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DNA IMMUNIZATION OF NEWBORN MICE WITH PLASMIDS EXPRESSING INFLUENZA VIRUS PROTEINS

BY

ADRIAN I. BOT

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1998

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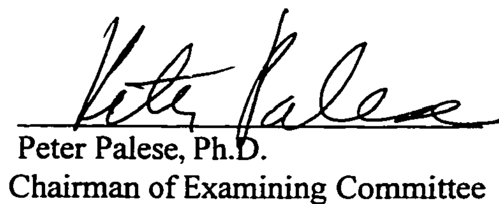

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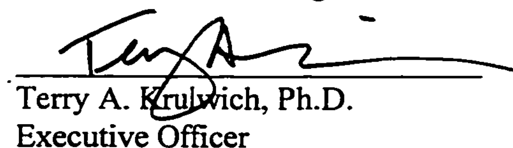

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ABSTRACT

DNA IMMUNIZATION OF NEWBORN MICE WITH PLASMIDS EXPRESSING INFLUENZA VIRUS PROTEINS

by

Adrian I. Bot

Adviser: Professor Constantin A. Bona

The effective immunization of neonates and infants is an essential goal for the human vaccination. Neonates and infants are more difficult to immunize since their immune system is not mature and they are more susceptible to tolerance induction.

We have investigated the plasmid-based immunization as a potential strategy for neonatal vaccination, using the preclinical model of the immune response in BALB/c mice to Influenza virus antigens. Newborn mice injected with three different plasmids expressing whole HA and NP antigens, or HA-derived dominant B and T cell epitopes developed significant virus-specific cross-reactive CTL, Th and B cell immunity. The lack of tolerance induction subsequent to neonatal inoculation of plasmid-based expression vectors, may be due to the continuous exposure of the peripheral lymphocytes to low doses of foreign antigen, associated with the adjuvant effect of the bacterial DNA. The neonatal plasmid-based vaccination elicited immune responses against four type-A Influenza virus strains, of different subtypes. The immune response was protective as demonstrated by the survival rate and/or pulmonary virus clearance subsequently to the infection with three different mouse-adapted strains of Influenza virus. The protection ability of the plasmid-based

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newborn immunization was significantly enhanced by the co-inoculation of plasmids expressing HA and NP. The naked DNA immunization of newborn mice displayed two advantages as compared to the inactivated vaccine: first, the plasmid immunization elicited cross-reactive CTLs and secondly, it was protective, in sharp contrast to the UV-inactivated virus that induced immune unresponsiveness.

The study of the relationship between the maternal immunity and the immune responsiveness of the offsprings, suggested that the plasmid-based immunization may circumvent to a certain extent the inhibitory effect of the maternal antibodies. Furthermore, rather than inducing central tolerance, the maternal plasmid-based immunization elicited protective antibodies that were transmitted to the progeny.

Experiments addressing the mechanism of protection against Influenza virus following the naked DNA immunization, suggested the involvement of *in vivo* transfected APC as well as of cytokines like IFNγ and IL-4.

Together, our results pinpoint to a potential application for the plasmid-based immunization of infants, against microbes responsible for infectious diseases with high morbidity and mortality in the young population.

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INTRODUCTION

A. Neonatal responsiveness

A properly operating immune system enables an organism to distinguish the antigens associated with invaders ('non-self antigens') from those associated with the organism itself ('self antigens').

Early studies showed that fetal and neonatal exposure to alloantigens was followed by immunological unresponsiveness. First, newborn animals injected with allogeneic hematopoietic cells failed to reject subsequent skin grafts (Billingham *et al.* 1956). Secondly, genetically non-identical bovine twins that shared placenta failed to reject allogeneic cells as adults (Owen 1958). Based on these observations, it was proposed that the ability of the immune system to distinguish between self and non-self results from the deletion of the self reactive clones during the fetal and neonatal stage of development (Burnet 1959). More recent studies underlining the susceptibility of the neonates to antigen-induced tolerance, supported this paradigm of self-nonself discrimination. Thus, neonatal inoculation of polysaccharides was followed by specific B cell unresponsiveness (Fernandez and Moller, 1978; Bona *et al.* 1978). Further, neonatal inoculation of T-dependent antigens induced specific tolerance (Etlinger and Chiller, 1979). Recent data obtained in transgenic animals showed that thymic exposure to foreign or self antigens resulted in the deletion of specific T cell precursors (Kisielow *et al.* 1988; Zinkernagel *et al.* 1991). Similarly, the exposure of immature B cells from

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B cell-receptor transgenic mice, to membrane-bound or soluble antigens results in deletion and anergy respectively, of the specific precursors (Hartly *et al.* 1991).

Two categories of data contributed to the revision of the above illustrated paradigm of self-nonself discrimination, as entirely based on neonatal tolerance. First, an increasing body of evidence suggested that under certain conditions, the newborns mounted immune responses. For example, the neonatal inoculation of minute amounts of bacterial polysaccharides was followed by a humoral response as well as lack of tolerance induction (Howard and Hale, 1976). Later, a few studies confirmed the ability of newborns to mount antibody responses to T-independent as well as T-dependent antigens and underlined the stepwise nature of the B cell repertoire acquisition (Sigal *et al.* 1977; Nutt *et al,* 1979; Bona, 1980). Recent studies suggested that the T cell unresponsiveness subsequent to neonatal exposure to moderate or high doses of antigens was not complete, since Th2 recall responses could be demonstrated (Forsthuber *et al.* 1996; Singh *et al.* 1996). However, these recall responses were characterized by impaired proliferation, explaining in part the previously encountered difficulty to demonstrate them. Furthermore, lower doses of antigens inoculated into neonates were shown to induce Th1 and CTL responses rather than Th2 responses (Sarzotti *et al.* 1996, Forsthuber *et al.* 1996). Presentation of the antigen by professional antigen presenting cells (APC) like dendritic cells (DC), was followed by the priming of immune responses rather than neonatal tolerance induction (Ridge *et al.* 1996). Thus, neonates are able, in certain circumstances, to mount humoral and cellular immune responses. This conclusion is strengthened by the fact that human newborns and infants

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mount immune responses following inoculation with Bacille Calmette-Guerin (BCG), oral poliovirus vaccine or hepatitis B virus (HBV) vaccine (reviewed by Bona and Bot, 1997).

The second category of data that contributed to the revision of the previous paradigm of self-nonself discrimination as consequence of neonatal tolerance, came from the study of transgenic animals lacking molecules involved in T cell signaling. Thus, mice deficient in CTLA-4, Fas or IL-2 develop early in their postnatal life fatal autoimmune diseases (Tivol *et al.* 1995; van Parijs *et al.* 1996; Sadlack *et al.* 1995). These studies showed that the mechanisms assuring down-regulation of the T cell responses to foreign antigens, as well as peripheral tolerance to self antigens not expressed in the thymus, are crucial for preventing autoimmunity (reviewed by Bluestone, 1994). In contrast and surprisingly, animals exhibiting defects in central negative selection, like certain T cell receptor (TCR) transgenic mice bred onto self Ag-bearing hosts, did not manifest overt autoimmunity or graft-versus-host syndrome (Rocha et al. 1993). Together, these studies indicate that the process of self-nonself discrimination at the level of T cells is the result of a stepwise restriction in the repertoire of lymphocytes, beginning with the thymic negative selection that predominantly takes place during the fetal and neonatal period and continuing with the peripheral tolerance after the priming events occurred, throughout the biological life. Thus, self-nonself discrimination is not solely due to the tolerance induction by antigens encountered during the neonatal period. Furthermore, the neonates are able to mount immune responses in certain circumstances, as mentioned above.

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The newborns mount poor antibody responses, in particular to T-independent antigens and are more susceptible to B and T cell tolerance induction. The differences between the immune responsiveness of neonates and adults, can be explained by taking into consideration both quantitative and qualitative factors (Bot *et al.* 1997c).

The neonates are characterized by a predominance of immature B cells with the phenotype sIgM^{+} sIgD^{-}, that undergo anergy or deletion subsequent to the engagement of the Ig receptors by soluble or membrane-bound antigens, respectively (Cambier *et al.* 1976; Teale *et al.* 1979). Recent studies showed that a large part of the peripheral B cells of newborns are recent emigrants from bone-marrow that retain the sensitivity to antigen-mediated negative selection and are sIgM^{+} sIgD^{+} (Carsetti *et al.* 1995). The poor B cell responsiveness of the neonates can be explained by the differences regarding the Ig-receptor signal transduction in the immature versus mature B cells, rather than the inability to receive T help or the differential expression of isotypes (Monroe, 1996). Studies that showed differences regarding the expression of *fyn* and *fgr* - *src* family tyrosine kinases (Wechsler and Monroe, 1995), as well as the inositol phospholipid signaling pathway in immature versus mature B cells (Yellen *et al.* 1991), supported this model. However, there are few mature B cells in the periphery of newborns that can be primed to become plasma or memory cells subsequent to the inoculation of certain antigens (reviewed in Bot *et al.* 1997c).

A large body of phenotypic and functional studies suggest that most of the peripheral T cells of neonates are immature. Thus, most of the peripheral T cells from newborn rodents display an immature phenotype as well as low responsiveness to policlonal

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stimulators (Ramsdell *et al.* 1991; Hosseinzadeh and Goldschneider, 1993; Bonomo *et al.* 1993). Functionally, the peripheral T cells from the newborn mice display a strong tendency to produce Th2 cytokines and impaired ability to secrete Th1 cytokines upon *in vitro* stimulation with mitogens (Adkins and Hamilton, 1992; Adkins *et al.* 1993) or antigens, even in the presence of professional APC (Bot *et al.* 1997c). This reflects into the tendency of neonates to mount Th2 responses that are refractory to a subsequent switch toward Th1 responses (Barrios *et al.* 1996). Furthermore, a recent study indicated that the peripheral T cells from neonates are more susceptible to apoptosis than the adult counterparts (Adkins *et al.* 1996).

Similar findings regarding the immaturity of the peripheral T cells as well as the tendency to produce Th2 cytokines, were also noted in human newborns (reviewed by Beverly, 1997). Interestingly, the peripheral T cells from human newborns lack the expression of CD40-L (Durandy *et al.* 1995). Recent studies showed the dependency of the IL-12 secretion and B7.2 expression by the professional APC, on the interaction between CD40 and CD40-L (Yang and Wilson, 1996; Kamanaka *et al.* 1996). Bypassing the requirement of CD40-L by in vitro stimulation of neonatal T cells with anti-CD3 together with anti-CD28 mAb, led to significant production of IFNγ, in an IL-2 dependent manner (Sornasse *et al.* 1996). Concordant with these result, strong co-stimulatory signals like IL-6 and anti-CD28 mAb were able to enhance the IL-2 response as well as the resistance to apoptosis of the peripheral T cells from newborn mice (Adkins *et al.* 1994; Adkins *et al.* 1996). Thus, most of the peripheral T cells from neonates are impaired in their ability to receive and transduce co-stimulatory signals.

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Besides the qualitative differences between the adult and neonatal immune systems, there are quantitative differences regarding the mature T cells and professional APCs, that may explain certain aspects of the neonatal responsiveness. We estimated the total number of splenic T cells with a certain specificity from adult versus newborn TCR-transgenic mice, using anti-clonotypic reagents and FACS analysis. Our analysis showed that the total number of transgenic-TCR expressing T cells is at least 100 fold reduced in neonates as compared to the adults (Bot *et al.* 1997c). Based on the experiments addressing the induction of CTL immunity to adult DCs or splenocytes bearing H-Y antigens, it was hypothesized that moderate doses of antigens that are immunogenic for adults, can induce tolerance in neonates simply because of the reduced number of T cells and professional APCs (Ridge *et al.* 1996). For example, a higher proportion of non-professional APCs like resting B cells (Fuchs and Matzinger, 1992), would be loaded with antigen, leading to tolerance rather than priming of the few specific T cells from newborns. Furthermore, there is evidence that the neonatal APCs are impaired in their ability to turn-on specific T cells. The newborn macrophages express lower levels of MHC class-II molecules (Lu, 1984), leading to impaired antigen presentation (Lu *et al.* 1979). The B cells from newborn mice display a reduced ability to stimulate the secretion of IL-2 by specific T cells in an antigen-dependent manner (Morris *et al.* 1992). Finally, DCs from newborns are reduced in number and may be deficient in IL-12 secretion as response to 'danger' signals, that induce increased IL-12 production by adult DCs (Beverly, 1997; Shirakawa *et al.* 1997).

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All the aspects mentioned above contribute to the decreased response of the newborns to most of the classical vaccines, that usually require multiple boosts in order to induce significant immunity. Besides the poor responsiveness of the newborns to antigens as well as the high susceptibility to tolerance, there is the problem of negative interference of maternal antibodies with the generation of immune responses to vaccines (Albrecht *et al.* 1977; Francis and Black, 1986; Harte *et al.* 1982). Recent studies showed that the maternal antibodies specific for the carrier of the immunogenic epitopes are responsible for this inhibition, since changing the carrier circumvented the down-regulation of the neonatal immune response (Wang *et al.* 1997).

Thus, an improved vaccination strategy for neonates would eventually have the following characteristics: (1) significant priming of Th1 cells as opposed to exclusive Th2 immunity, that would allow the development of protective immunity against intracellular pathogens and would minimize allergy as a potential side-effect; (2) priming of CTLs in the absence of side-effects due to the vector replication, that is the case of the live-attenuated vaccines; (3) a minimal inhibition by the maternal antibodies, that would allow early vaccination to common microbes; (4) long lasting immunity, that would preclude the necessity of many boosts or larger doses, potentially tolerogenic in some of the recipients.

B. Naked DNA immunization

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DNA immunization is a relatively new strategy consisting in the induction of cellular and humoral responses, following the injection by various routes of mammalian expression-vectors consisting in bacterial plasmids encoding foreign antigens (Wolff *et al.* 1990; Tang *et al.* 1992).

The plasmid can be injected intramuscularly (i.m.), subcutaneously (s.c), intradermally (i.d.) or intravenously (i.v.), in saline or coated on gold microparticles (Eisenbraun *et al.* 1993). Independently on the means of inoculation, the plasmid is taken up by certain cells at the site of injection (Raz *et al.* 1994) and presumably, by cells located remotely from the inoculation site (Torres *et al.* 1997), subsequently to the transport via the blood or lymph. The *in vivo* transfected cells express the foreign protein for various intervals of time, depending on the type of tissue inoculated with the plasmid. For example, i.m. inoculation leads to foreign antigen expression in approximately 1% of the myocytes at the injection site, for intervals of time usually longer than 30 days (reviewed by Pardoll and Beckerleg, 1995). In contrast, i.d. inoculation of plasmid or gene-gun immunization with plasmid-coated gold particles, leads to foreign antigen expression in the epithelial cells that is more limited in time, because of the continuous process of differentiation and shedding of the aged keratinocytes (Raz *et al.* 1994). Two types of cells take up the plasmid and express the foreign antigen: non bone-marrow derived (non-BMD) cells, that are not professional APC (i.e. myocytes, fibroblasts, keratinocytes) and bone-marrow derived (BMD) cells, that are professional APC (i.e. Langerhans cells - LC; dendritic cells -DC; macrophages - Mf). Whereas most of the cells that take up the plasmid and express the foreign

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antigen are non-BMD cells, only few professional APC are *in vivo* transfected. Recent studies showed that the Langerhans cells or DCs take up the plasmid and express the foreign antigen following i.d. or i.m. inoculation of plasmids (Condon *et al.* 1996; Casares *et al.* 1997). Other studies, regarding bone-marrow chimera obtained by reconstitution of parental mice with F1 bone-marrow, showed that the T cell response induced by the DNA vaccines is restricted to the BMD APCs (Corr *et al.* 1996; Doe *et al.* 1996). Recent studies suggested that nBMD cells that express the foreign antigen can secrete or liberate the protein that is further taken up, processed and presented by BMD APC (Ulmer *et al.* 1996; Fu *et al.* 1997).

Whereas there is consensus regarding the priming of the B cells that require the presence of soluble antigen released by the transfected cells, the exact mechanism of T cell priming following DNA immunization is still controversial. There are two main models that are not mutually exclusive. One of them, based on studies carried out by adoptive cell transfer experiments of non-professional APCs (Ulmer *et al.* 1996) and indirectly supported by the data regarding the cross-priming phenomenon (Huang *et al.* 1996), explains the activation of Th and CTLs as a consequence of antigen transfer from *in vivo* transfected nBMD cells to professional APCs. The other model, based on the data that showed the uptake of the plasmid and expression of the foreign antigen by professional APC (Condon *et al.* 1996) and indirectly, on previous studies indicating that few APC migrating to secondary lymphoid organs can induce significant immune responses (Kundig *et al.* 1995), explains the activation of the T cells as a result of antigen processing and presentation by the transfected BMD APC. These mechanisms

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may play different roles according to the strategy and site of inoculation, as well as the type of epitopes expressed by the plasmid, namely class I or class II-restricted. Independently on the validity of the above mentioned models, it is widely accepted that the professional APC are critically involved in the priming of naïve $CD4^+$ or $CD8^+$ T cells following DNA immunization. Another indirect but strong argument for the involvement of professional APCs, is the fact that i.d. or gene-gun immunization requires less plasmid in order to induce significant immune responses (Eisenbraun *et al.* 1993), presumably because of the presence in large numbers of Langerhans cells in the skin.

