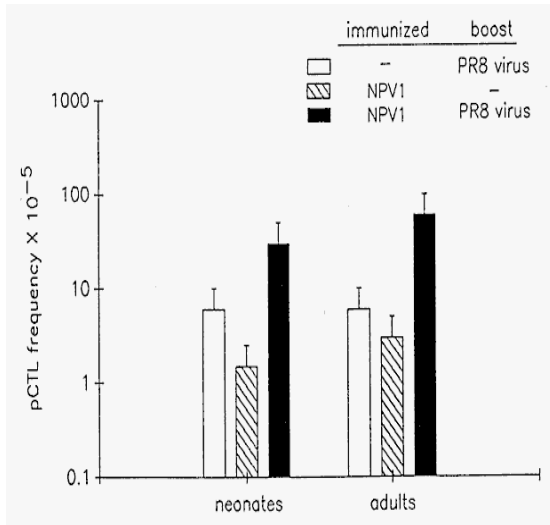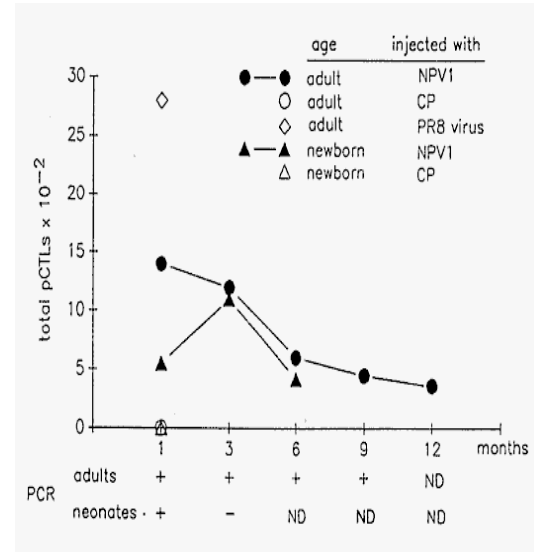
Figure 5. Frequency of the PR8 specific pCTLs in the spleen of mice immunized as newborns or adults with NPV1, at one month after the completion of immunization. Some mice were boosted with live-PR8 virus one week previous to pCTL estimation. Control mice were immunized only with live-virus one week before the sacrifice. The frequency of pCTLs was estimated by limiting dilution analysis. Results were expressed on a $\log_{10}$ scale, as mean of pCTL frequency x 10^{-5} in groups of 3-4 mice.

cells from animals immunized with virus only (71); (e) animals immunized as newborns or adults with NPV1 showed enhanced protection in terms of pulmonary virus titer reduction and survival, subsequent to lethal infection with PR8 (H1N1) or HK (H3N2) viruses (17,71).

Mice immunized as newborns with NPV1 displayed significant virus-specific CTL immunity at least 6 months after the completion of immunization (Fig.6 and ref.71). Interestingly, the kinetics of CTL induction was slower in the case of animals immunized with NPV1 as newborns, indicating that the expansion of the memory pool paralleled the development of the T cell repertoire. The plasmid as detected by PCR, persisted at least 1 month but not more than 3 months in mice inoculated at birth (Fig.6 and ref.71), indicating that the continuous antigenic exposure primed newly emerging T cells and expanded the memory pool.

Thus, the CTL response of neonates and adults to NPV1 were qualitatively identical, although some quantitative differences occurred. Whereas NP encodes immunodominant CTL epitopes, HA encodes the major B and Th epitopes of influenza virus (reviewed in ref.72). We investigated the T helper response of mice immunized as neonates or adults with pHA that expressed HA of WSN virus. It was already mentioned


Figure 6. Kinetics of the total number of PR8 specific pCTLs in spleens of animals immunized as newborns or adults with NPV1. The number of pCTLs was estimated from the frequency obtained by limiting dilution analysis and the total number of splenocytes. Results were expressed as mean of total pCTL/spleen x 10^{-2} in groups of 3-4 mice. Control mice were inoculated with the plasmid that lacks the NP open reading frame (CP) or with live PR8 virus, one week previous to the sacrifice. PCR results regarding the persistence of NPV1 at the site of injection were considered positive (+) if at least one mouse in a particular group was positive and negative (-) if no mouse was positive. Each PCR group consisted of 6-10 mice. ND - not done.

that pHA immunization induced HI antibodies specific for WSN virus, in animals inoculated as neonates or adults. The study of the isotypes profiles was carried out by RIA and showed: (a) the predominance of IgG_2 antibodies in mice immunized as neonates or adults with pHA, compared to adult mice immunized only with virus (Fig.7A and ref.44); (b) the predominance of IgG_2 antibodies in mice immunized as adults with pHA and boosted with WSN virus; (c) the predominance of IgG_1+IgG_3 antibodies in mice immunized as newborns with pHA and boosted with WSN virus.

These observations suggested that whereas pHA immunization of adult mice led to the development of Th1 cells, immunization of neonates was followed by a mixed Th1/Th2 response: the Th1 profile predominated in mice that were not exposed to WSN virus and the Th2 response predominated after the exposure to live WSN virus. The study of IFNgamma and IL-4 secretion by T cells from mice immunized as neonates or adults with pHA and boosted with live-virus, supported this hypothesis: while the T cells from mice immunized as adults produced significantly more IFNgamma
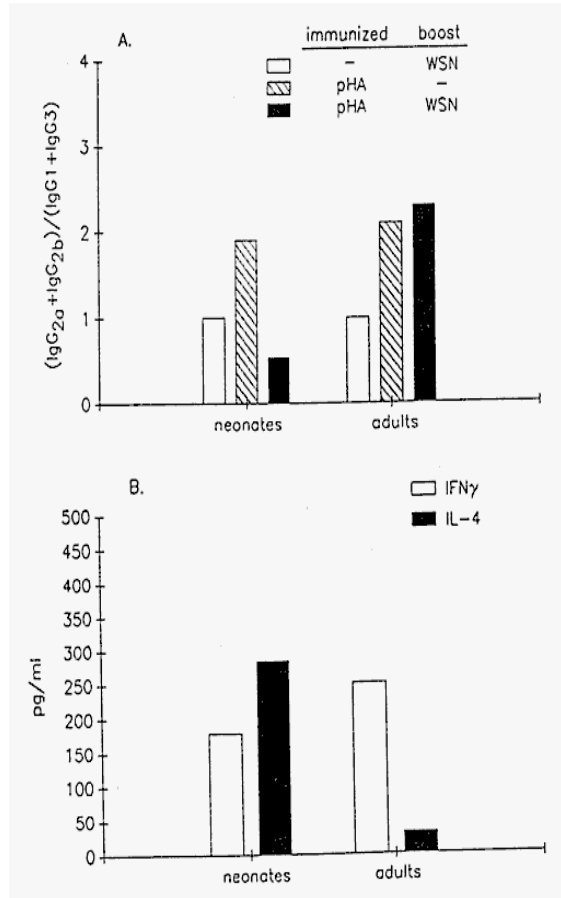
Figure 7. The helper profile of the WSN specific T lymphocytes primed by pHA inoculation of newborn or adult mice. A: The ratio between IgG$_2$ and IgG$_1$+IgG$_3$ WSN specific antibodies in the sera of mice immunized with pHA one month previously and boosted or not with the live-virus one week previous to the blood harvest. The concentration of the specific antibodies bearing a particular isotype was estimated by RIA using isotype-specific reagents. B: The concentration of IFNgamma and IL-4 in culture supernatants of WSN restimulated T cells, from mice immunized with pHA as newborns or adults and boosted with live-virus one week before the sacrification. The concentration of cytokines was assessed by ELISA and the results were expressed as means of triplicate estimations.

than IL-4, the T cells from mice neonatally immunized produced comparable amounts of IFNgamma and IL-4 (Fig.7B and ref.44). Thus, antigenic exposure during the early postnatal stage of development led to a qualitatively different Th response comprising virus-specific Th2 commited precursors, that differentiated to Th2 effector cells after restimulation with antigen.

There are two mutually exclusive scenarios that could define the kinetics of antigen exposure following the neonatal inoculation with plasmid vaccines: (a) plasmid inoculation leads to immediate and continous exposure of neonatal and post-neonatal T cells to the antigen or (b) the exposure occurrs later on, when the T cell repertoire is more developed. Three lines of evidence support the first scenario: (a) mice immunized with NPV1 at birth mounted enhanced CTL responses when boosted with virus three weeks later (Fig.5 and refs.17,71); (b) the Th response of mice immunized as neonates with pHA and boosted with virus 1 month later is 'newborn-like', comprising a significant Th2 component (Fig.7 and ref.44); (c) it was previously shown that the protein synthesis and antigen accumulation begins after a short lag-interval of 24-72 hours following the intracellular delivery of the expression vectors by various means (40,73-75). Thus, it is more likely that an early continuous exposure to low doses of antigens occurred following neonatal inoculation with mammalian expression vectors. A consequence of this model would be that increasing the exposure of the neonatal immune system to antigens expressed by plasmids leads to the induction of unresponsiveness. In spite of the fact that we have not encountered such a phenomenon in the case of plasmids expressing influenza proteins that were inoculated in a wide dose-range (10ug - 100ug/dose), another group noted in certain circumstances neonatal tolerance following the inoculation of plasmids expressing the circumsporozoite antigen of the malaria parazite (D.M. Klinman, personal communication). This observation supports the model of early antigen exposure following neonatal immunization with plasmids, but suggests that there are some important parameters that may determine whether the immunization is effective or not: (a) the efficacy of transcription and the adjuvant-like properties (76,77) of the vector; (b) the route, schedule and dose of inoculation; (c) the genetic background of the animal; (d) the type of protein encoded by the expression vector (i.e. secreted or cellular); etc.. In contrast to adults, increasing the vaccine dose or the transcriptional efficacy of the vector may lead to rather deleterious effects in neonates, like T cell unresponsiveness. However, the tremendous efficiency of DNA immunization in generating Th1 immune responses not only in adults but to a certain extent in neonates as well (Fig.7 and ref.44), correlates well with both the notion that a continued stimulation with antigen must occurr in order to maintain the T cell expression of the beta-2 chain of IL-12R (78,79) and that bacterial DNA displays Th1 promoting adjuvant properties (76).