Two particular aspects make the DNA immunization an appealing strategy of vaccination. First, there is significant generation of CTL immunity (Ulmer *et al.* 1993), presumably due to the uptake of the plasmid and expression of the protein in the professional APCs, allowing the presentation by the classically described endogenous pathway (Townsend and Bodmer, 1989). Secondly, because of the adjuvant effect of the plasmid DNA due to the presence of unmethylated CpG motifs (Pisetsky, 1996; Klinman *et al.* 1997). The immunostimulatory motifs are responsible for the strongly biased Th1 response subsequent to DNA immunization (Roman *et al.* 1997) as well as for the increased B cell response, due to their mitogenic effect (Krieg *et al.* 1995). Controversy still persists on why various routes of inoculation induce distinct Th profiles (Pertmer *et al.* 1996) and how the immune system recognizes the 'danger' signal associated with unmethylated CpG motifs (Pisetsky, 1997). Presumably, certain APC like macrophages and DCs are activated subsequent to the bacterial DNA

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exposure and up-regulate their functions, like the expression of co-stimulatory molecules (i.e. B7, CD40) as well as the secretion of cytokines (i.e. IL-12, IFNγ, TNFα). This model is indirectly supported by previous studies demonstrating the rapid secretion of Th1-promoting cytokines by splenocytes exposed to naked bacterial DNA (Klinman *et al.* 1996).

Since the first studies demonstrating that the mammalian expression vectors are immunogenic (Wolff *et al.* 1990; Tang *et al.* 1992), numerous reports addressed the immunogenicity and protection ability of the DNA vaccines in adults of various species, including rodents and non-human primates. The prototype DNA vaccines employed in preclinical studies were plasmids expressing one foreign antigen known to encode protective epitopes from a certain microbe. Among the first infectious disease models addressed by DNA immunization was Influenza virus. A plasmid encoding the nucleoprotein (NP) of a type A Influenza virus was found to elicit cross-reactive CTL immunity in rodents (Ulmer *et al.* 1993). Other studies, while confirming this result, showed the long persistence of the immune memory, as well as the plasmid itself, at the injection site (Yankauckas *et al.* 1993). Further, other groups demonstrated the immunogenicity in terms of humoral responses, of plasmids expressing the hemagglutinin (HA) of Influenza virus (Robinson *et al.* 1993; Fynan *et al.* 1993). An important step was completed by demonstrating the immunogenicity and the protective ability of a prototype vaccine composed of a mixture containing plasmids expressing HA, NP and the matrix protein of Influenza virus, after the inoculation in ferrets and non-human primates (Donnelly *et al.* 1995). This study addressed two important

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aspects of the DNA immunization: (1) the immunogenicity and protection ability of DNA vaccines in outbred species and in particular, non-human primates and (2) the effectiveness of vaccines consisting in mixtures of plasmids expressing antigens that bear protective epitopes. The last point was elegantly illustrated by another study demonstrating the enhanced protection against Mycoplasma pulmonis conferred by immunization of mice with a DNA expression library (Barry *et al.* 1995).

Other early studies demonstrated the immunogenicity of plasmids expressing the gp160 protein of HIV (Wang *et al.* 1993), the surface antigen of Hepatitis B virus (HBsAg) (Davis *et al.* 1993) and the glycoprotein of the bovine herpesvirus (Cox *et al.* 1993). Later studies demonstrated the immunogenicity of plasmids encoding virus antigens derived from the Herpes Simplex Virus (HSV) (Rouse *et al.* 1994; Manickan *et al.* 1995), Rabies virus (Xiang *et al.* 1994), Hepatitis C virus (Lagging *et al.* 1995) and the Lymphocytic Choriomeningitis virus (LCMV) (Martins *et al.* 1995). Significantly, plasmids expressing bacterial and parasite antigens were demonstrated to be immunogenic as well. For example, plasmids expressing Mycobacterial antigens (Lowrie *et al.* 1994), the Plasmodium yoelii circumsporozoite protein (Sedegah *et al.* 1994; Hoffman *et al.* 1994), and the Leishmania major surface glycoprotein (Xu and Liew, 1995), were shown to be immunogenic in rodents.

Recent studies addressed the immunogenicity of plasmids encoding the Measles virus nucleocapsid and hemagglutinin antigens (Cardoso *et al.* 1996), the Newcastle-disease virus F protein (Sakaguchi *et al.* 1996), the Papillomavirus major capsid protein L1 (Donnelly *et al.* 1996), the Rotavirus envelope proteins VP4,6 and 7 (Hermann *et al.*

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1996) and the simian immunodeficiency virus (SIV) *env* and *gag* antigens (Lu *et al.* 1996). Several recent studies tested the immunogenicity and in certain cases, the protective abilities of plasmids expressing non-viral antigens like the OspA antigen of Borrelia burgdorferi (Luke *et al.* 1997), the paramyosin of Schistosoma japonicum (Yang *et al.* 1995), the p30 protein of Toxoplasma gondii (Angus *et al.* 1996), the fragment C of Tetanus toxin (Anderson *et al.* 1996) and even the cellular prion protein (PRNP), in PRNP gene-targeted mice (Krasemann *et al.* 1996).

Thus, a large body of data demonstrate the immunogenicity of the plasmid vectors expressing foreign antigens. Some infectious disease models allowed an evaluation of the protective ability of prototype DNA vaccines. Besides the case of Influenza virus mentioned above (Donnelly *et al.* 1995), recent studies demonstrated the protective ability of DNA vaccines following the inoculation into non-human primates, against the challenge with HIV (Boyer *et al.* 1997) or HBV (Prince *et al.* 1997).

The DNA immunization may be applied in other directions than the induction of protection against subsequent microbial challenge. For example, based on the fact that plasmid immunization induces strongly-biased Th1 responses, some groups attempted to prevent Th2 allergic responses to common allergens, by inoculation of DNA vectors expressing those particular antigens (Hsu *et al.* 1996). Further, DNA based vectors expressing tumoral antigens were shown to induce immune responses, probably bypassing certain defects in the antigen presentation of tumor-derived antigens (Conry *et al.* 1994). Finally, there were few attempts to induce tolerance to alloantigens using i.m. plasmid-inoculation (Geissler *et al.* 1994), based on the assumption that the

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presentation of foreign antigens by non-professional APCs leads to unresponsiveness. However, until now, these attempts failed, probably because of the strong adjuvant effects of the bacterial DNA as well as the involvement of professional APCs recruited at the site of injection.

C. Influenza virus model

The influenza viruses are enveloped orthomyxoviruses with a segmented RNA genome of negative polarity, containing eight segments that encode ten proteins in the case of type A and B viruses, or seven segments encoding nine proteins in the case of type C viruses (reviewed in Kilbourne, 1987 and Krug, 1989). Eight of the proteins are structural and are present in the virion: hemagglutinin (HA), neuraminidase (NA), membrane associated matrix proteins (M1 and M2), nucleoprotein (NP) and polymerases (PA, PB1 and PB2). The other two proteins are non-structural (NS1 and NS2) and are present only in infected cells.

The influenza viruses are divided into three major types (A,B,C) based on structural differences among the internal proteins. In addition, the Influenza A viruses are divided into subtypes based on the sequence differences in the HA and NA, reflected into antigenic variation. HA and NA glycoproteins undergo natural genetic variation due to antigenic shift that is a major change caused by the reassortment between the segmented genomes of different strains, or antigenic drift, that is a minor change caused by point mutations followed by immune selection. Whereas the shift results in a

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progeny virus of a different subtype, the drift does not usually change the subtype of a particular strain of Influenza virus. The process of reassortment occurs in cells infected by at least two different strains of Influenza virus. Major reservoirs for new strains of Influenza virus, mostly shift variants, are represented by pigs, birds and horses.

The HA and NA glycoproteins bear dominant B and Th epitopes. The HA molecule is a trimer, each monomer being composed of two chains: HA1 and HA2 (Laver, 1971). X-ray crystalographic studies showed that the HA1 chain is made of two main regions: a tripled stranded coil of α helices and a globular region of anti-parallel β sheets (Wilson *et al.* 1981). In the endoplasmic reticulum (ER), the HA is assembled into a trimeric complex and is glycosylated in the Golgi before the export to the surface through the secretory pathway. During the secretory pathway, the HA molecule is cleaved by a cellular trypsin-like protease located in the epithelial cells of the respiratory tract, that are permissive cells (reviewed in Krug, 1989). This is a step required for a normal function of HA in the nascent virion, endowing the virus particle with the ability to infect other cells. Mutations in the glycoproteins as described in the case of the NA of the A/WSN/33 strain, may circumvent the requirement for trypsin-like proteases and may lead to virus replication in other types of cells, associated with increased virulence (Li *et al.*, 1993). Anti-HA antibodies prevent the binding of the virion to the cell receptors (sialoproteins) and the fusion of the virus with plasma membrane inside the endosomal-lysosomal compartment (Schultze, 1975). The localization of B and T cell epitopes on HA was established by the use of monoclonal antibodies, synthetic peptides, laboratory and natural variants that differ from wild type

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strains by point mutations. The major B cell epitopes on HA of the H1 subtype were designated Sa, Sb, Ca and Cb (Caton *et al.* 1982) and in the case of the H3 subtype, A, B, C, D and E, respectively (Wiley *et al.* 1981). A dominant Th epitope was defined corresponding to the HA 110-120 peptide of the PR8 virus, recognized in the context of mouse I-E^d class-II molecules (Haberman *et al.* 1990). B cell epitopes were defined on NA and the NA-specific antibodies were shown to prevent the spreading of Influenza virus by inhibiting the enzymatic function of NA (Kilbourne, 1976). Thus, while the HA-specific antibodies are inhibitory, the NA-specific antibodies may be permissive but limit the extent of the virus infection. Some of the B and particularly Th cell epitopes on HA and NA are conserved among the strains of the same subtype and contribute to the protection against drift variants. The dominant B and Th epitopes on HA and NA are different among shift variants, resulting in reduced protection against challenge with strains of distinct subtypes, depending mostly on CTL epitopes.

The dominant CTL epitopes are located on the internal proteins, namely the NP and the matrix proteins. The CTL epitopes were defined using short synthetic peptides and in vitro cytotoxic assays (Zinkernagel and Doherty, 1979) with effector cells from virus immunized animals. The epitopes recognized by CTLs are generated from cytoplasmic proteins by proteasomes and are translocated into the ER through TAP transporters (Townsend and Bodmer, 1989). They bind nascent MHC class-I molecules in the ER and are transported as peptide-class I complexes to the plasma membrane. Consequently, only peptides generated from proteins synthesized in the cell can efficiently load the MHC class-I molecules, that will interact with the CTL receptors.

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Recent studies described a minor exogenous pathway that is able to generate class-I restricted peptides from exogenous proteins in a subset of professional APC (Huang *et al.* 1996). A dominant CTL epitope was defined on the NP of the A/PR/8/34 strain of Influenza virus, at the position NP 147-155 (Bastin *et al.* 1987). This epitope that is recognized in the context of mouse K^d class-I molecules, is conserved among strains of different subtype. It was previously shown that CTLs mediate the clearance of the pulmonary virus, by destruction of the infected cells, thus limiting the spreading of the virus (Taylor and Askonas, 1986). Thus, the CTL immunity does not prevent the infection but mediates the recovery. Consequently, the CTL epitopes suffer less selective pressure than the B and Th cell epitopes that are involved in the protection from infection. The dominant CTL epitopes on the internal proteins are conserved among various strains of Influenza virus from different subtypes, being responsible for the heterologous protection against shift variants. Besides dominant epitopes there are minor CTL epitopes on NP (Fu *et al.* 1997a), other internal proteins, as well as HA (Vitiello *et al.* 1996; Milligan *et al.* 1991). Interestingly, recent studies suggested that there is ongoing competition among multiple CTL epitopes derived from NP for a particular class-I haplotype, so that by removal of a dominant epitope, a non-dominant one will replace it (Fu *et al.* 1997a).

Previous studies indicated that in certain circumstances, the cytokines secreted by the virus-specific T cells influence the outcome of the viral infection. For example, while a Th1 response facilitate the clearance of the virus, a Th2 response, particularly due to the IL-4, is detrimental for the virus clearance (Graham *et al.* 1994; Moran *et al.* 1996). A

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recent study indicated that the cytokines secreted by virus specific Th cells may directly contribute to the clearance of the virus (Topham *et al.* 1996). While a previous study failed to involve the IFNγ in the resistance against the primary infection with the A/Japan/H2N2 strain of Influenza virus (Graham *et al.* 1993), more recent data directly implicated IFNγ in the defense against other strains of Influenza virus (Sarawar *et al.* 1994; Bot *et al.* in preparation).

Influenza viruses are notable among other viruses in the ability to produce annual epidemics following antigenic drift and sometimes, pandemics caused by antigenic shift, that represent a major health problem (Lui and Kendal, 1987). Children and persons over 60 years of age as well as immunodepressed individuals are at higher risk for infection and serious complications, including death (Hellman and LaMontagne, 1990; Nobel *et al.* 1977). Most of the epidemics are produced by a single strain of Influenza virus, although outbreaks caused by two types (i.e. A and B) or two subtypes (i.e. H1N1 and H3N2) are not uncommon (Couch *et al.* 1986). The epidemic strain for a given year is sometimes but not always detected during the later part of the preceding epidemic (Glezen *et al.* 1982), a fact that is reflected in the antigenic structure of the Influenza vaccine.

Several strategies of vaccination against Influenza virus have already been explored at various levels.

The licensed vaccine is obtained by formalin inactivation of virions grown in allantoic fluids of embryonated chicken eggs. This vaccine induces protective antibodies against HA and NA that are highly specific for the homologous strain, but no

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cross-reactive CTL responses. Due to this aspect and to the limited ability to forecast the strains of future epidemics, the currently available killed Influenza vaccines contain antigens from each of the major strains of contemporary Influenza viruses (i.e. A/H1N1, A/H3N2 and B). Protection against Influenza virus infection is currently provided by means of annual immunization with trivalent inactivated vaccines particularly of the individuals at high risk for developing serious complications. There are two important drawbacks of the currently licensed vaccine: first, its limited immunogenicity especially in the high-risk groups (i.e. children lacking memory immunity and elderly that are immunodepressed) and secondly, allergic side-effects due to chicken egg contaminants. In order to circumvent the later aspect, there are variants of killed vaccine composed of purified antigens from multiple strains (split vaccine). However, even in high responder individuals, the immunity induced by the killed vaccines declines rapidly and is limited in terms of cross-protection, requiring annual vaccination. Dramatic shifts that can occur during interspecies transmission of viruses may lead to the preferential spreading of the emergent variant into the youngest segment of the population, that is more prone to developing aggravated forms of disease due to the lack of cross-reactive immunological memory. Notably, such a case has been recently described and although isolated, it underlined the potential inefficiency of the conventional vaccination, (Webster) since the killed virus confer no significant immunity against unpredictable shifts.

The peptide-based vaccines are at preclinical stage and represent a variant of the subunit vaccines. They present a major drawback, namely their immunogenicity is

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restricted by the availability of certain MHC haplotypes. However, the peptides were used to dissect and understand the specific cellular immune responses (Taylor *et al.* 1987; Bastin *et al.* 1987). Interestingly, several peptides derived from HA, NP, NS or M proteins, when injected with certain adjuvants, generated significant CTL responses (Yewdell and Hackett, 1989). Whereas the adult mice were primed by inoculation of an NP peptide recognized by CTLs, the newborns were neither primed nor tolerized (Kuzu *et al.* 1993).

The anti-idiotypic vaccines are another variant of subunit vaccines and are based on the ability of the internal image immunoglobulins to mimic viral epitopes. In animals, previous studies showed that anti-idiotypic (anti-Id) antibodies can stimulate an antibody response against HA or NA (Dinca *et al.* 1993; Mayer *et al.* 1989). Interestingly, whereas high doses of anti-Id antibodies administered to neonates caused a long lasting unresponsiveness of the B cells bearing the corresponding Id (Bona *et al.* 1979), minute amounts primed specific B cells and led to idiotypic dominance (Hiernaux *et al.* 1981).

The live attenuated vaccines have a theoretical advantage over the killed vaccines since they can induce humoral and cellular immunity due to the mimicking of the natural infection. Attenuation of vaccine viruses has been achieved by several means: passage in non-human hosts, generation of avian-human reassortants, identification of temperature sensitive strains following chemical mutagenesis and cold adaptation. Early studies with host range mutants obtained by serial egg passage revealed that mutants generated by this method were not reliably attenuated, producing disease in

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some seronegative vaccinees (Beare *et al.* 1968). Similarly, the avian-human reassortants caused some serious side effects during the clinical trials in children and infants, consisting in high fever and flu-like ilness in one quarter of the vaccinees (Steinhoff *et al.* 1991). Thus, the internal proteins of avian origin failed to reliably confer attenuated phenotypes to the human strains. The advent of reverse genetics in the manipulation of negative strand RNA viruses (Li *et al.* 1993), opened an avenue for the engineering of attenuated strains by molecular biology means. The temperature sensitive mutants replicate well at 34-35°C, that is the temperature of the nasopharynx, but not at 37°C, the temperature of the lower respiratory tract. However, some of the children vaccinated with temperature sensitive mutants shed virulent Influenza virus, demonstrating phenotype reversion (Tolpin et al. 1982). The single form of live attenuated Influenza viruses presently receiving serious considerations as vaccines are the cold adapted viruses. They are obtained by serial passage of the virus at progressively lower temperatures, down to 25°C. Reassortants of cold adapted Influenza virus were extensively studied in clinical trials and have been shown to be relatively safe in high risk categories like institutionalized children (Tanaka *et al.* 1993), elderly (Powers *et al.* 1991) and chronically ill (Gorse *et al.* 1986). Furthermore, the protection ability is comparable to that of the inactivated vaccine (Belshe *et al.* 1984). A cold-adapted Influenza vaccine containing two attenuated strains derived from A/Kawasaki/9/86(H1N1) and A/Beijing/352/9 (H3N2) has recently been tested in human infants and children of age between 2 months to 3 years (Gruber *et al.* 1997). Whereas only approximately 10-15% of the vaccinees developed fever, more than 50%

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developed respiratory symptomes like rhinorrhea and cough. The vaccine was immunogenic in more than half of the vaccinated seronegative infants but not in the seropositive infants, most probably due to the inhibition by maternal antibodies.