In conclusion, the data regarding neonatal immunization with naked DNA support the concept that functionally mature T cells are present during the early period of life, but do not rule out possible qualitative differences between the dominant populations of peripheral T cells in neonates and adults, respectively. Thus, it becomes clear that the classical view of self-nonself discrimination as consequence of neonatal tolerance (1-3) has restricted validity. Instead, neonatal lymphocytes are able to
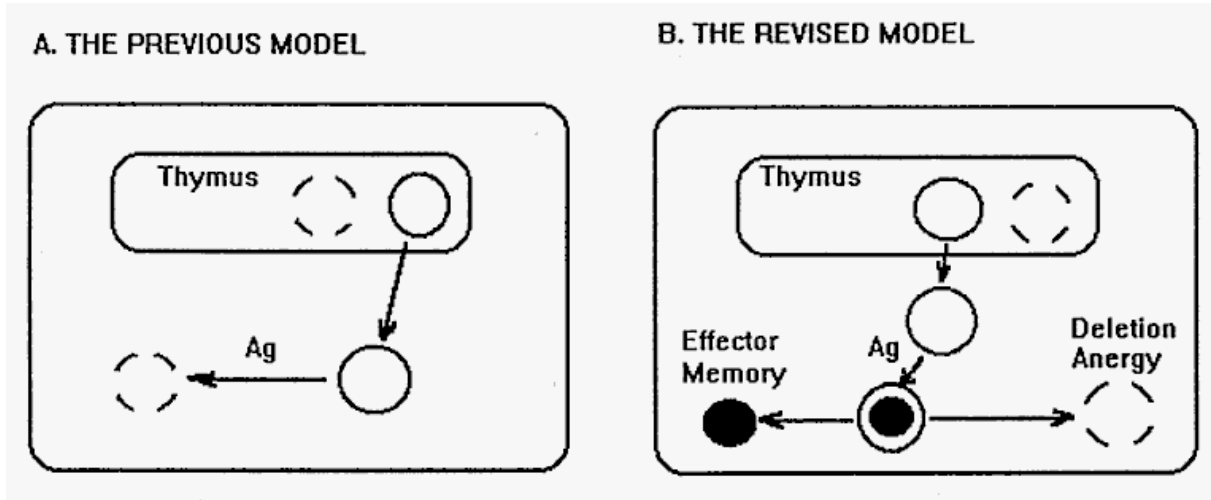
Figure 8. Two alternative models addressing the neonatal responsiveness of peripheral T cells. A: The classical model views the inability of the neonatal T cells to respond to antigens as the basis of the 'learned' process of self-nonself discrimination. This would be accomplished by the deletion of all T cells that encounter their antigens during the neonatal time-window. B: The revised model takes into account the ability of neonatal T cells to mount immune responses in certain circumstances. The inactivation of autoreactive T cells that escape into periphery would be accomplished by 'downstream' mechanisms mediated through Fas/Fas-L, IL-2/IL-2R and B7/CTLA-4 interactions.

mount effector and memory responses in certain conditions. It can be inferred from these data that some forms of self-nonself discrimination must follow the T cell priming events. Indeed, besides the central tolerance that occurs in the thymus (10), peripheral tolerance assures that autoreactive T cells which escaped in the periphery (reviewed in ref.80) are eventually deleted or anergized. The best evidence to support such a model is that animals deficient in Fas/Fas ligand, IL-2 or CTLA-4 (81-83), develop early in their postnatal life fatal autoimmune diseases. A model that assimilates the responsiveness of the neonatal T cells and the self-nonself discrimination mechanisms, is schematically shown in Figure 8.