A new potential strategy for immunization against Influenza virus is the plasmid based vaccination. Its advantage over the currently killed vaccine is that it elicits B, Th as well as CTL immunity (Ulmer *et al.* 1993). Its obvious advantage over the live-attenuated vaccine is the absence of side-effects due to the phenotype reversion. However, there are still other issues regarding the safety of DNA based vaccines that remain to be resolved, namely the possibility of genomial insertion, induction of autoimmunity or tolerance, toxicity. Presently, these issues are addressed through preclinical and clinical trials.

D. Aims of the study

The general aim of the present study is to test the immunogenicity of plasmid-based expression vectors in newborn mice. We compare the immune responses of neonates and adults immunized with plasmids from a qualitatively and quantitatively point of view. The use of the Influenza virus model allows us to test the protective ability conferred by plasmids expressing HA and NP of type A Influenza viruses, subsequent to neonatal inoculation. Furthermore, we explore the advantages of plasmid-based versus conventional immunization of neonates, as well as aspects regarding the interaction between the maternal immunity and the responsiveness of offspring. Finally,

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we address some aspects regarding the mechanisms of immunogenicity and protection conferred by DNA based immunization, in particular the potential role of Langerhans cells and the requirement of IFNγ and IL-4.

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MATERIALS AND METHODS

1. Mice

BALB/c (H-2^d) mice were obtained from Jackson Laboratories (Bar Harbor, MA) and bred in the Mount Sinai Animal Facility. TCRα/β transgenic mice that express a transgenic TCR specific for HA 110-120 peptide in the context of I-E^d (TCR-HA Tg), were obtained by cloning the α and β chains from a T cell hybridoma (14.3.d; Weber *et al.* 1992) and injecting the genomic DNA into fertilized (B6 x DBA/2J) F2 eggs (Kirberg *et al.* 1994). The transgenic offsprings were back-crossed for five generations to mice of BALB/c background. The phenotype of the transgenic mice was monitored by FACS analysis with monoclonal anti-TCR antibodies (clonotype specific mAb 6.5) and by PCR, with insert-specific primers.

IFNγ deficient mice lacking functional IFNγ genes (Dalton *et al.* 1993) and IL-4 knock-out mice (Kopf *et al.* 1993) were purchased from Jackson Laboratories (Bar Harbor, MA). They were housed in the nude mouse area of the Mount Sinai Animal Facility.

2. Viruses

The influenza virus strains A/PR8/34 (H1N1), A/HK/68 (H3N2), A/Japan/305/57

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(H2N2), and B Lee/40 were grown in the allantoic cavity of 10 day-old embryonated hen eggs incubated at 37°C. Allantoic fluids were harvested 48 hours later and stored at -80°C. A/WSN/33 virus (H1N1 subtype) was grown in Madin-Darby bovine kidney cells (MDBK) in the presence of DMEM supplemented with 1% BSA, at 37°C and 5% CO_2 atmosphere. The supernatants were harvested 48 hours later and stored at -80°C. The virus titer was determined by hemagglutination of chicken red blood cells using supernatants obtained after 48 hours incubation of log_{10} dilutions of seed virus with Madine Darby Canine Kidney (MDCK) carcinoma cells. Some experiments required the virus purified on sucrose-gradient and inactivated by exposure to an UV-light source (Germicidal Lamp - 30 Watts, General Electric) for 20 minutes at a distance of 50 cm, under continuous stirring.

3. Cell lines

As target cells for cytotoxic experiments we used P815 mastocytoma cells ($H\text{-}2^d$) or M12 B lymphoma cells (class-I and class-II positive). P815 cells express MHC class I molecules but lack the expression of class-II molecules. They were grown in DMEM medium (Bio Whittaker) supplemented with 10% FCS (Hyclone L-Glutamine (Gibco),1mM sodium -pyruvate (Gibco) and Gentamycine (Sigma).

T cell hybridoma cells (TcH) 14-3-1 were obtained by transfecting an NF-AT/β-gal construct into a Th hybridoma cell line (14-3-d), which recognizes the HA 110-120

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peptide of PR8 virus in the context of I-E^d MHC class II molecules (Bot *et al.* 1996c). The construct is composed of an upstream regulatory element containing an NF-AT binding site trimer fused downstream to the IL-2 promoter (fragment -70 to +47). Downstream, the construct has a LacZ open reading frame attached. Its transcription is regulated in an all or none manner, by the upstream regulatory element (Fierling *et al.* 1990). Activation of transcription occurs when the TCR is properly occupied by the immunogenic peptide - MHC class-II complex. The construct contains a hygromycin resistance gene under the control of TK promoter. The TcH cells were maintained in IMDM supplemented with 10% FCS, L-Glutamine (1mM), sodium pyruvate (1mM), 2-ME (50μM) and hygromycin (0.5mg/ml), in 5% CO_2 atmosphere and 37°C.

2PK3 B lymphoma cells that are professional APC, expressing both class-I and II molecules, were grown in DMEM medium (Bio Whittaker) supplemented with 10% FCS (Hyclone), 1mM L-Glutamine (Gibco), 1mM sodium-pyruvate (Gibco) and Gentamycine (Sigma).

4. Reagents

The synthetic peptides NP-K^d 147-155 TYQRTRALV, NP-D^b 366-374 ASNENMETM, HA I-E^d 110-120 SFERFEIFPKE and HA 150-159 with the sequence WLTEKEGSYPR, were synthesized in the Protein Core Facility of Mount Sinai School of Medicine. The first two sequences were derived from the nucleoprotein and the last

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two from the hemagglutinin of the PR8 strain of Influenza virus. BSA covalently coupled to the HA 150-159 synthetic peptide, was used as coating antigen in ELISA assays aimed to detect HA-specific antibodies.

Ig-gal-HA was obtained by coupling the HA 110-120 peptide with a mouse IgG2b monoclonal antibody (Brumeanu *et al.* 1996a). Ig-HA was engineered by replacing through PCR mutagenesis the CDR3 segment with an insert encoding the HA 110-120 peptide (Zaghouani *et al.* 1993). BHA is a fragment of hemagglutinin from the A/PR8/34 virus, obtained by enzymatic digestion of the virus with bromelain.

As reagents for in vitro or in vivo depletion of $CD4^+$ and $CD8^+$ T cells, we used rat anti-mouse CD4 mAb (GK1.5, ATCC) and CD8 mAb (TIB210, ATCC). The TCR-specific 6.5 mAb was obtained by immunization with soluble TCR (Weber *et al.* 1992). As HA-specific reagents, we used polyclonal antibody against HA 110-120 peptide and PY201 mouse anti-PR8 mAb (kindly donated by Drs. J. Schulman and T. Moran, Mount Sinai School of Medicine - New York). As reagent against NP, we used anti-X31 polyclonal antibodies (kindly donated by Dr. P. Palese, Mount Sinai School of Medicine - New York).

5. Plasmids

The NPV1 plasmid, here designated pNP, was constructed at Merck Research Laboratories by inserting the open reading frame of the nucleoprotein gene of A/PR8/34

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virus into the *Bgl II* site of a mutated pBR322 plasmid containing upstream 1.96 kb of the enhancer, promoter and intron A of the initial early gene of CMV (IE1) and downstream, 0.55 kb of the bovine growth hormone (BGH) poly(A) signal sequence (Fig. 1 and Ulmer *et al.* 1993). The pNP plasmid was kindly donated by Dr. Margaret A. Liu (Chiron Corp., Emeryville -CA). As negative control we used the V1 plasmid without the NP insert (control plasmid - CP).

The pRc/CMV-HA/WSN plasmid (here designated pHA; Bot *et al.* 1997b) was constructed by inserting the HA cDNA of A/WSN/33 (subtype H1N1) strain of Influenza virus into the pRc/CMV mammalian expression vector (Invitrogen) and kindly donated by Dr. Peter Palese (Mount Sinai School of Medicine - New York) (Fig. 1). Transcription of the WSN-HA gene is driven by the CMV initial-early promoter and terminated by the BGH polyadenylation signal. As control, we used the pRc/CMV vector without the HA insert.

The VH-TB plasmid was generated in two steps: first, the VH-TB open reading frame was constructed using the variable region of the heavy chain of 91A3 anti-arsonate antibody, in which the CDR3 and CDR2 were replaced by PCR mutagenesis with the HA 110-120 (T epitope) and the HA 150-159 (B epitope) of the HA of A/PR8/34 virus (Zaghouani *et al.* 1993; Brumeanu *et al.* 1996).

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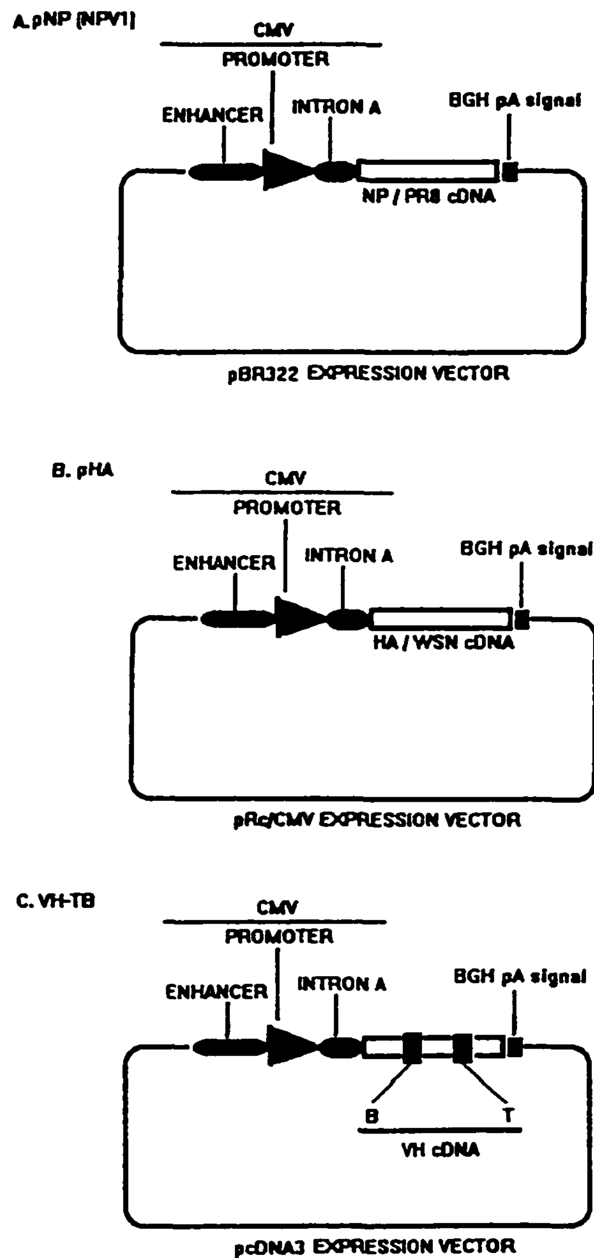

Fig. 1. The structure of the three plasmids, namely pNP (NPV1), pHA and VH-TB, used for the inoculation of newborn BALB/c mice.

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Secondly, the VH-TB open reading frame was inserted into the pcDNA3 mammalian expression vector (Invitrogen) driven by the CMV initial-early promoter and containing the BGH polyadenylation signal (Fig. 1; Casares *et al.* 1997).

The pcNP plasmid was constructed by inserting the cDNA of the NP from the PR8 virus into a pcDNAI expression vector (Invitrogen), containing the CMV promoter and the SV40 polyadenylation sequence. HeLa cells transfected with pcNP expressed NP in the nucleus as revealed by immunohistochemistry (Wang *et al.* 1997b). The pcNP74 plasmid was obtained by inserting a truncated cDNA lacking the first 74 codons of the open reading frame into the pcDNAI vector. HeLa cells transfected with pcDNAI expressed the NP in cytoplasm as revealed by immunohistochemistry (Wang *et al.* 1997). The pcNP and pcNP74 plasmids were kindly donated by Dr. Peter Palese (Mount Sinai School of Medicine - New York).

The plasmids were propagated in *Escherichia coli* and purified on Qiagen tips (Qiagen Inc., Santa Clarita, CA). The concentration and purity was estimated by spectrophotometry and confirmed by analytical gel electrophoresis.

6. Immunization of mice

Adult mice were immunized with live virus by intraperitoneal injection (i.p.) at a dose of $1X10^4$ $TCID_{50}$/mouse. Newborn mice were immunized in the gluteal muscle with

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various amounts of sucrose-purified virus in 50μl of saline, one day after the birth. Since the neonatal inoculation of live-virus was mostly lethal, the majority of the experiments were carried out with UV-inactivated virus.

Adult mice were immunized intramuscularly with plasmid according to two protocols. Some of the mice were previously injected with 100μl of cardiotoxin solution (LATOXAN / France - a protein kinase C inhibitor from Naja nigricollis, diluted at 0.5μg/ml in 0.9% in saline solution). The immunization and the boost were carried out at five days interval in the same place, namely the anterior tibial muscle of the right foot, with disposable 28-gauge insulin syringes with a penetration of 2mm controlled by a rubber stopper. Alternatively, the adult mice were injected three times with plasmid at three weeks interval. Pilot experiments were aimed to determine the dose and the number of inoculations required to induce optimal CTL responses. They showed that in the case of cardiotoxin pretreatment, two inoculations with pNP plasmid at a dose of at least 30μg followed by virus boost, induced detectable primary cytotoxicity of the freshly harvested splenocytes, like three inoculations of 30μg of pNP at three weeks interval (data not shown). We chose the second protocol, without cardiotoxin pretreatment, when we compared the immunity of mice immunized as adults with that of newborns, since the neonatal inoculation with cardiotoxin was avoided.

The newborn mice were immunized with 30μg of plasmid in 50μl of saline, into the right gluteal muscle, at day 1, 3 and 6 after the birth. Pilot experiments showed that

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three immunizations with pNP were required for the induction of detectable primary cytotoxicity against PR8 virus, following the virus infection at the age of three weeks (data not shown). A single inoculation of 1-day old mice with 20µg of pNP, was sufficient to induce secondary cytotoxicity, detectable after *in vitro* stimulation of the splenocytes harvested 4 weeks later (data not shown).

7. Challenge of mice

Mice were challenged via the aerosol route with various lethal doses of A/PR8/34, A/WSN/33 or A/HK/68 virus in the form of allantoic fluid or supernatant diluted in saline. The infection was carried out for 30 minutes in an aerosol chamber to which a nebulizer (Ace Glass Inc.) was attached, connected to a vacuum/pressure system pump (35 l/min and 15lb/inch^2). Immunized and control mice were always infected simultaneously. Mice were observed each day after infection, for at least 16 days and their survival expressed as percentage. Their clinical status was assessed daily by noting the mobility, the respiratory pattern and the weight loss. The surviving mice were sacrificed and the pulmonary virus titers estimated, in order to verify the complete clearance of the virus.

8. Measurement of the pulmonary virus titer

For the lung titer estimation, at least three mice from each group were sacrificed by

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cervical dislocation, at three or seven days after the infection. The lungs were harvested and homogenized in 1.8ml of PBS-0.1% gelatin. Log_{10} dilutions of lung homogenates were carried out in DMEM supplemented with 1% BSA and gentamicin (Sigma). Triplicate aliquots of 25µl from each dilution were added to 96-well flat bottom plates containing confluent monolayers of MDCK cells briefly washed with trypsin supplemented media (0.05% Trypsin and 0.53mM Na-EDTA). After 1 hour of incubation at 37°C in 5% CO_2 atmosphere, 175µl of DMEM-FCS 10% maintenance medium was added to each well and the plates were incubated for 48 hours at 37°C and 5% CO_2. From each well, 50µl of supernatant was harvested and co-incubated for 30 minutes at room temperature with 50µl of PBS 1x and 100µl 0.5% chicken red blood cells in 96-well round-bottom flexible RIA plates (Falcon). The virus titers were determined by interpolation of the virus dilution that showed hemagglutination in 50% of wells (Reed and Muench, 1938).

9. Purification of lymphocytes and negative selection

Spleens, lungs or lymph nodes were aseptically removed from immunized or infected mice and single cell suspensions were prepared by mincing and passing the tissue through cell strainers (Falcon). In the case of lungs, the tissue was pretreated with collagenase (8U/ml) for 90 minutes at 37°C (Bot *et al.* 1996b; Stein-Streilein *et al.* 1983). The bronchial lavage lymphocytes (BAL) were obtained by tracheal canulation

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and successive washings with 1ml of saline, after a method described by Allan *et al.* 1990. Harvested cells were treated with hypotonic shock for 5 minutes on ice to remove the erythrocytes ($5x10^7$ cells in 4ml Tris lysis-buffer). Mononuclear cells were enriched on Histopaque-1083 (Sigma, St. Louis-MO). The T cells were eventually enriched on 10 ml nylon-wool columns (Unisorb T&B, Nycomed - Norway). Sometimes, the adherent cells consisting of B cells and macrophages were harvested after washing the T cells off the column, in order to be used as APCs. Alternatively, the T cells were enriched by removing the plastic adherent cells, after 1 hour incubation in Petri dishes at 37°C and 5% CO_2. The T cells were used for primary CTL assays, lymphocyte cultures or further negative selection of $CD4^+$ and $CD8^+$ T cells.

For the negative selection, the enriched T cells were resuspended in PBS-BSA 1% at a concentration of $8x10^6$ cells/ml and incubated with 80µg/ml of anti-CD4 or anti-CD8 rat anti-mouse monoclonal antibodies, for 30 minutes at 4°C with gentle rocking. The cells were washed three times with cold PBS-BSA 1% and incubated on ice with iron beads coupled to goat anti-rat IgG (BioMag, PerSeptive Diagnostics, MA) for 30 minutes, at a concentration of $4x10^6$ cells/1ml of the suspension of magnetic-beads. After incubation, two 5-minute rounds of magnetic depletion were performed on the magnetic separator (PerSeptive Laboratories, MA). Alternatively, the $CD4^+$ T cells were enriched by negative selection on immunoaffinity columns coupled with polyclonal goat anti-mouse IgG (Biotex Laboratories, Edmonton - Canada), after cell

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coating with rat anti-mouse CD8a mAb (YTS 169.4). The cells separated from supernatant were washed with DMEM-FCS 10% and incubated or used for cytotoxic assays.

10. Cytotoxic assay

The primary cytotoxic assay was carried out by incubating freshly harvested effector cells with $5x10^3$ ^{51}Cr-labeled target cells at different effector to target ratios in 96-well V or U bottom plates. Target cells were sometimes infected with virus for 1 hour at 37°C ($1x10^5$ $TCID_{50}$ / $1x10^6$ cells in DMEM-BSA 1%) previous to the labeling with ^{51}Cr-saline (0.1 mCi / $1x10^6$ cells in 250μl PBS 1x, at 37°C for 1 hour). Alternatively, the cytotoxic assay was carried out in the presence of 5-10 μg/ml of specific peptides or after 30-minutes coating of the ^{51}Cr-labeled target cells with excess peptide, at 37^0C. After 4 hours incubation at 37°C in 5% CO_2, the plates were centrifuged for 5 minutes at 1900 rpm and the supernatants harvested and counted in a γ-counter (Automatic/Wallac-Finland).

For the secondary cytotoxic assays, mixed lymphocyte cultures were prepared and incubated for 3-5 days in RPMI supplemented with FCS 10%, 50μM 2-mercaptoethanol, 1% non-essential amino acids (GibcoBRL) and 2% HEPES buffer (GibcoBRL), at a density of $4x10^6$ cells/ml with a ratio of responder to stimulator cells equal to 1. Before incubation, the stimulator cells prepared from non-immunized

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BALB/c mice were irradiated with 2000 Rads and infected with virus (10^8 cells in 1.5ml DMEM-BSA1%+0.5ml virus in allantoic fluid, at 37°C in water bath for 1 hour) or co-incubated with responder cells in the presence of peptides at 5 to 15μg/ml. The secondary CTL assay was carried out as the primary assay, after carefully washing and centrifuging on Histopaque-1083 (Sigma, St. Louis - MO) the effector cells obtained from the lymphocyte cultures. The results were expressed as % specific lysis = {[(actual - spontaneous release)/(maximum-spontaneous release)x100] - background release} ±SD of triplicates, for each E:T ratio. Sometimes, the secondary CTL assays were carried out after *in vitro* stimulation of responder cells in 96-well flat bottom plates at various R/S ratios, followed by cell transfer into V-bottom plates together with target cells. This protocol takes into consideration the expansion ability of the pCTL precursors, in the same manner as the limiting dilution analysis protocol does.

11. Limiting dilution analysis

To evaluate the number of specific CTL precursors in the spleens of immunized mice, single cell suspensions were prepared and $1\text{-}5 \times 10^5$ responder cells were incubated in 24 wells of 96 well-flat bottomed tissue culture microtiter plates and titrated in six to eight steps of twofold dilutions. Irradiated and infected, or peptide-coated syngeneic normal cells were added in a number of 2.5×10^5/well. Micro-mixed lymphocyte cultures were incubated for 3-5 days in complete RPMI media and individual cultures were tested in a

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^{51}Cr release assay on MHC-matched target cells infected with virus, coated with peptide or left uninfected as control. Positive wells were considered those that displayed a higher ^{51}Cr release than the matched background+3xSD. The percentage of cultures in one dilution step that were negative regarding the specific cytotoxicity was logarithmically plotted against the number of responder cells/well. After linear regression by the least square method, the frequency of the CTL precursors was read at 37% negative wells according to Poisson's formula: -ln(negative well index)/(number of responder cells/well) = 1/(number of responder cells/well at 0.37 negative well index). The number of precursor cells was represented as frequency or 1/frequency for comparison purposes. The total number of specific pCTLs was estimated by multiplying the total number of cells/organ with the pCTL frequency.

12. T cell proliferation assay

The T cells were incubated in IMDM-FCS 10% or RPMI-FCS 10% media supplemented with 50μM 2-mercaptoethanol, in 96-well round bottom plates ($2x10^4$/well) or flat bottom plates ($2x10^5$/well) at 37°C and 5% CO_2 in the presence of irradiated APCs (1:1). Sometimes the stimulator cells were pre-infected with virus and in other cases the incubation was carried out in the presence of peptides (5-30 μg/ml) or sucrose-purified UV-inactivated virus. After 3-5 days of incubation, 1mCi of (^3H)-thymidine (Amersham) was added to each well and after other 18 hours, the cells were

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harvested on glass filter paper (Skatron Instruments, Norway) using a cell harvester (Skatron / Sterling, VA). The filters were dried at 37°C and the radioactivity measured in a β–counter (Pharmacia Biotechnology) was expressed as cpm/well±SE of triplicates.

13. Cytokine measurement

For cytokine detection, the T cells were incubated with stimulator cells for various intervals, in 96-well flat bottom plates. In some experiments, 1-2 U/ml of exogenous IL-2 (Boehringer Mannheim, Germany) was added, since it greatly increased the ratio of noise versus the background (data not shown). The supernatants were harvested and the concentrations of IFNγ, IL-4 and in some experiments of IL-2, were measured by sandwich ELISA using cytokine-specific reagents provided with standardized kits (Cytoscreen/BioSource International, Camarillo-CA). Samples with known cytokine concentration were included in order to construct calibration curves. The optical densities were read at 450nm after blanking the ELISA reader (Cambridge Technology Inc.) on the null-concentration wells. The concentrations of the samples were interpolated from the standard curve obtained by the least square method. The results were expressed as means±SE (pg/ml) of duplicates or triplicates. Values lower than the background+3xSE were considered below the sensitivity of detection and assigned the 0 value. Generally, the sensitivity of the ELISA assays was below 5pg/ml for IL-4 and IIFNγ and 7pg/ml for IL-2.

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14. Radioimmunoassay and isotyping

To estimate the serum titer of specific antibodies as well as their isotypes, microtiter plates were coated for 18 hours at 4^0C with 50μl of carbonate buffer (0.1M at pH of 9.6), containing 1-5μg/ml of antigens in PBS-BSA1% or with PBS-BSA1% alone. The plates were extensively washed with PBS 1x and then blocked with 3% BSA in PBS at room temperature. After 4 hours the plates were washed again with PBS and serial (log_{10}) dilutions of sera (50μl in 1% BSA-PBS) were added for two hours at 37°C. After extensive washing with PBS 0.05%-Tween 20, bound antibodies were revealed by incubating the plates for two hours at room temperature with 5×10^4 cpm/well of ^{125}I-rat anti-mouse k chain monoclonal antibodies. The bound antibodies were measured in a γ-counter (Automatic/Wallac - Finland). The concentration of antibodies was determined by interpolation on the linear segment of a standard curve, constructed with various dilutions of Influenza virus-specific monoclonal antibodies (kindly donated by Dr. T. Moran, Mount Sinai School of Medicine, New York).

For the determination of isotypes, the microtiter plates were coated with PBS-BSA1%, or sucrose-purified WSN virus dissolved in PBS-BSA1% and then incubated with 1:100 dilution serum samples. After extensive washing with 1% NP-40 in PBS, the plates were developed with 50,000 cpm ^{3}H-labeled anti-mouse isotype antibodies (BioRad Laboratories, Hercules-CA). After 3 hours of incubation, the plates were

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extensively washed with distilled water and the radioactivity was counted in a β-scintillation counter. Since no virus-specific reagents were available to construct standard curves for each isotype, the results were expressed as means of triplicates (cpm) and the background activity was subtracted.

15. ELISA for specific Ab detection.

The 96-well Nunc Immuno plates were coated overnight at 4^0C with 4μg/ml of sucrose-purified PR8 virus, or HA 150-159 synthetic peptide coupled to BSA (BSA-B) and dissolved in coating buffer (0.1M $NaHCO_3$ with pH of 8.2). After the washing with PBS- Tween-20 (0.5%), the wells were blocked with PBS-BSA3% at room temperature for 1 hour. After the washing, serial three-fold dilutions of mouse sera were added for 2 hours at room temperature. The specific binding activity of the mouse antibodies was estimated after the addition of biotin-coupled, goat anti-mouse IgG antibodies (1:1000; Sigma Immunochemicals; St. Louis, MO). Streptavidin-Peroxidase (HRP) and Tetramethylbenzidine (TMB) were successively added in order to develop the reaction. The enzymatic reaction was stopped with acid solution and the optical densities were read at 450nm (Cambridge Technology Inc., MA). The results were expressed as means±SE of ODs at particular serum dilutions. Sera from naïve mice was always used as negative control.

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16. HI assay

The hemagglutination inhibition assay was carried out after treatment of sera with receptor destroying enzyme (RDE / neuraminidase; Sigma, St. Louis-MO) overnight at 37°C, in order to remove the non-specific hemagglutination-inhibiting activity due to the serum sialoproteins. The RDE was inactivated by incubation with sodium-citrate 2.5% at 56°C, for 30 minutes. The two-fold serial dilutions of RDE-treated sera were incubated with 0.5% human erythrocyte saline suspension in the presence of agglutinating titers of WSN or PR8 virus. The experiment was carried out in triplicate wells. After 45 minutes incubation in 96-well round bottom flexible plates (Falcon) at room temperature, the results were read and expressed as $\log_2$ of the last inhibitory dilution. Negative controls (blank sera) and positive controls (HA specific monoclonal antibodies) were included in the experiment.

17. Plasmid detection by PCR

Injected and non-injected muscles were removed at various time intervals after the last immunization, rapidly frozen in ethanol-dry ice and kept at -80°C. They were thawed and homogenized (Tissue Homogenizer - Kimble) in lysis buffer [25mM Tris-H_3PO_4 (pH 8), 2mM CDTA (trans-1:2 diamenocyclohexantetra-acetic acid), 2mM dithiothreitol (DTT), 10% glycerol and 1% Triton X -100]. The DNA was extracted by 2-3 successive treatments with phenol-chloroform-isoamyl alcohol (Sigma) and the

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traits of phenol were removed by ether-extraction. The DNA was precipitated by treatment with 2.5 vol. of 100% ethanol, 1/10 vol. of 3M Na-acetate and 1μl glycogen solution (Boehringer Mannheim) and kept at least 45 minutes at -80°C. After 20 minutes spinning at 4°C, the pellets were briefly washed with 70% ethanol and vacuum-dried for 5 minutes. The DNA was dissolved in 10-15 μl sterile water. Alternatively, the DNA was extracted from muscles or tail tissue by overnight incubation at 56°C with 500μl of proteinase K supplemented lysis buffer (0.1M Tris-Cl, 0.2% SDS, 5mM EDTA, 200mM NaCl, 100μg/ml proteinase K; adjusted to the pH of 8.2). The DNA was precipitated with isopropanol and resuspended in TE buffer overnight at room temperature, under shaking.

The PCR reaction was carried out using a PCR Reagent System Kit (Life Technologies) for 30-40 cycles in a DNA Thermal Cycler (Perkin Elmer). In the case of pHA injected muscles, a hot-start approach was used in order to avoid non-specific PCR products.

As primers for NP we used the following sequences: 5'-CATTGTCTAGAATTTGAACTCCTCTAGTGG and 5'-GGCCGTCGACCATG ATGATCTGGCATTCC of the NP gene, that amplify a 699-bp PCR product. For pHA detection, we used the primers 5'-CCCAAGGAAAGTTCATGG and 5'-GAACACCCCATAGTACAAGG of HA, amplifying a PCR product of 198-bp. For the detection of TCR-specific inserts corresponding to the transgenic Vβ and Vα of the

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TCR-HA Tg mice, we used the following pairs of specific primers: 5'-GTACCTGGTATAACACTCAG with 5'-ATTGCCTCCACTCAGAGCAC and 5'-AATGAACCTTTATTCTGAAC with 5'-TAGGAGAAAGCAATGGAGAC, respectively.

The DNA was extracted from Langerhans cells by lysis with 20μl of proteinase K supplemented buffer, after washing and centrifugation of various numbers of crawl-out cells (10^5 to 800) in 96-well V bottom plates. The hot-start PCR reaction was carried out using the NP-specific primers described above and the DNA from cell lysates.

The PCR products were visualized on an ethidium bromide-stained agarose gel. As positive controls for the PCR reactions we used 5ng of pNP or pHA plasmid and as negative controls we used control vectors without the viral genes.

18. Hystochemistry of β-galactosidase$^+$ TcH

The T cell hybridoma (TcH) expressing a TCR specific for HA 110-120 of the PR8 virus, in context of I-E^d, were incubated with infected, peptide-coated or antigen-pulsed APCs for at least 4 hours. Pilot experiments showed that optimal stimulation of the TcH occurred when the ratio of responders/stimulators was 1/2 (data not shown). The cells were centrifuged at 1900rpm for 10 minutes, the supernatants were discarded and the cells were mildly fixed with a glutaraldehyde/formaldehyde solution (2% formaldehyde

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and 0.2% glutaraldehyde in PBS), for 5 minutes at 4^0C. After briefly washing with PBS 1x, the cells were incubated overnight with substrate (X-gal substrate in a PBS solution containing 5mM potassium ferrocyanide, 5mM potassium ferricyanide and 2mM $MgCl_2$). The percentage of the β-gal positive cells was counted by optic microscopy and normalized to the number of TcH. In certain experiments, the APCs were pulsed with antigens or antigen-antibody complexes previously to the incubation with TcH, according to a protocol desribed elsewhere (Bot *et al.* 1996c).

19. Epidermal tissue culture

The epidermal tissue cultures were set up in order to separate the migratory cells, comprising a large percentage of Langerhans cells, as previously shown (Ortner *et al.* 1996). After the intradermal inoculation of the plasmid or virus into the dorsal side of the ear, the ears were harvested, briefly rinsed in ethanol 70% and dried for 30 minutes at room temperature. Dorsal and ventral flaps were prepared by ear dissection and incubated overnight in Petri dishes, with the inner part facing the medium consisting in RPMI supplemented with 10% FCS as well as 0.5μg/ml of Amphotericine B (GibcoBRL). The next day, the ear flaps were transferred into Petri dishes or 12-well plates containing fresh medium and the procedure was repeated over the next 2-4 days. The migratory cells that were harvested each day from the bottom of the wells or Petri dishes respectively, by forceful pipetting, were passed through cell strainers (Falcon)

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and washed twice in DMEM. Usually, approximately $5x10^4$ migratory cells were separated from each mouse, the majority of them migrating out during the first day of incubation. The presence of Langerhans cells was assessed by two methods: first, by immunohistochemistry with fluorescent anti-MHC class-II antibodies, we showed that approximately 30-50% of the migratory cells are class II^+, result that is concordant with previous studies (Ortner *et al.* 1993). Secondly, we incubated migratory cells from epidermal layers in vivo exposed to PR8 virus by intradermal injection, with TcH for 4 hours at 37^0C and $5\%CO_2$. The histochemistry showed a significant percentage of β-galactosidase$^+$ TcH, demonstrating the presence of APC able to process the virus and to present the HA 110-120 peptide in the context of $I\text{-}E^d$ molecules. The migratory cells were used for two purposes: to investigate the uptake of the plasmid and expression of the foreign antigen and secondly, to test their priming ability in adoptive cell transfer experiments.

20. Immunohistochemistry

After overnight incubation in Petri dishes containing RPMI-FCS 10% supplemented with Amphotericine B in order to remove blood and tissue debris, the ear flaps were incubated in 12-well plates with collagen-coated glass cover-slips on the bottom. After 48 hours, the flaps were removed, the migratory cells adherent to the slips were washed with PBS 1x and fixed and permeabilized with cold methanol:acetone (1/1 vol.), for 10

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minutes at 4^0C. After fixation, the liquid was removed and the glass-slides were dried at room temperature for 15 minutes. After brief treatment with PBS, the cells were incubated with rabbit anti-X31 Influenza virus anti-serum (1:1000) and rat anti-mouse class-II antiserum (1:5; clone H-21-A; Biosource International, Camarillo-CA), in PBS-gelatin 1% supplemented with NaN_3 0.01% overnight, at 4^0C. The next day the cells were washed with PBS and incubated with fluorescent secondary antibodies (DTAF-conjugated donkey anti-rabbit and Cy3-conjugated goat anti-rat polyclonal antibodies at 1:100 dilution; Jackson Immunoresearch Labs, West Grove-PA), overnight at 4^0C. The following day the cells were washed again and the nuclei were stained by 30 minutes incubation with 5μg/ml DAPI (4',6-Diamidino-2-Phenylindole; Sigma, St. Louis - MO). The cells were studied with an UV-microscope (Carl Zeiss, Germany) using filters for each of the three fluorescent dyes.

21. Intrasplenic cell adoptive transfer

Migratory cells were harvested and washed twice in cold RPMI. They were resuspended in DMEM and injected into the spleen of the recipient mice, in a total volume of 50μl. The recipient mice were prepared by general anesthesia with an i.m. injected cocktail of 0.2mg Xylazine (Rompun) and 1mg of Ketamine (Ketaset) and surgically incised in the upper-left part of the abdomen. The inoculation was carried out through the omentum, into the anterior extremity of the spleen, after the immobilization of the organ to the

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posterior wall of the abdominal cavity, by applying mild mechanical pressure with a blunt forceps. The incisions were sutured and the mice were rested for seven days. The spleens were harvested and the splenocytes were restimulated with virus-infected APC for four days in a limited dilution assay manner, before being tested in cytotoxic assays.