In essence, an important idea emerged from these studies, namely that there are potentially effective immunization strategies, that do not require a live or live-attenuated vector, yet they are still able to induce broad humoral and cellular responses even during the earliest stages of postnatal development.

5. PERSPECTIVE: TOWARDS THE DEVELOPMENT OF A NEW GENERATION OF EFFICIENT VACCINES FOR INFANTS

Lower responsiveness to antigens and high susceptibility to tolerance are the two main characteristics of the immune response of neonates. Earlier and more recent studies showed that in certain conditions, the immunization of newborns led to protective humoral and cellular responses. This is in sharp contrast to the previous paradigm that the neonatal lymphocytes inevitably become tolerant subsequent to the antigen exposure. Recent studies suggested that: (a) the classical 'neonatal tolerance' is in fact a shifted response that comprises mainly the Th2 cell induction; (b) protective responses can be obtained following neonatal immunization by decreasing the dose and prolonging the exposure to antigens, or by co-injecting certain adjuvants.

Our experimental results suggest that naked DNA vaccines can have potential broad benefits as immunization strategy for neonates and young adults. The magnitude of the immune responses obtained by vaccination with plasmids encoding individual proteins of influenza virus, was lower than in that observed with the live-virus immunization. A method to enhance the efficacy of plasmid immunization, is to associate in the same vaccine formulation, multiple epitopes with synergic effects on the magnitude and protectivity of the immune response. In light of the latest developments in the field of neonatal immune responsiveness, this would be a better strategy than to merely increase the dose of antigens. Immunization of newborn mice with a mixture of plasmids encoding NP and HA proteins of influenza virus, confered complete protection upon later challenge with a 100% lethal dose (Table 3 and manuscript in preparation).

We can explain this synergy in the following way: first, Th epitopes encoded by the HA plasmid enhanced the generation of CTLs and second, neutralizing antibodies, cytokine producing T cells and cross-reactive CTLs against type A influenza viruses participated together in the response to the infection. Thus, neonatal immunization with naked DNA has the ability to induce broad and protective immune responses.

Table 3. Protection against lethal challenge with $1LD_{100}$ of A/WSN/33 influenza virus, by neonatal immunization with plasmids encoding HA of WSN and NP of the drift variant, A/PR8/34 influenza virus

Age of immunization	Vaccine[a]					
	-	ctrl. plasmid	WSN virus	NPV1	pHA	NPV1+pHA
Adult	0/17[b]	0/7	13/13	0/4	4/7	5/5
Neonate	0/10	0/7	0/3[c]	0/9	5/12	10/10

[a] Mice were injected according to the schedule depicted in Fig.1, with 50 µg individual plasmid/dose when inoculated separately or 25 µg+25 µg of each when administered in mixtures;
[b] Mice were challenged by aeorsol infection at 5 weeks after the completion of immunization; results were expressed as surviving/total number of mice in a given group;
[c] Since the inoculation of live WSN virus is lethal for BALB/c newborn mice, they were immunized with UV-attenuated WSN virus.

The naked DNA vaccines have important advantages like stability, low-cost and the ability to generate CTL responses in the absence of potential side-effects due to the injection of live-attenuated vaccines. The long persistence of the plasmid associated with continous stimulation of the lymphocytes may lead to good memory responses and Th1 induction. Furthermore, neonatal immunization with naked DNA may bypass the inhibitory effect of the specific antibodies of maternal origin on the generation of broad immune responses following the vaccination with classical vectors. Due to this particular reason combined with direct side-effects of some formulations, most of the present vaccination schedules are carried out later during the postnatal life.

In conclusion, plasmid vaccination may be a potentially useful strategy of neonatal immunization against common pathogens that cause major infectious diseases in newborns and infants, such as Hepatitis B virus, Herpes simplex virus, HIV, Rotaviruses, Plasmodia, Ortho and Paramyxoviruses, etc..

6. ACKNOWLEDGMENTS

7. REFERENCES

1. R. Billingham, L. Brent & P. Medawar: Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance. *Proc Roy Soc Lond* 239, 44-5 (1956)

2. R.D. Owen: Immunological tolerance. *Fed Proc* (Balt.) 16, 581-91 (1958)

3. M. Burnet: Immunological tolerance and related phenomena. In: *The clonal selection theory of acquired immunity.* The Abraham Flexner Lectures, Vanderbilt Univ. Press, Nashville, TN (1959)

4. C. Fernandez & G. Moller: Immunological unresponsiveness to native dextran B512 in young animals of dextran high-responder strains is due to lack of Ig-receptor expression. *J Exp Med* 147, 645-55 (1978)

5. C. Bona, R. Lieberman, C.C. Chien, J. Mond, S. House, I. Green & W.E. Paul: Immune response to levan. I. Kinetics and ontogeny of anti-levan and anti-inulin antibody response and expression of cross-reactive idiotype. *J Immunol* 120, 1436-42 (1978)

6. A. Nisonoff & S.A. Bangasser: Immunological suppression of idiotypic specificities. *Transpl Rev* 27, 100-34 (1975)

7. S. Dray: Effect of maternal isoantibodies on the quantitative expression of two allelic genes controlling gamma-globulin allotypic specificities. *Nature* (London) 195, 677-80 (1962)

8. A.R. Lawton, R. Asofsky, M. Hylton & M.D. Cooper: Suppression of immunoglobulin class synthesis in mice. I. Effects of treatment with antibody to mu-chain. *J Exp Med* 135, 277-97 (1972)

9. H.M. Etlinger & J.M. Chiller: Maturation of the lymphoid system. *J Immunol* 122, 2564-69 (1979)

10. P. Kisielow, H. Bluthman, U.D. Staerz, M. Steinmetz & H. von Boehmer: Tolerance in T cell receptor transgenic mice involves deletion of non-mature CD4$^+$CD8$^+$ thymocytes. *Nature* 333, 742-46 (1988b)

11. S.B. Hartly, R. Croslic, R. Brink, A.B. Kantor, A. Basten & C.C. Goodnow: Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens. *Nature* (London) 353, 765-69 (1991)

12. Q.D. Sun, M. Gots, G. Meyermann & H. Wekerle: Resistance to experimental autoimmune encephalomyelitis induced by neonatal tolerization to myelin basic protein: clonal elimination vs. regulation of autoagressive lymphocytes. *Eur J Immunol* 19, 373-80 (1989)

13. J.P. Clayton, G.M. Gammon, D.G. Ando, D.H. Kono, L. Hood & E.E. Sercarz: Peptide-specific prevention of experimental allergic encephalomyelitis. *J Exp Med* 169, 1681-89 (1989)

14. J.S. Petersen, A.E. Karlsen, H. Markholst, A. Worsaae, T. Dyrberg & B. Michelsen: Neonatal tolerization with glutamic acid decarboxylase but not bovine serum albumin delays the onset of diabetes in NOD mice. *Diabetes* 43, 1478-84 (1994)

15. R.R. Singh, B.H. Hahn & E.E. Sercarz: Neonatal peptide exposure can prime T cells and upon subsequent immunization induce their immune deviation: implications for antibody vs T cell-mediated autoimmunity. *J Exp Med* 183, 1613-21 (1996)

16. K.M. Garza, N.D. Griggs & K.S.K. Tung: Neonatal injection of an ovarian peptide induces autoimmune ovarian disease in female mice: requirement of endogenous neonatal ovaries. *Immunity* 6, 89-96 (1997)

17. A. Bot, S. Bot, A. Garcia-Sastre & C. Bona: DNA immunization of newborn mice with a plasmid expressing nucleoprotein of influenza virus. *Viral Immunol* 9, 207-10 (1996)

18. T. Forsthuber, H.C. Yip & P.V. Lehman: Induction of Th1 and Th2 immunity in neonatal mice. *Science* 271, 1728-31 (1996)

19. M. Sarzotti, D.S. Robbins & P.M. Hoffman: Induction of protective CTL responses in newborn mice by a murine retrovirus. *Science* 271, 1726-8 (1996)

20. J.P. Ridge, E.J. Fuchs & P. Matzinger: Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. *Science* 271, 1723-6 (1996)

21. J.G. Howard & C. Hale: Lack of neonatal susceptibility to induction of tolerance by polysaccharide antigens. *Eur J Immunol* 6, 486-92 (1976)

22. C. Fernandez, L. Hammarstrom, G. Moller, D. Primi & J.E. Smith: Immunological tolerance affects only a subpopulation of the antigen-specific B lymphocytes: evidence against clonal deletion as the mechanism of tolerance induction. *Immunol Rev* 43, 3-41 (1979)

23. C. Bona: Sequential activation of V genes during postnatal life. *Amer J Repr Immunol* 1, 35-9 (1980)

24. C. Bona: Modulation of immune responses by Nocardia immunostimulants. *Prog Allergy* 26, 97-102 (1979)

25. N.H. Sigal, A.R. Pickard, E.S. Metcalf, P.J. Gearhart & N.R. Klinman: Expression of phosphorylcholine-specific B cells during murine development. *J Exp Med* 146, 933-48 (1977)

26. N.B. Nutt, A.N. Wiesel & A. Nisonoff: Neonatal expression of the cross-reactive idiotype associated with anti-phenyl arsonate antibodies in strain A mice. *Eur J Immunol* 9, 864-8 (1979)