22. Statistical analysis

We performed statistical analysis to compare independent samples with normal or binomial distributions (Rosner, 1995). In the case of normal distributions (lung titers, specific lysis values, etc.), we used a statistical test for the sample variances (F test) before applying the two-sided t test for equal or unequal variances.

In the case of binomial distributions (survival experiments), we used a test for normal approximation validity before applying the chi-square test. If the normal approximation was not valid or more stringency in evaluating statistical data was required, we used the Fisher's exact test.

For limiting dilution analysis, Poisson's distribution laws were applied. In order to construct standard curves for pCTL frequency estimation, antibody or cytokine concentration measurement, we used the linear interpolation by the least square method.

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RESULTS

1. Neonatal immunization with a plasmid expressing NP

We investigated the ability of a plasmid expressing NP of influenza virus strain A/PR8/34 (PR8), to induce cytotoxic immune responses following i.m. inoculation of newborn BALB/c mice. Previous studies showed that injection of the same plasmid in adult mice, primed a strong CTL response that protected the animals against challenge with a heterologous strain of Influenza virus (Ulmer *et al*, 1993). It is known that while HA encodes the major B and Th epitopes responsible for the generation of protective antibodies (Virelizier, 1975), NP and other inner proteins encode CTL epitopes that participate to the clearance of virus (Yap *et al*, 1978; Lin and Askonas, 1981; Lukacher *et al.* 1984). It was shown that anti-NP antibodies do not mediate any protective effects (Epstein *et al.* 1993). Thus, we asked the question if persistent antigen exposure following DNA immunization of newborn mice primes a protective cellular immune response or alternatively, induces immunological tolerance.

1.1. CTL priming by neonatal immunization with a plasmid expressing NP

To determine the response of BALB/c mice immunized as newborns with pNP plasmid, we measured the primary cytotoxicity of freshly isolated splenocytes, as well as the secondary cytotoxicity following *in vitro* stimulation of splenocytes from

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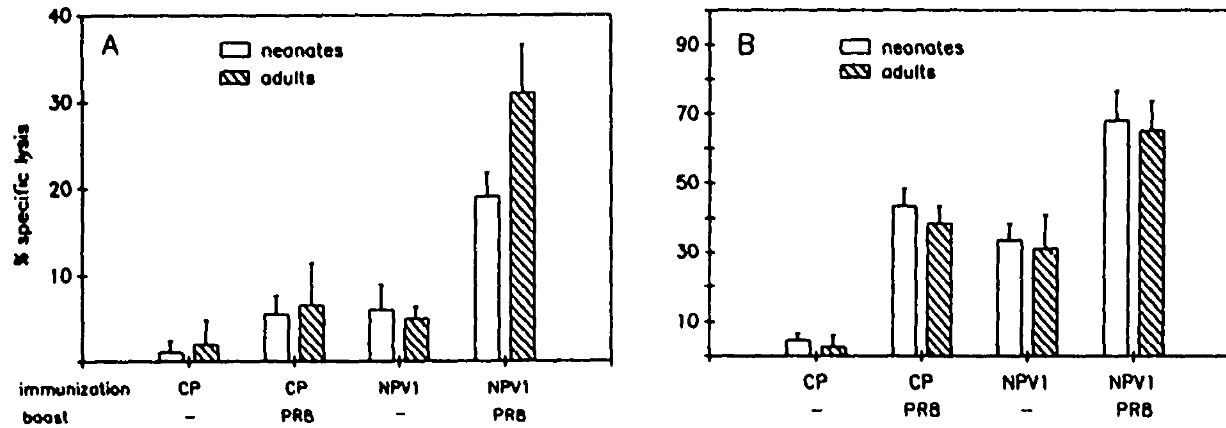

Fig. 2. Primary (A) and secondary (B) cytotoxic activity of splenocytes from mice injected with NPV1 (pNP), at one month after the completion of immunization. A subgroup of mice were boosted with PR8 virus 1 week previous to the sacrification. As controls, we used mice inoculated with nil plasmid (CP) and mice injected with CP and boosted with live PR8 virus. Freshly isolated splenocytes (A) or effector cells subsequent to in vitro stimulation with PR8 virus (B) were incubated at E/T ratio of 160:1 and 100:1, respectively. The results were expressed as means of individual % specific lysis values ± SEM, in groups of three mice.

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animals sacrificed at ages of one (Fig. 2) and three months (Fig. 3), respectively. Half of the animals were boosted with live-PR8 virus one week previous to the sacrification, in order to assess the responsiveness of mice immunized with pNP plasmid. We carried out in parallel, analogous experiments in adult BALB/c mice and we included controls in both age groups. As shown in Fig. 2A, both the neonates and the adult mice immunized with pNP and boosted with live PR8 virus displayed significant primary cytotoxicity against the NP 147-155 peptide, that is the major CTL epitope in the H-2^d haplotype. In contrast, the mice injected with a control plasmid (CP) and boosted with PR8 virus one week prior to the sacrification (Fig. 2A), displayed no primary cytotoxicity against the same peptide. After the *in vitro* expansion with PR8-infected BALB/c splenocytes, the splenocytes from adult and newborn mice immunized with pNP displayed significant secondary cytotoxicity for the NP 147-155 peptide, like the mice immunized with live-PR8 virus one week previous to the sacrification (Fig 2B). Mice immunized with pNP and boosted with PR8 virus consistently exhibited the highest specific lysis values. No primary or secondary CTL activity was observed in the case of animals injected only with CP, which lacks the NP open reading frame (Fig. 2). These results indicate that pNP inoculation had a significant priming effect on the generation of NP specific CTLs, both in neonate and adult BALB/c mice. Similar results were obtained in animals studied three months after the completion of immunization with pNP (Fig. 3A and B),

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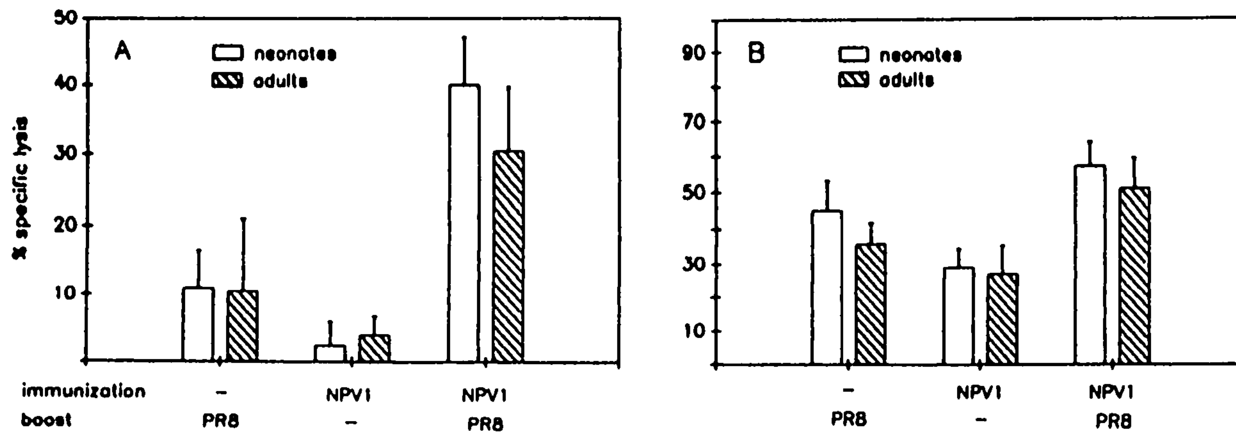

Fig. 3. Primary (A) and secondary (B) cytotoxic activity of splenocytes from mice immunized with NPV1 (pNP), at three months after the completion of immunization. The assay was carried out in a similar manner with the one described in Fig. 2. The results were expressed as means of individual % specific lysis values ± SEM, in groups of three mice.

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showing that CTL immunity primed by pNP injection of neonates persists at least three months.

1.2. Cross-reactivity of CTLs primed by neonatal immunization with pNP

We further investigated the cross-reactive pattern of the CTLs induced subsequently to the pNP immunization of newborn mice, by performing secondary cytotoxic assays after *in vitro* expansion of splenocytes harvested from 3 month-old mice. As target cells, we used P815 cells infected with A/PR8/34 (H1N1), A/HK/68 (H3N2), A/Japan/57 (H2N2) or B/Lee/40, respectively. It was previously established that whereas NP is relatively conserved among various subtypes of type A Influenza viruses, major differences occur in HA (reviewed by Krug, 1989). As shown in Fig. 4, splenocytes from mice immunized with pNP, PR8 virus or immunized with pNP and boosted with PR8 virus, displayed significant CTL activities against type A Influenza viruses but not against B/Lee virus, which is a type B Influenza virus. Again, the mice immunized with pNP and boosted with PR8 virus showed the highest secondary cytotoxic activities, confirming the priming ability of pNP in neonates (Fig. 4B). Concordant with previous studies showing that pNP induces type A cross-reactive CTLs in adult mice (Ulmer et al., 1993), these results demonstrate the same cross-reactive ability of CTLs primed by pNP injection of newborns.

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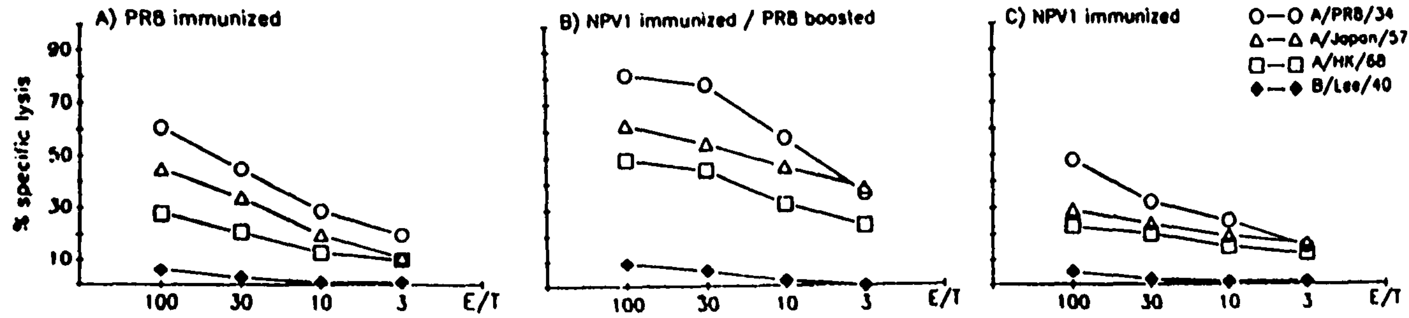

Fig. 4. Cross-reactivity of CTLs from 3 month-old mice immunized as neonates with NPV1 (pNP) plasmid. The effector cells were obtained by in vitro expansion of splenocytes harvested from three animals in each group, with PR8 infected stimulator cells. Responder cells were obtained from mice immunized with PR8 virus one week before sacrification (A), with pNP as neonates and boosted with PR8 virus (B), or immunized with pNP as newborns (C). As target cells, we used non-infected P815 cells and P815 cells infected with type A Influenza virus (open symbols) or B/Lee/40 virus (closed symbols). The results were expressed as means of triplicates of % specific lysis values, at various E/T ratios.

1.3. IFNγ production and proliferation of T cells primed by neonatal immunization with pNP

As an independent means to assess the priming of virus-specific T cells following the neonatal injection of plasmid, we measured the production of IFNγ by the T cells. One month after the completion of immunization with pNP, the T cells were harvested and restimulated *in vitro* with NP 147-155 peptide in the presence of irradiated, haplotype-matched splenocytes. Estimation of the IFNγ concentration in the supernatants showed significantly higher values in the case of mice immunized with pNP and boosted with PR8 virus, compared to the mice immunized with PR8 virus alone (Fig. 5A). No significant IFNγ production was noted in the case of non-fractionated T cells from mice immunized as newborns with pNP alone. Enrichment of $CD8^+$ T cells by negative selection previously to *in vitro* stimulation, revealed significant amounts of IFNγ produced by the T cells from mice immunized as neonates with pNP alone (Fig. 5B). No IL-4 was detected in culture supernatants following restimulation of T cells with NP 147-155 peptide, irrespective of the immunization protocol (data not shown).

We tested the proliferation ability of the T cells from mice immunized as neonates with pNP. In the presence of NP 147-155 peptide and without IL-2 added to the culture medium, the T cells from mice immunized with pNP and boosted with PR8 virus displayed significant proliferation, as compared to the T cells from mice

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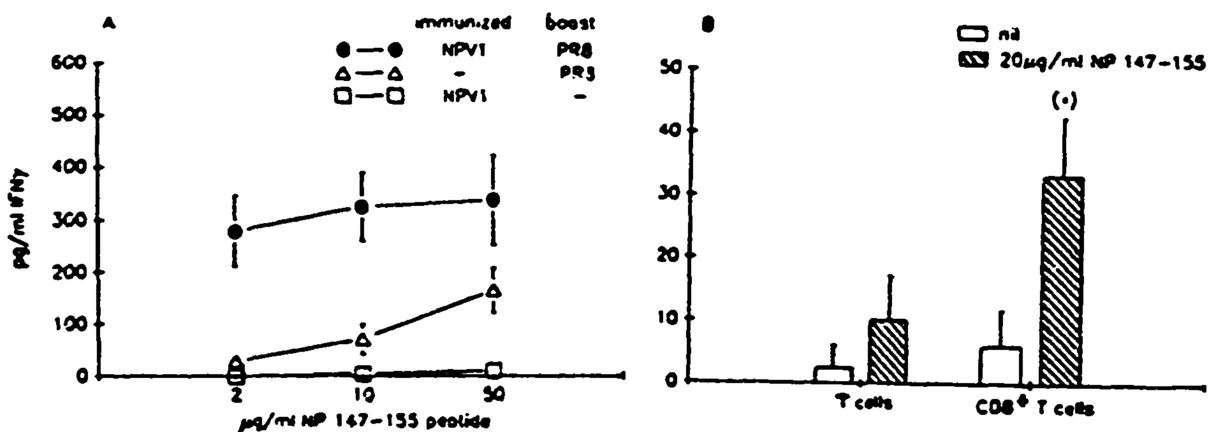

Fig. 5. IFNγ production by T cells from mice immunized as newborns with NPV1 (pNP) plasmid. T cells purified from spleens of mice immunized as newborns with pNP and boosted or not with live PR8 virus were incubated with APC in the presence of various concentrations of NP 147-155 peptide and 4U/ml of IL-2 (A). As control, we used T cells from PR8 immunized adult mice. In a parallel experiment (B), we separated by negative selection $CD8^+$ T cells from the spleens of mice immunized as newborns with pNP and restimulated them with NP 147-155 peptide. The concentration of IFNγ was estimated after 4 days by ELISA and the results were expressed as means of triplicates ± SD. (*) p of the t test < 0.05.

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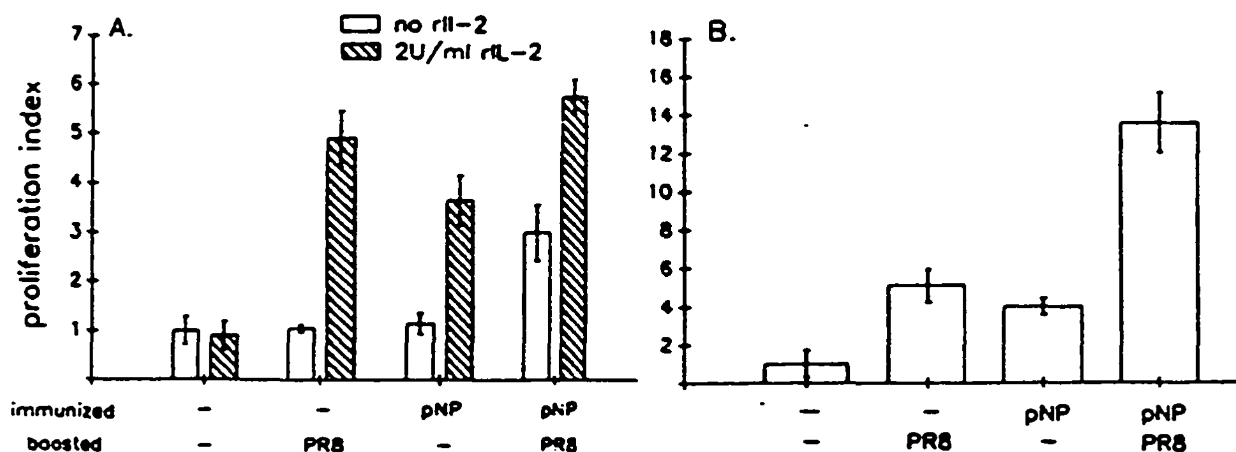

Fig. 6. Proliferation index of the T cells from mice immunized as newborns with pNP plasmid. Nylon wool purified T cells from mice immunized as neonates with pNP and boosted or not with live PR8 virus were in vitro stimulated with NP 147-155 synthetic peptide (10μg/ml), in the presence of APC with or without adding rIL-2 (2U/ml) (A). As controls, we used T cells from naïve mice or adult mice immunized with PR8 virus. In a parallel experiment, we stimulated the T cells with 4U/μg of UV-inactivated PR8 virus, in the absence of rIL-2 (B). ^{3}H-Thymidine was added after 72 hours of incubation and the radioactivity incorporated was measured next day. The results were expressed as means of proliferation index values ± SD of triplicates.

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immunized with PR8 virus or pNP alone (Fig. 6A). The addition of IL-2 to the culture medium, improved the proliferation index of the NP 147-155 specific T cells from neonates immunized with pNP alone. When restimulated with UV-attenuated PR8 virus, the T cells from mice immunized with pNP and boosted with PR8 virus displayed a high proliferation index (Fig. 6B). Furthermore, the T cells from PR8 or pNP immunized mice displayed lower but significant proliferation when stimulated with UV-attenuated PR8 virus (Fig. 6B), indicating that pNP also primed class-II, besides MHC class-I restricted T cells. This was further supported by the fact that the proliferation of T cells from mice immunized with pNP was significantly inhibited in the presence of anti-I-E^d + anti-I-A^d rat anti-mouse antibodies (data not shown).