27. H.C. Morse III, B. Prescott, S.S. Cross, P.W. Stashak & P.J. Baker: regulation of the antibody response to type II pneumococal polysaccharide V. Ontogeny of factors influencing the magnitude of the PFC response. *J Immunol* 16, 274-88 (1976)

28. D.G. Osmond: Population dynamics of bone marrow B lymphocytes. *Immunol Rev* 93, 103-24 (1986)

29. J. Teale, J.E. Layton & G.J.V. Nossal: *In vitro* model for natural tolerance to self antigens. *J Exp Med* 150, 205-12 (1979)

30. J.C. Cambier, J.R. Kettman, E.S. Vitetta & J.W. Uhr: Differential susceptibility of neonatal and adult murine spleen cells to *in vitro* induction of B cell tolerance. *J Exp Med* 144, 293-7 (1976)

31. J.R. Kettman, J.C. Cambier, J.W. Uhr, F. Lipler & E.S. Vitetta: The role of receptor IgM and IgD in determining triggering and induction of tolerance in murine B cells. *Immunol Rev* 43, 69-95 (1979)

32. A.J. Yellen, W. Glenn, V.P. Sukhatme, X. Cao & J.G. Monroe: Signaling through surface IgM in tolerance-susceptible immature murine B lymphocytes: Developmentally regulated differences in transmembrane signaling in splenic B cells from adult and neonatal mice. *J Immunol* 146, 1446-54 (1991)

33. R.J. Wechsler & J.G. Monroe: Immature B lymphocytes are deficient in expression of the src-family

kinases p59[fyn] and p55[fgr1]. *J Immunol* 154, 1919-29 (1995)

34. R. Brink, C.C. Goodnow, J. Crosbie, E. Adams, J. Eris, D.V. Mason, S.B. Hartleg & A. Basten: Immunoglobulin M and D antigen receptors are both capable of mediating B lymphocyte activation, deletion or anergy after interaction with specific antigen. *J Exp Med* 176, 991- 1005 (1992)

35. R. Carsetti, G. Kohler & M.C. Lammers: Transitional B cells are the target of negative selection in B cell compartemnt. *J Exp Med* 181, 2129-40 (1995)

36. A. Durandy, G. De Saint Basile, B. Lisowska-Grospierre, J.-F. Gauchat, M. Forveille, R.A. Kroczek, J.-Y. Bonnefoy & A. Fischer: Undetectable CD40 ligand expression on T cells and low B cell responses to CD40 binding agonists in human newborns. *J Immunol* 154, 1560-8 (1995)

37. A. Aruffo, M. Farrington, D. Hollenbaugh, X. Li, A. Milatovich, S. Nonoyama, J. Bajorath, L.S. Grosmaire, R. Stenkamp, M. Neubaer, R.L. Roberts, R.J. Noelle, J.A. Ledbetter, U. Francke & H.D. Ochs: The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper IgM syndrome. *Cell* 72, 291-300 (1993)

38. I.S. Grewal, J. Xu & R. Flavell: Impairment of antigen-specific T cell priming in mice lacking CD40 ligand. *Nature* 378, 617-20 (1995)

39. Y. Yang & J.M. Wilson: CD40 ligand-dependent T cell activation: requirement of B7-CD28 signaling through CD40. *Science* 273, 1862-4 (1996)

40. D.L. Montgomery, J.W. Shiver, K.R. Leander, H.C. Perry, A. Friedman, D. Martinez, J.B. Ulmer, J.J. Donnelly & M.A. Liu: Heterologous and homologous protection against influenza A by DNA vaccination: optimization of DNA vectors. *DNA Cell Biol* 12, 777-83 (1983)

41. E.F. Fynan, H.L. Robinson & R.G. Webster: Use of DNA encoding influenza hemagglutinin as an avian influenza vaccine. *DNA Cell Biol* 12, 785-9 (1993)

42. E.F. Fynan, R.G. Webster, D.H. Fuller, J.R. Haynes, J.C. Santoro & H.L. Robinson: DNA vaccines: protective immunization by parental, mucosal and gene-gun inoculations. *Proc Natl Acad Sci USA* 90, 11478-82 (1983)

43. J.J. Donnelly, A. Friedman, D. Martinez, D.L. Montgomery, J.W. Shiver, S.L. Motzel, J.B. Ulmer & M.A. Liu: Preclinical efficacy of a prototype DNA vaccine: enhanced protection against antigenic drift in influenza virus. *Nature Medicine* 1, 583-7 (1995)