Together, these data show that pNP inoculation of newborn mice primed NP 147-155 specific $CD8^+$ T cells with the ability to produce IFNγ. Subsequent exposure to PR8 virus resulted in significant expansion of the NP-specific T cell population.

1.4. Estimation of CTL precursor frequency subsequent to neonatal immunization with pNP

To estimate more precisely the priming effect of pNP injection in neonates and adult mice, we carried out CTL precursor (pCTL) frequency estimation by limiting dilution analysis using splenocytes from animals immunized with pNP or PR8 virus, or immunized with pNP and boosted with PR8 virus. As shown in Fig.7, the mice

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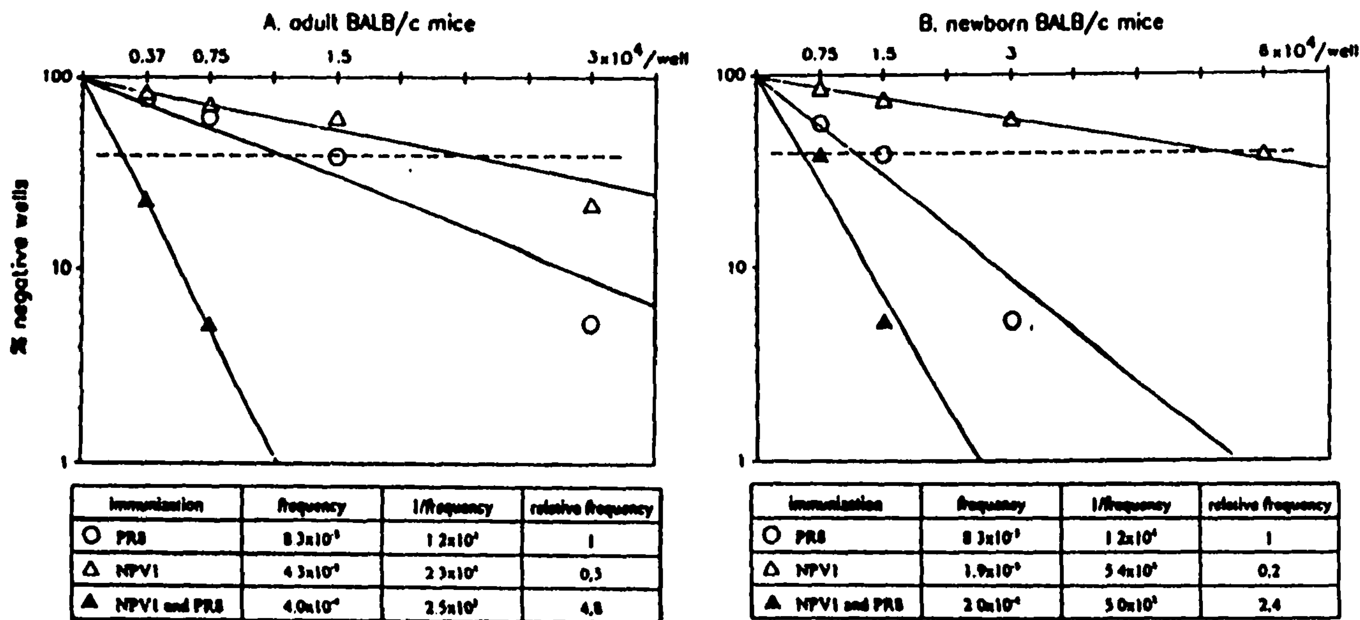

immunization	frequency	1/frequency	relative frequency
○ PR8	$8.3x10^{-5}$	$1.2x10^{4}$	1
△ NPV1	$4.3x10^{-5}$	$2.3x10^{4}$	0,3
▲ NPV1 and PR8	$4.0x10^{-4}$	$2.5x10^{3}$	4,8

immunization	frequency	1/frequency	relative frequency
○ PR8	$8.3x10^{-5}$	$1.2x10^{4}$	1
△ NPV1	$1.9x10^{-5}$	$5.4x10^{4}$	0,2
▲ NPV1 and PR8	$2.0x10^{-4}$	$5.0x10^{3}$	2,4

Fig. 7. CTL precursor frequency analysis of mice immunized as newborns or adults with NPV1 (pNP) plasmid. The frequency of PR8 specific pCTL was estimated by limiting dilution assay of splenocytes from mice immunized as adults (A) or neonates (B) with pNP, at 1 month after the completion of immunization. Part of the mice were boosted with live-PR8 virus 1 week previous to sacrification. As controls, we used adult mice immunized with PR8 virus one week previous to the study. The results are expressed as % negative wells versus number of responder cells/well. The pCTL frequency was estimated after linear interpolation, as described in Materials and Methods.

immunized as neonates or adults with pNP and boosted with PR8 virus displayed higher frequencies of PR8 virus-specific pCTLs, as compared to animals immunized with PR8 virus or pNP alone. Immunization with pNP induced a lower expansion of specific pCTLs, as compared to the live virus. Notably, the efficacy of immunization with pNP was approximately two times higher in adult mice versus neonates, as shown by the comparison of the relative frequencies (Fig. 7). Thus, neonatal injection of pNP was followed by the priming of a CTL memory population that expanded subsequently to the virus stimulation.

We studied the kinetics of the total number of PR8-specific pCTLs in the spleen of animals immunized as neonates or adults with pNP (Fig. 8). The total number of pCTLs was estimated using the pCTL frequency data from limiting dilution analysis and the total number of splenocytes. The kinetics of the pCTL expansion was slower in the case of neonates immunized with pNP (Fig. 8), indicating that the expansion of the memory pool paralleled the development of the T cell repertoire. At 1 month following the completion of immunization, the adult mice displayed the highest number of pCTLs, that was approximately half compared to the number of pCTLs induced by live-virus immunization. The mice immunized as newborns with pNP, reached the maximum number of pCTLs at three months following the completion of immunization. The CTL memory cells persisted at least 12 months in the case of mice immunized as adults and at least 6 months in that of neonates (Fig. 8). No pCTLs were detectable in the control mice injected with the plasmid lacking the viral insert (Fig. 8).

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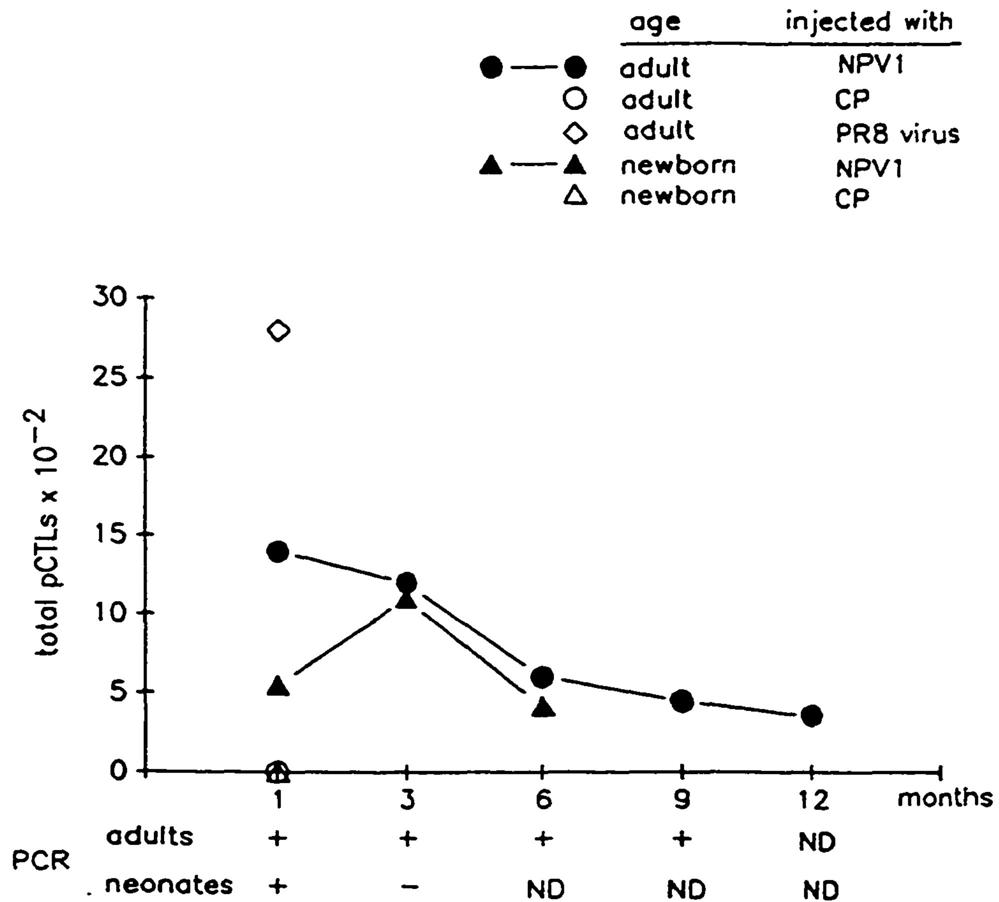

Fig.8. Kinetics of the total number of PR8 specific pCTLs in spleens of mice immunized as newborns or adults with NPV1 (pNP) plasmid. Mice immunized as newborns or adults with pNP were studied at various intervals after the completion of immunization. The total number of splenic pCTL was estimated based on the means of total number of splenocytes/mouse and the PR8-specific pCTL frequencies in splenic cell populations pooled from three mice in each group. Controls: adult or newborns inoculated with nil plasmid (CP) and PR8-immunized adult mice. In parallel, the persistence of pNP at the site of inoculation is represented: (+) if at least one mouse in that particular group was positive and (-), no positive mouse.

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1.5. Persistence of the pNP plasmid at the site of injection

We studied the presence and persistence of the pNP plasmid in the gluteal muscle and anterior tibial muscle of mice immunized as newborns and adults, respectively. While the plasmid was easily detectable at the site of injection in adult mice one month after immunization, neonates immunized with pNP displayed weak PCR signals at the age of one month (Fig. 9). At three months after the completion of immunization, some adult mice still presented detectable plasmid at the site of injection. In contrast, pNP was not detected in the mice immunized as newborns and studied three months later (Fig. 9).

These results suggest that the persistence of the plasmid at the injection site is more limited in mice injected as neonates. Whereas the plasmid persisted more than three months in some mice immunized as adults, it persisted less in all of the mice immunized as newborns. Thus, the plasmid persistence at the site of immunization did not perfectly correlate with the kinetics of pCTL number (Fig. 8).

1.6. T help dependency of CTLs primed by neonatal inoculation of pNP

Mice immunized with pNP as neonates displayed significant CTL activity following *in vitro* stimulation with PR8-infected splenocytes from naïve BALB/c mice in the presence of exogenously added IL-2 (Fig. 2,3,4). Even in the absence of exogenous

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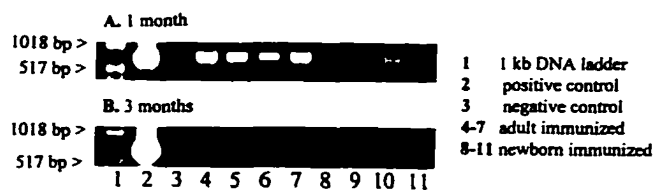

Fig. 9. Persistence of the pNP plasmid at the site of injection. The plasmid was detected by PCR analysis of DNA extracted from the tibial anterior muscle (adults, lanes: 4-7) and gluteal muscle (neonates, lanes: 8-11), at one month (A) or three months (B) since the completion of immunization. As marker we used 1kb DNA ladder (lane 1), as positive control pNP that gives a 711bp PCR product (lane 2) and as negative control, pBR322 plasmid (CP) (lane 3). The results were confirmed by a second PCR.

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IL-2, the CTLs primed by pNP were able to expand following virus stimulation and to lyse PR8 infected or peptide-coated target cells (Fig. 10). However, the *in vitro* expansion of CTLs by PR8 virus and the secondary activity against the virus and NP 147-155 peptide, were significantly increased in the presence of T cells bearing a transgenic TCR (TCR-HA) specific for HA 110-120 peptide in the context of I-E^d MHC class-II molecules (Fig. 10C and D). It is noteworthy to mention that the spleen cells from transgenic mice that were used as stimulator cells, express the TCR-HA on approximately 10% of the T cells (Kirberg *et al.* 1994). Furthermore, they secrete large amounts of cytokines when *in vitro* or *in vivo* stimulated with PR8 virus (Table 1). The transgenic mice infected with PR8 virus display a faster recruitment into the lungs and a more pronounced expansion of virus-specific MHC class-I restricted CTLs (Table 2), that is responsible for their resistance against lethal challenge (Bot *et al.*, submitted).

Depletion of $CD4^+$ or $TCR\text{-}HA^+$ T cells resulted in diminished activation of CTLs as well as impaired clearance of the pulmonary virus by the transgenic mice (Table 2). Thus, both the priming and the secondary stimulation of Influenza virus specific CTLs are under the regulation of T helper cells.

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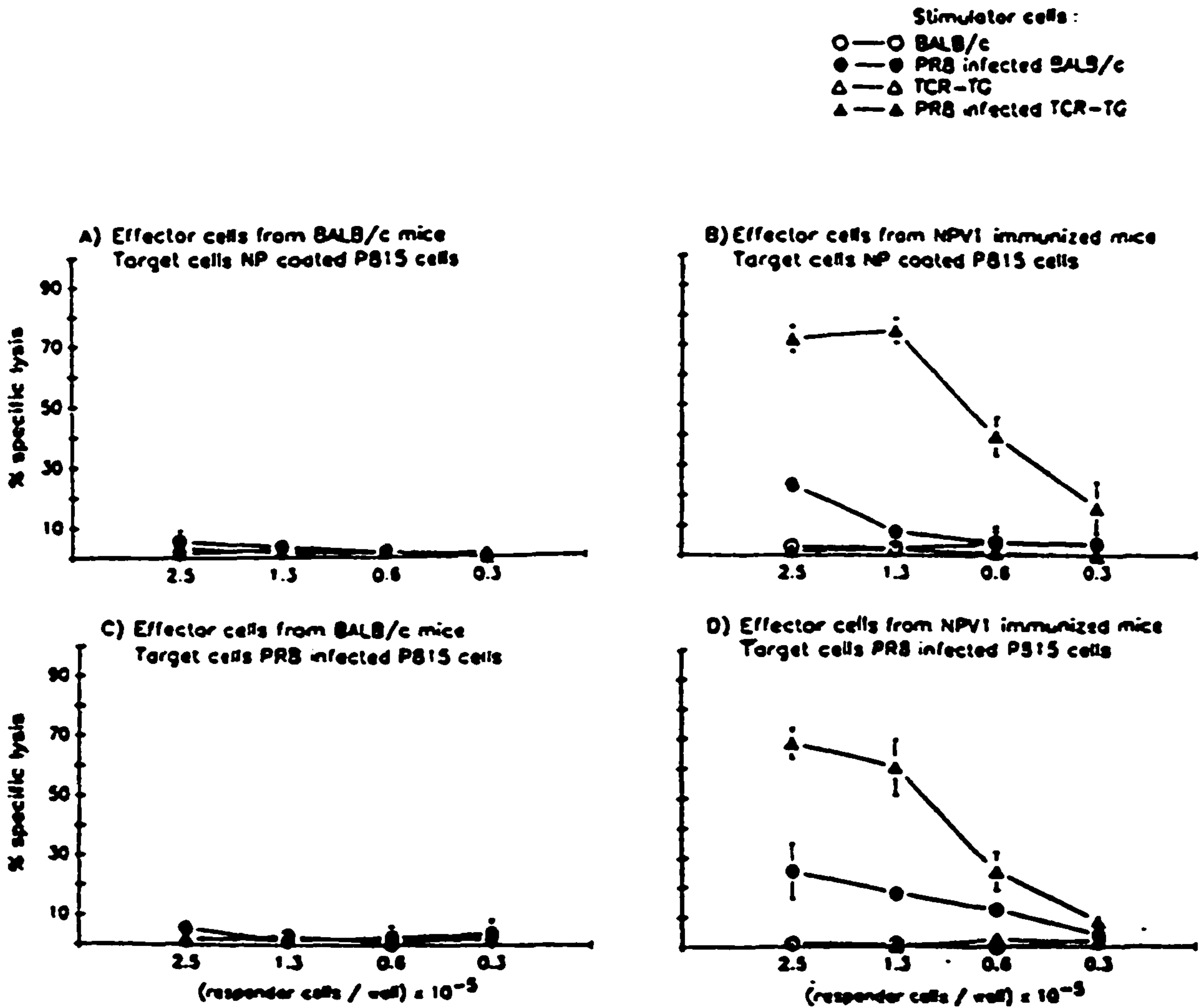

Fig. 10. T help requirement for effective expansion of CTLs induced by neonatal immunization with NPV1 (pNP) plasmid. Various numbers of splenocytes from naïve mice (A,C) or mice immunized as neonates with pNP (B,D), were stimulated with PR8-infected or non-infected splenocytes from BALB/c or TCR-HA transgenic mice. The cytotoxicity was measured against P815 target cells infected with PR8 virus (C,D) or coated with NP 147-155 peptide (A,B).

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Table 1. IFNγ and IL- 4 production following *in vitro* or *in vivo* priming of TCR-HA^{+} T cells with PR8 virus

Group	In vitro[a]		In vivo[b]			
	IFNγ	IL-4	IFNγ		IL-4	
			day3	day7	day3	day7
BALB/c mice	6±5[c]	0[d]	0	74±34	8±2	24±20
TCR-HA transgenic mice	140±25	127±18	355±2	478±70	390±48	436±88

[a] 2×10^5 T cells from transgenic mice were incubated for 72 hours with a similar number of irradiated APCs in the presence of 20μg/ml of sucrose-purified PR8 virus.