44. A. Bot, S. Antohi, S. Bot, A. Garcia-Sastre & C. Bona: Induction of humoral and cellular immunity against influenza virus by immunization of newborn mice with a plasmid bearing a hemagglutinin gene. (submitted)

45. M.P. Cancro, D.E. Wylie, W. Gerhard & N.R. Klinman: Patterned acquisition of the antibody repertoire: Diversity of the hemagglutinin specific B cell repertoire in neonatal BALB/c mice. *Proc Natl Acad Sci USA* 76, 6577-81 (1979)

46. L.J. Rubinstein, M.Yeh & C. Bona: Idiotype-antiidiotype network. II. Activation of silent clones by treatment at birth with idiotypes is associated with the expansion of idiotype-specific helper T cells. *J Exp Med* 156, 506-21 (1982)

47. P. Matzinger: Tolerance, danger and the extended family. *Annu Rev Immunol* 12, 991-1045 (1994)

48. E.J. Fuchs & P. Matzinger: B cells turn off virgin but not memory T cells. *Science* 258, 1156-9 (1992)

49. M.G. Otsyula, C.J. Miller, A.F. Tarantal, M.L. Marthas, T.P. Greene, J.R. Collins, K.K. van Rompay & M.B. McChesney: Fetal or neonatal infection with attenuated Simian immunodeficiency virus results in protective immunity against oral challenge with pathogenic SIVmac[251]. *Virology* 222, 275-8 (1996)

50. C.Y. Lu, E.G. Calamai & E.R. Unanue: A defect in the antigen presentation function of macrophages from neonatal mice. *Nature* 282, 327-9 (1979)

51. C.Y. Lu, D.Y. Beller & E.R. Unanue: During ontogeny, Ia - bearing accessory cells are found early in the thymus but late in the spleen. *Proc Natl Acad Sci USA* 77, 1597-1601 (1980)

52. C.Y. Lu: The delayed ontogenesis of Ia - positive macrophages: implications for host-defense and self-tolerance in the neonate. *Clin Invest Med* 7, 263-7 (1984)

53. J.F. Morris, J.T. Hoyer & S.K. Pierce: Antigen presentation for T cell interleukin 2 secretion is a late acquisition of neonatal B cells. *Eur J Immunol* 22, 2923-8 (1992)

54. B. Adkins & K. Hamilton: Freshly isolated, murine neonatal T cells produce IL-4 in response to anti-CD3 stimulation. *J Immunol* 149, 3448-55 (1992)

55. B. Adkins, A. Ghanei & K. Hamilton: Developmental regulation of IL-4, IL-2 and IFNg production by murine peripheral T lymphocytes. *J Immunol* 151, 6617-26 (1993)

56. B. Adkins, K. Chun, K. Hamilton & M. Nassiri: Naive murine neonatal T cells undergo apoptosis in

response to primary stimulation. *J Immunol* 157, 1343-50 (1996)

57. C. Barrios, P. Brawand, M. Berney, C. Brandt, P.-H. Lambert & C.-A. Siegrist: Neonatal and early life immune responses to various forms of vaccine antigens qualitatively differ from adult responses: predominance of a Th2-biased pattern which persists after adult boosting. *Eur J Immunol* 26, 1489-93 (1996)

58. F. Ramsdell, M. Jenkins, Q. Dinh & B.J. Fowlkes: The majority of CD4$^+$CD8$^-$ thymocytes are functionally immature. *J Immunol* 147, 1779-85 (1991)

59. H. Hosseinzadeh & I. Goldschneider: Recent thymic emigrants in the rat express a unique antigenic phenotype and undergo post-thymic maturation in peripheral lymphoid tissues. *J Immunol* 150, 1670-9 (1993)

60. A. Bonomo, P.J. Kehn & E.M. Shevach: Premature escape of double positive thymocytes to the periphery of young mice: possible role in autoimmunity. *J Immunol* 152, 1509-14 (1993)

61. T. Sornasse, P.V. Larenas, K.A. Davis, J.E. de Vries & H. Yssel: Differentiation and stability of Th1 and 2 cells derived from naive human neonatal CD4+ T cells analyzed at the single cell level. *J Exp Med* 184, 473-81 (1996)

62. M. Kamanaka, P. Yu, T. Yasui, K. Yoshida, T. Kawabe, T. Horii, T. Kishimoto & H. Kikutami: Protective role of CD40 in Leishmania major infection at two distal phases of cell-mediated immunity. *Immunity* 4, 275-81 (1996)

63. B. Adkins, A. Ghanei & K. Hamilton: Up-regulation of murine neonatal T helper cell responses by accessory cell factors. *J Immunol* 153, 3378-85 (1994)