[b] 2×10^5 T cells from transgenic mice infected with PR8 virus, harvested 3 and 7 days after the challenge, were incubated with similar numbers of APC in the presence of 20μg/ml of sucrose-purified PR8 virus.

[c] Results are expressed as mean of duplicates ± SE (pg/ml).

[d] Values under the sensitivity of the assay (3 pg/ml) were considered 0.

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Table 2. T help dependency of activation and pulmonary recruitment of MHC class-I restricted CTLs

Group	Total number of MHC-I restricted pCTLs[a]				Pulmonary virus titer[b]	
	Spleen		Lungs			
	day3	day7	day3	day7	day3	day7
BALB/c	10^2	$9.8x10^2$	10	$6.4x10^2$	4.6±0.2	2.9±0.5
TCR-HA mice pretreated:						
Nil	$3.0x10^2$	$5.5x10^3$	$6.0x10^2$	$3.2x10^3$	4.3±0.6	<1.0
CD4 mAb	$1.1x10^2$	10^2	0[c]	$2.9x10^2$	3.3±0.4	3.2±0.4
CD8 mAb	0	0	0	0	3.4	2.1±0.4
6.5 mAb	$1.2x10^2$	$3.1x10^2$	20	$4.2x10^2$	3.1±0.4	3.7±0.5

[a] The total number of virus-specific pCTLs / organ was estimated by limiting dilution analysis in groups of three pooled mice.

[b] The pulmonary virus titers were estimated individually at day 3 and 7 after challenge with PR8 virus and expressed as means ± SE of $\log_{10}TCID_{50}$.

[c] No CTL activity was detected.

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1.7. Pulmonary virus titers in mice immunized with pNP as neonates and challenged with lethal doses of Influenza viruses

The protection induced by pNP in neonates and adult mice was investigated following aerosol infection with LD_{100} of PR8 or HK virus, one and three months after the completion of immunization. Pulmonary virus titers were estimated in groups of three mice at day 3, 7 and 16 after infection. As shown in Table 3, whereas the PR8 immunized adult mice displayed no virus on day 3 after homologous infection, significant pulmonary virus titers were noted on day 3 following the challenge with HK virus. Adult mice immunized with PR8 and challenged with HK virus cleared the pulmonary virus between day 3 and 7 after infection. In contrast, naïve mice or mice injected with CP failed to clear the virus and did not survive until day 16 following the challenge. In terms of virus lung titers, no significant differences were noted between mice immunized with pNP and naive mice on day 3 following homologous or heterologous challenge. In contrast, at day 7 following PR8 virus infection, significant decrease of pulmonary virus titer was noted in case of adult and newborn mice immunized with pNP, challenged at one or at three months after the completion of immunization (in all cases, p of t test <0.05). All mice immunized with pNP that survived until day 16 after infection, completely cleared the virus from their lungs.

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Table 3. Effect of immunization with pNP on pulmonary virus titer after lethal aerosol challenge with PR8 or HK influenza viruses

Animal groups	Immunization	Pulmonary virus titer ($log_{10}TCID_{50}$)		
		day 3	day 7	day 16
Challenge with PR8:				
Adult 1 month after immunization	nil	4.6±0.5	3.8±0.1	+[b]
	PR8 virus[a]	<1.0	<1.0	ND[c]
	control plasmid	4.8±0.1	3.7±0.5	+
	pNP plasmid	4.0±0.3	0.9±1.5	<1.0
Adult 3 months after immunization	pNP plasmid	4.8±0.1	<1.0	<1.0
Newborn 1 month after immunization	control plasmid	5.9±0.0	4.6±0.2	+
	pNP plasmid	4.5±1.2	1.2±2.1	<1.0
Newborn 3 months after immunization	pNP plasmid	4.1±0.5	0.9±1.2	<1.0
Challenge with HK:				
Adult 1 month after immunization	nil	6.4±0.7	5.7±0.3	+
	PR8 virus	5.7±0.3	<1.0	ND
	control plasmid	6.8±0.1	5.7	+
	pNP plasmid	5.8±0.1	<1.0	<1.0
Adult 3 months after immunization	pNP plasmid	4.3±0.4	<1.0	<1.0
Newborn 1 month after immunization	control plasmid	ND	ND	ND
	pNP plasmid	6.6±0.3	5.1±0.6	+
Newborn 3 months after immunization	pNP plasmid	5.6±0.2	<1.0	<1.0

[a] Mice were immunized i.p. with live PR8 virus 7 days previous to sacrification.

[b] No surviving mice at day 16 after challenge.

[c] *Measurement of pulmonary virus titer was not carried out.*

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Similar results were noted following heterologous challenge, except the group of mice immunized as newborns and challenged one month later, that failed to clear the virus. Interestingly, when infected at the age of three months, mice immunized with pNP as neonates and challenged with HK virus cleared the virus by day 7. Thus, the kinetics of virus reduction in lungs of mice immunized with pNP as adults or neonates is consistent with a protective role for T cell immunity, in particular the virus-specific CTLs.

I.8. Survival of mice immunized as neonates with pNP and challenged with lethal doses of Influenza viruses

We also followed the survival of pNP immunized adult and neonates after the challenge with LD_{100} of PR8 or HK virus (Fig. 11). Adult mice immunized with pNP and challenged one or three months later with PR8 virus, displayed a statistically significant survival rate (p of Fisher's test <0.05) compared with control mice non-immunized or injected with CP (Fig. 11 A,B,C). While the PR8 immunization conferred 100% protection, the percentage of survivors was lower in the case of pNP immunized mice, underlining the role of virus-specific protective antibodies. Newborns immunized with pNP displayed no significant survival one month later, after the lethal challenge with PR8 virus ($p>0.10$; Fig. 11B). In contrast, three month-old mice immunized as neonates with pNP plasmid showed a statistically significant

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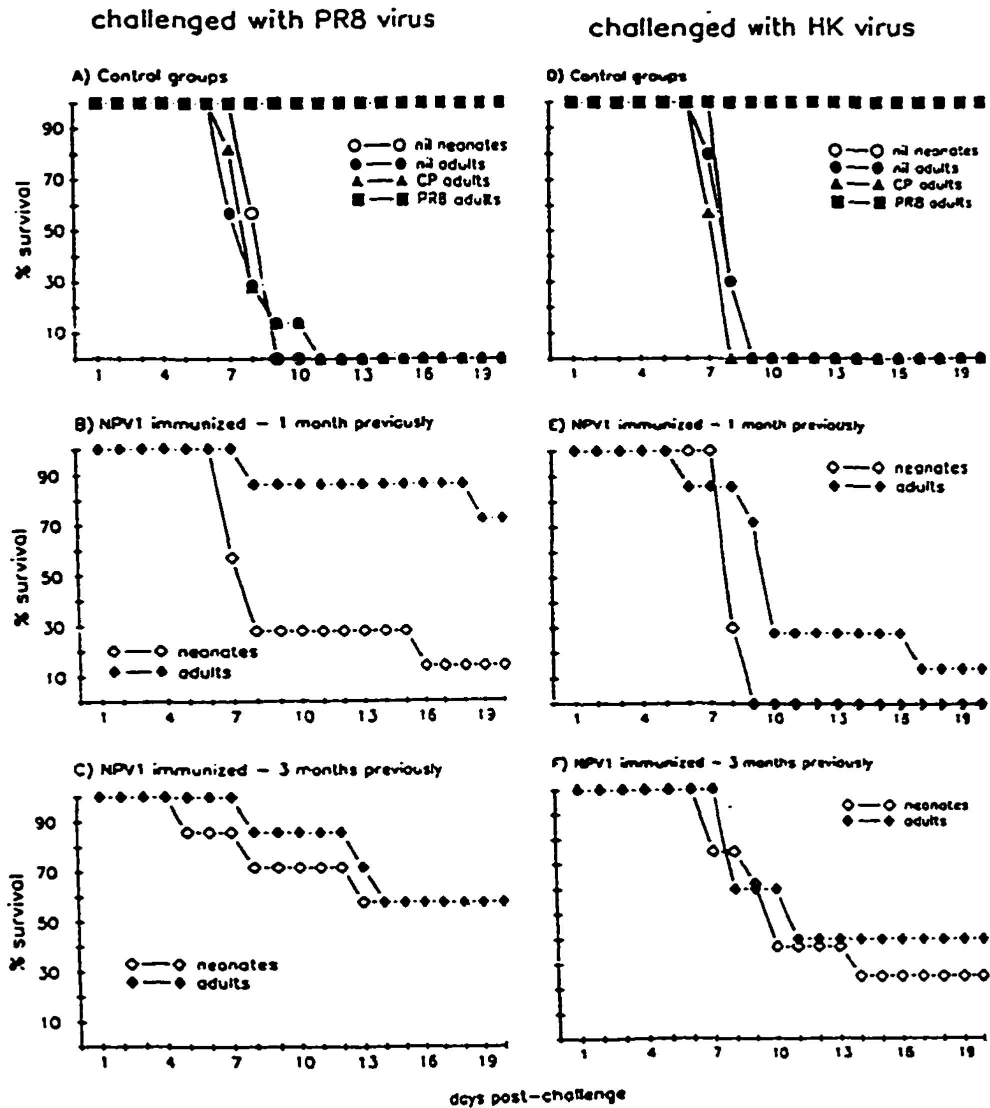

Fig.11. Survival of mice immunized with NPV1 (pNP) plasmid and challenged with lethal doses of PR8 or HK viruses. Controls are shown in the panels A and D: naïve mice, mice inoculated with control plasmid, or with live-PR8 virus one week before the challenge. The pNP immunized mice were infected with lethal doses of PR8 (B,C) or HK virus (E,F), at one (B,E) or three months (C,F) subsequent to the completion of immunization. Each group was composed of at least 7 mice.

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survival rate after infection with the same dose of PR8 virus, comparable to that noted in adult mice immunized with pNP plasmid ($p<0.05$; Fig. 11C). Lower survival rates were displayed by the mice immunized as neonates or adults with pNP and challenged with LD_{100} of HK virus (Fig. 11E, F).

1.9. Local recruitment of effector cells in mice immunized as newborns with pNP and challenged with PR8 virus

In order to define the local protective mechanisms triggered by the pNP immunization of newborn mice, we estimated the number of PR8 specific pCTLs in the lungs of 3 month-old mice following aerosol challenge. Compared with non-immunized age-matched BALB/c mice, the mice immunized as neonates with pNP plasmid displayed increased numbers of specific pCTLs both in lung and spleen (Table 4). On a ratio basis, the difference was more important in lungs at day 3 after infection, suggesting that an early local recruitment of specific CTLs plays important roles in effective recovery from viral pneumonia and limitation of DTH lesions that otherwise lead to severe respiratory insufficiency and death.

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Table 4. Frequency of CTL precursors in lungs and spleen of 3 month-old mice challenged with a lethal dose of PR8 Influenza virus

Group	Day post-challenge	Spleen		Lung	
		1/frequency[a]	total[b]	1/frequency[a]	total[b]
BALB/c immunized with pNP[c]	3	1.3×10^5	10^3	6.3×10^4	2×10^2
	7	2.4×10^4	4.2×10^3	5.2×10^3	2×10^3
BALB/c non-immunized	3	6.6×10^5	10^2	5.5×10^5	10
	7	6.3×10^4	9.8×10^2	9.4×10^3	6.4×10^2

[a] Responder cells were pooled from 3 mice in each group and pCTL frequency was estimated by limiting dilution analysis.

[b] Total pCTL number/organ was estimated taking into account the total number of separated cells.

[c] Mice were immunized with 3x30μg of pNP at day 1, 3 and 6 after birth.

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1.10. Conclusion

Injection of newborn mice with pNP led to the priming of CTLs with the ability to lyse virus-infected cells (Fig. 2 and 3) and to secrete IFNγ following restimulation with NP 147-155 peptide in context of MHC-I molecules (Fig. 5). The estimation of virus-specific pCTL frequency in spleens of mice immunized with pNP and boosted with virus (Fig. 7) indicated that CTLs primed during the early period of life have the ability to proliferate upon subsequent antigen exposure. The pCTL frequency of mice immunized with pNP and boosted with live PR8 virus was on the same order of magnitude with the virus specific pCTL frequency of mice immunized with an H3N2 subtype of Influenza virus and boosted with an H1N1 virus (Bot *et al.* submitted), namely $1/10^4$, indicating an optimal priming by the plasmid. This conclusion was confirmed by in vitro proliferation of the T cells from mice immunized as newborns with pNP, following restimulation with PR8 virus or NP 147-155 peptide (Fig. 6). Furthermore, the CTLs primed by neonatal immunization with plasmid, were promptly recruited into the lungs of mice infected with virus and contributed to the viral clearance and to the increased survival following lethal infectious challenge (Table 4). Th cells displayed the ability to greatly enhance the expansion of MHC class-I restricted CTLs (Fig. 10). Taken together, the data show that newborn mice immunized with a plasmid encoding NP of PR8 virus, develop specific CTL immunity like the adult mice.

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CTLs primed by neonatal immunization with pNP displayed a similar specificity to those induced by adult immunization. This conclusion is supported by the presence of CTLs specific for the immunodominant NP 147-155 epitope, previously characterized in adult mice (Taylor *et al.* 1987) and by the cross-reactivity against two virus-strains of a distinct subtype (Fig. 4).

Quantitative rather than qualitative differences in terms of CTL immunity were noted between mice immunized with pNP as neonates or adults, respectively. The estimation of pCTL frequency in spleens of mice immunized as adults or neonates with pNP, showed a slower expansion of the virus-specific population in the later (Fig. 8). This seems to be due to the immaturity of the neonatal immune system, since the plasmid was detected at 1 month after the completion of immunization, in both age groups (Fig. 9). Furthermore, the 3 month-old mice immunized as neonates with pNP displayed an enhanced protection that correlated with the increased number of virus-specific pCTLs in the spleen, compared to the 1 month-old mice (Fig. 8 and 11). A slower expansion of the virus-specific CTL pool in the immunized neonates may explain the reduced protection of the 1 month-old mice compared to the 3 month-old mice as well as the adult mice challenged with LD_{100} of PR8 or HK virus.

In spite of the cross-reactivity of CTLs induced by pNP immunization, a reduced heterologous protection was demonstrated in mice immunized with pNP as adults or neonates (Fig. 11). This may be due to a reduced magnitude of the cross-reactive immunity conferred by the whole NP or more probably, to a faster replication rate of the HK virus in the lungs. The discrepancy with a previous study which

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showed complete heterologous protection (Ulmer *et al.* 1993), may be explained by differences in the method and the dose of infection. It is known that aerosol challenge leads to an extensive infection of upper and lower respiratory tract that causes fatal DTH lesions of the lungs (Sullivan et al., 1976), even in the case of virus clearance. Thus, our results showed that even if most of the mice immunized with pNP cleared the pulmonary virus by day 7 following lethal homologous or heterologous challenge (Table 3), few of them survived and completely recovered from a clinical standpoint (Fig. 11). Effective early recruitment of the virus-specific CTL at the site of infection leads to rapid limitation of infection, prompt decrease of pulmonary virus titer and reduced DTH lesions. While a slower recruitment of specific CTLs may be still followed by a reduction of virus titer, DTH lesions tend to be more extensive and may cause the death of the animal.

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2. Neonatal immunization with a plasmid expressing HA

We investigated the ability of a plasmid (pHA) expressing HA of the A/WSN/33 strain of Influenza virus to induce humoral and cellular immune responses following neonatal inoculation into BALB/c mice. Influenza virus HA expresses four dominant B cell epitopes (Canton *et al.* 1982) known to induce protective antibodies (Haberman *et al.* 1990) and at least three immunodominant epitopes recognized by $CD4^+$ T cells (Eisenlohr *et al.* 1988), as well as additional minor epitopes recognized by $CD4^+$ or $CD8^+$ cytotoxic T cells (Braciale *et al.* 1987). We inoculated the newborn mice three times on day 1, 3 and 6 after birth, with a dose of 30µg of plasmid. As control plasmid (CP) for this study we used the pHA vector lacking the viral insert. Some 1 day-old newborn mice were inoculated with live WSN virus and we found that at doses higher than 1µg/mouse, the live virus is constantly 100% lethal. At doses equal or less than 1µg/mouse, the number of surviving mice varied with the batch of virus. Adult mice were inoculated with 30µg of plasmid/dose, three times at three weeks interval. The immunization of adult mice with live virus was carried out as shown in *Materials and Methods.*

2.1. Humoral response subsequent to neonatal inoculation with pHA

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The antibody responses of mice injected with pHA as adults or newborns were studied at various intervals following the completion of immunization, as well as 7 days after the boost with live WSN virus. The data depicted in Table 5 show that the majority of adult animals immunized with pHA mounted significant hemagglutination inhibiting (HI) titers at 1 and 3 months after the immunization (12 out of 16 at 1 month and 5 out of 8 at 3 months following completion of immunization) but only 2 out of 9 mice displayed HI antibodies 6 months after immunization. No HI antibodies were detected 9 months after i.m. immunization with plasmid, although the mice responded normally to the virus boost (data not shown). Injection of adults or newborns with CP did not elicit the production of anti-WSN antibodies. In the case of animals immunized as newborns with pHA, significant HI titers of antiviral antibodies were observed in 12 out of 19 mice at one month and 3 out of 4 mice at 3 months after immunization (Table 5).

All mice immunized with pHA as neonates or adults, were able to mount significant secondary humoral responses following the boost with WSN virus, indicating that the prolonged exposure to antigen did not compromise the B cell repertoire. Similar results were obtained by RIA that estimated the titer of the specific antibodies, using microtiter plates coated with WSN virus (Casares *et al.* 1997b).