64. J. Kirberg, A. Baron, S. Jakob, A. Rolink, K. Karjalainen & H. von Boehmer: Thymic selection of CD8 single positive cells with a class II major histocompatibility complex restricted receptor. *J Exp Med* 180, 25-34 (1994)

65. L. Bruno, H. von Boehmer & J. Kirberg: Cell division in the compartment of naive and memory T lymphocytes. *Eur J Immunol* 26, 3179-84 (1996)

66. A. Bot, A. Reichlin, H. Isobe, S. Bot, J. Schulman, W.M. Yokoyama & C. Bona: Cellular mechanisms involved in protection and recovery from influenza virus infection in immunodeficient mice. *J Virol* 70, 5668-72 (1996)

67. P.M. Taylor & B.A. Askonas: Influenza nucleoprotein-specific T cell clones are protective *in vivo*. *Immunology* 58, 417-20 (1986)

68. D.J. Topham, R.A. Tripp, S.R. Sarawar, M.Y. Sangster & P.C. Doherty: Immune CD4+ T cells promote the clearance of influenza virus from major histocompatibility complex class II$^{-/-}$ respiratory epithelium. *J Virol* 70, 1288-91 (1996)

69. M.B. Graham, V.L. Braciale & T.J. Braciale: Influenza virus-specific CD4$^+$ T helper type 2 T lymphocytes do not promote recovery from experimental virus infection. *J Exp Med* 180, 1273-82 (1994)

70. J.B. Ulmer, J.J. Donnely, S.E. Parker, G.H. Rhodes, P.L. Felgner, V.J. Dwarki, S.H. Gromkowski, R.R. Reck, C.M. DeWitt, A. Friedman, L.A. Hawe, K.R. Leander, D. Martinez, H.C. Perry, J.W. Shiver, D.L. Montgomery & M.A. Liu: Heterologous protection against influenza virus infection by injection of DNA encoding a viral protein. *Science* 259, 1745-9.

71. A. Bot, S. Bot, A. Garcia-Sastre: Protective cellular immunity against influenza virus induced by plasmid inoculation of newborn mice. (submitted).

72. A. Bot: Immunoglobulin deficient mice generated by gene targeting as models for studying the immune responses. *Intern Rev Immunol* 13, 327-40 (1996)

73. M.D. Eisenbraun, D. Heydenburg Fuller & J.R. Haynes: Examination of parameters affecting the elicitation of humoral immune responses by particle bombardment-mediated genetic immunization. *DNA Cell Biol* 12, 791-7 (1993)

74. M. Yokoyama, J. Zhang & J.L. Whitton: DNA immunization confers protection against lethal lymphocytic choriomeningitis virus infection. *J Virol* 69, 2684-8 (1995)

75. L.P. Martins, L.L. Lau, M.S. Asano & R. Ahmed: DNA vaccination against persistent viral infection. *J Virol* 69, 2574-82 (1995)

76. D.M. Klinman, A.-K. Yi, S.L. Beaucage, J. Conover & A.M. Krieg: CpG motifs present in bacterial DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12 and interferon-gamma. *Proc Natl Acad Sci USA* 93, 2879-83 (1996)

77. A.M. Krieg, A.-K. Matson, T.J. Waldschmidt, G.A. Bishop, R. Teasdale, G.A. Koretzky & D.M. Klinman: CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374, 546-9 (1995)

78. L. Rogge, L. Barberis-Maino, M. Biffi, N. Passini, D.H. Presky, U. Gubler & F. Sinigaglia: Selective expression of an IL-12 receptor component by human Th1 cells. *J Exp Med* 185, 825-31 (1997)

79. S.J. Szabo, A.S. Dighe, U. Gubler & K.M. Murphy: Regulation of the IL-12R beta-2 subunit expression in developing Th1 and Th2 cells. *J Exp Med* 185, 817-24 (1997)

80. J.A. Bluestone: Is CTLA-4 a master switch for peripheral T cell tolerance? *J Immunol* 158, 1989-93 (1997)

81. L. van Parijs, A. Ibraghimov & A.K. Abbas: The roles of costimulation and Fas in T cell apoptosis and peripheral tolerance. *Immunity* 4, 321-8 (1996)

82. B. Sadlack, J. Lohler, H. Schorle, G. Klebb, H. Haber, E. Sickel, R.J. Noelle & I. Horak: generalized autoimmune disease in interleukin 2 deficient mice is triggered by an uncontrolled activation and proliferation of CD4+ T cells. *Eur J Immunol* 25, 3053-9 (1995)

83. E.A. Tivol, F. Boriello, A.N. Schweitzer, W.P. Lynch, J.A. Bluestone & A.H. Sharpe: Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical regulatory role of CTLA-4. *Immunity* 3, 541-7 (1995)