These results suggested that the immunization with pHA of adult as well as newborn mice not only generated antibody-producing cells, but B memory cells as well. This hypothesis is supported by the isotype analysis of the virus-specific antibodies at 1 month after the plasmid immunization (Fig 12). The isotype profile

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Table 5. Hemagglutination inhibiting titers of sera from BALB/c mice immunized with pHA plasmid or WSN virus

Mice immunized as	Immunization with	No. of mice	Preimmunization titer		Time of bleeding (months)	Titer against		No. of responders		Titer at 7 days after WSN boost, against	
			WSM	PR8		WSN	PR8	WSN	PR8	WSN	PR8
Adults	WSN	5	0[a]	0	1	8.2 ± 1[b]	1.2 ± 0.8[b]	5/5	5/5	8.2 ± 1.3	2.2 ± 1.6
	CP[c]	3	0	0	1	0	1.0 ± 0.7	0/3	1/3	0	0
	CP	3	0	0	1	0	0	0/3	0/3	7.3 ± 5.3	1.3 ± 2.3
	pHA	16	0	0	1	5.5 ± 3.4	0	12/16	0/16	8.3 ± 1.5	1.0 ± 1.9
	pHA	8	0	0	3	8.7 ± 3.8	0	5/8	0/8	8.3 ± 1.5	2.0 ± 2.0
	pHA	9	0	0	6	1.0 ± 0	0	2/9	0/9	8.3 ± 0.6	1.3 ± 0.6
Newborns	CP	5	ND[d]	ND	1	0	0	0/5	0/5	7.0 ± 0.8	0
	pHA	19	ND	ND	1	5.2 ± 2.7	0	12/19	0/19	9.4 ± 0.9	2.0 ± 1.6
	pHA	4	ND	ND	3	3.3 ± 1.5	0	3/4	0/4	8.8 ± 2.9	3.2 ± 2.5

[a]0 = HI titers less than 1:40.
[b]Data expressed as mean ± SD of log_2 dilution of HI titer. Between three and 19 mice in each group were assessed before and following the virus boost.
[c]This control group was not boosted with WSN virus.
[d]ND, not done.

following primary immunization with WSN virus is clearly distinct from that of animals immunized with pHA and boosted with WSN virus, that is characteristic of a secondary response, thus demonstrating the priming effect of pHA administered to newborn or adult mice. While IgM dominated the primary response of animals immunized with virus, Ig(G2a+G2b) dominated the response of adults and newborns immunized with pHA. Significant differences regarding the isotype profile of WSN-specific antibodies were noted between the mice immunized with pHA as adults or neonates and boosted with virus. Whereas Ig(G2a + G2b) still dominated the response of the mice immunized with pHA as adults and boosted with WSN virus, Ig(G3+G1) predominated in the mice injected with pHA as neonates and boosted with virus 3 weeks later (Fig. 12). Furthermore, the IgG2b antibody titer was significantly suppressed in mice immunized as neonates with pHA and boosted with virus. Interestingly, the titer of IgG2a antibodies was no suppressed after the virus boost, in mice immunized as neonates with pHA. These results suggested the involvement of distinct but overlapping subpopulations of virus-specific Th cells in adults and neonates immunized with pHA and boosted with WSN virus.

It is noteworthy that small, but significant titers of IgA anti-WSN antibodies were observed in animals immunized with pHA as adults or newborns, both before as well as after the virus boost.

Thus, immunization of adult and newborn mice with a plasmid expressing the HA of WSN virus, elicited a humoral response that was characterized by WSN-specific HI antibodies of various isotypes, including α.

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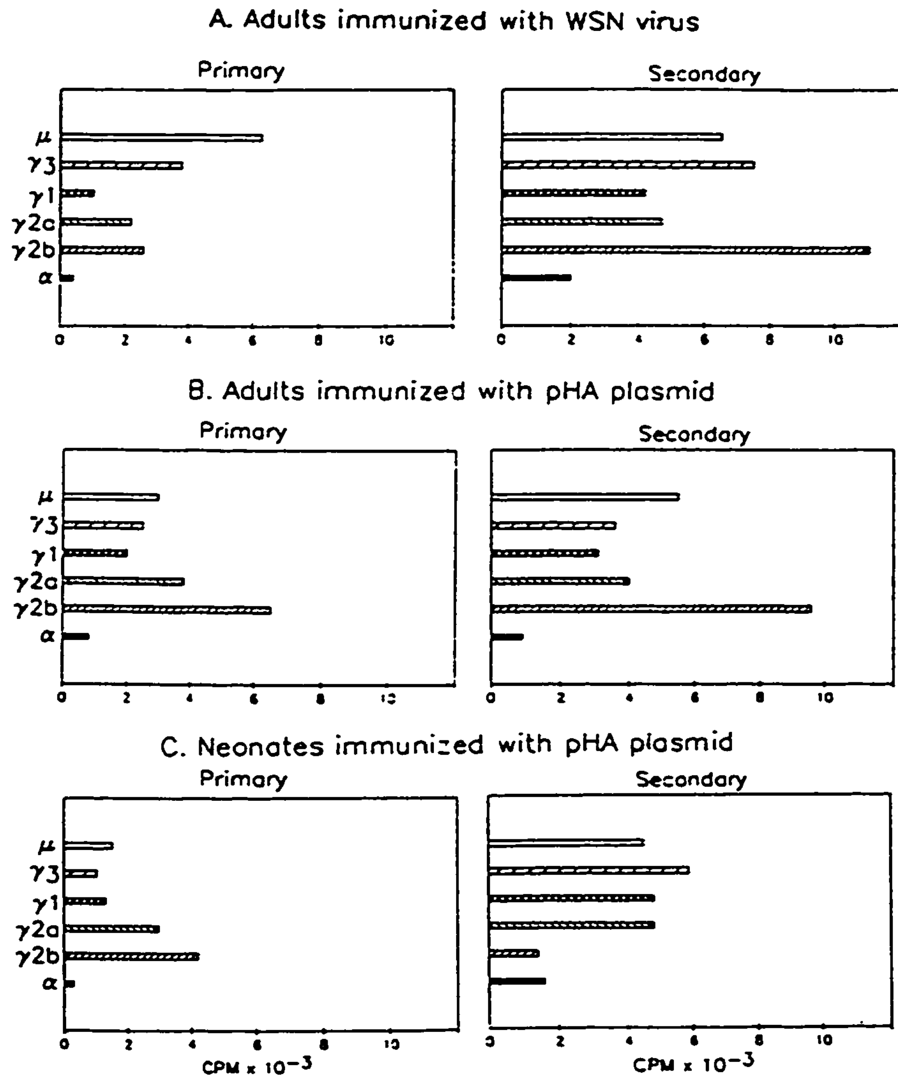

Fig. 12. Isotype profiles of anti-WSN antibodies in sera of mice immunized as adults (B) or newborns (C) with pHA plasmid. As controls, we used adult mice immunized with live WSN virus (A). Blood was harvested 7 days after primary immunization with virus (A - left panel) or three weeks after the completion of immunization with plasmids (B,C - left panels). The second blood harvest was carried out 7 days after the virus boost (A,B,C - right panels). Three to five mice in each group were studied and the isotypes were determined by RIA. The results are expressed as means of cpmx10^{-3} of individual values. SE was smaller than 25% of the mean, in each case.

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2.2. T helper immunity induced by neonatal inoculation of pHA

In addition to yielding epitopes recognized by B cells, influenza virus HA also carries epitopes recognized by T cells (Braciale *et al.* 1987). The lymphokines produced by Th cells play an important role in the regulation of growth and differentiation of B cells and in recruitment of various effector cells mediating host defense reactions (Mosmann *et al.* 1986). Thus, we investigated the cellular responses elicited by the immunization of newborns and adults with pHA. We analyzed the lymphokine production by negatively selected $CD4^+$ T splenocytes from adults or newborns immunized with CP, pHA or WSN virus and boosted or not with virus three weeks later (Table 6). Seven days after the boost with WSN virus, the $CD4^+$ T cells from adult animals injected with CP produced both IFN-γ and IL-4 and the amount of IFN-γ was significantly enhanced subsequent to *in vitro* stimulation with virus. Even without *in vitro* antigen stimulation, the T cells from mice immunized with WSN live-virus continued to produce cytokines, consistent with the presence of Th effector cells in the spleens. The animals immunized as adults with pHA displayed a Th1-biased profile following the *in vitro* stimulation with virus, which persisted after the virus boost. The *in vivo* exposure of adult mice to WSN virus led to the induction of both Th1 and Th2 cells and a subsequent boost favored the expansion of Th2 cells that secreted IL-4 (Table 6).

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Table 6. Lymphokine production by CD4$^+$ T cells from mice immunized with pHA plasmid or WSN virus

Group		Lymphokines	Adult mice		Newborn mice	
Immunization	Boost		Nil[a]	WSN[a]	Nil[a]	WSN[a]
Nil	-	IFN-γ	0	0	ND	ND
	-	IL-4	0	0	ND	ND
CP	-	IFN-γ	0	11 ± 5[b]	14 ± 5	22 ± 3
	-	IL-4	0	0	0	0
	WSN	IFN-γ	24 ± 1	158 ± 4	89 ± 28	261 ± 26
	WSN	IL-4	236 ± 11	79 ± 19	198 ± 5	141 ± 39
pHA	-	IFN-γ	9 ± 1	60 ± 2	0	29 ± 18
	-	IL-4	0	0	2 ± 2	6 ± 3
	WSN	IFN-γ	19 ± 3	284 ± 10	38 ± 8	179 ± 50
	WSN	IL-4	54 ± 3	31 ± 4	138 ± 4	257 ± 24
WSN	-	IFN-γ	52 ± 2	214 ± 11	103 ± 30	51 ± 8
	-	IL-4	48 ± 3	181 ± 3	132 ± 6	248 ± 20
	WSN	IFN-γ	10 ± 1	127 ± 3	9 ± 5	61 ± 12
	WSN	IL-4	218 ± 4	235 ± 12	228 ± 8	594 ± 5

[a]Nylon wool non-adherent splenocytes (1.5×10^5) from three mice in each group were incubated for 4 days with 1.5×10^5 irradiated BALB/c splenocytes with or without UV-inactivated WSN virus (5 μg/ml) in the presence of 1 U/ml exogenous IL-2.

[b]Concentration of cytokines in supernatant was determined by ELISA and expressed as pg/ml. Values below background +3 SD were considered 0.

The inoculation of neonates with live WSN virus, induced a Th2 response that was enhanced following the virus boost. Interestingly, the pHA immunization of neonates induced the differentiation of Th cell precursors into both Th1 and Th2 cells, the latter becoming dominant following the virus boost. Thus, whereas pHA immunization primed Th1 cells in the adult mice, it led to a mixed Th1/Th2 response in the neonates. In contrast, the administration of low doses of highly-replicative neurovirulent WSN virus (Li *et al.* 1993), led to the priming of a mixed Th1/Th2 response in adults and to a strongly-biased Th2 response in neonates.

2.3. CTL responsiveness of mice immunized with pHA

In order to assess the effect of pHA immunization on the generation of specific CTL activity, we studied the primary and secondary cytotoxicity of splenocytes against WSN-infected target cells. Figure 13 (A and C) depicts the results of primary cytotoxicity, namely the ability of freshly separated CTLs to lyse target cells. Lymphocytes from mice immunized with WSN virus, pHA or CP and not boosted, were largely unable to lyse WSN-infected target cells in the primary assays. In contrast, the animals boosted with WSN virus displayed significant primary cytotoxicity. Subsequently to the *in vitro* exposure to WSN virus (Fig. 13D), lymphocytes from all groups of adult mice except those immunized with CP or pHA and not boosted, displayed significant CTL activities. Interestingly, the neonates immunized with WSN virus, whether boosted or not with virus, displayed

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significantly decreased secondary CTL activities (Fig. 13B). This suggested that neonatal inoculation with 1μg of WSN virus led to a reduced responsiveness of the specific pCTL precursors. This was not due to the induction of neutralizing antibodies since the pCTL precursors differentiated to effector cells in adult mice (Fig. 13) and secondly, because neonatal immunization with WSN virus induces B cell unresponsiveness (Antohi *et al.* submitted). Significantly, the pHA immunization of neonates was not followed by CTL unresponsiveness to WSN virus. A more quantitative assessment of cytotoxicity carried out by pCTL frequency estimation, confirmed the results shown in Fig. 13 (Bot *et al.* 1997b).

Thus, whereas WSN virus inoculation of neonates had detrimental effects on the subsequent generation of specific CTLs, pHA immunization did not impair the cytotoxic response in the case of newborn as well as adult mice.

2.4. Persistence of pHA plasmid at the site of inoculation

In order to assess the persistence of pHA plasmid at the site of injection, we harvested the inoculated muscles at various intervals following the completion of immunization. The plasmid was still detected at 6 months after the completion of immunization, in the mice immunized as adults with pHA (Fig. 14). Interestingly, pHA persisted at least 3 months in the mice immunized as newborns. This contrasts with the limited persistence of the pNP plasmid following neonatal inoculation (Fig. 10).

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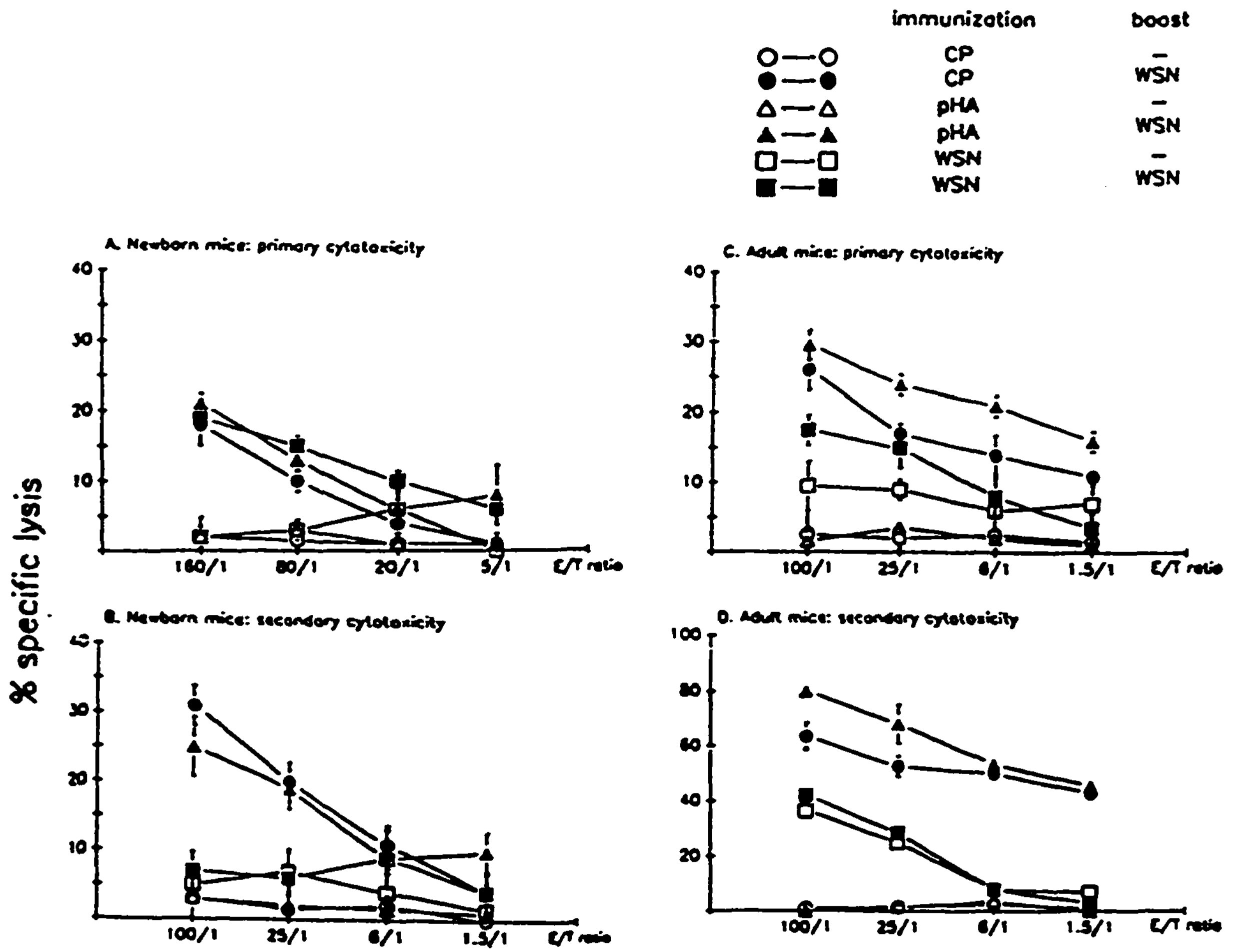

Fig.13. Primary and secondary CTL profiles of splenocytes from mice immunized as newborns (A,B) or adults (C,D) with pHA plasmid or WSN virus. Primary cytotoxicity (A,C) was carried out using as effectors, freshly isolated splenocytes harvested 4 weeks after the completion of immunization with pHA. Part of the mice were boosted with live WSN virus, one week previous to the sacrification. Secondary cytotoxicity (B,D) was carried out with effector cells in vitro stimulated with WSN virus. Results are expressed as means of % specific lysis values ± SE, for 3-5 mice in each group.

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2.5. Protection against lethal challenge with Influenza virus subsequent to neonatal immunization with pHA

The antibody and cellular responses correlated with decreased viral titers in the lungs of animals immunized with WSN virus or pHA and challenged with LD_{100} of WSN virus or the PR8 drift variant (Table 7). Only slight decreases of virus lung titers were evident at day 3 following WSN or PR8 virus challenge of mice immunized with pHA, compared to non-immunized mice or those injected with CP. In contrast, significant decreases of virus titers were noted at day 7 following the challenge of mice immunized with pHA as neonates or adults ($p<0.05$). Moreover, the animals immunized with pHA that survived by day 16 after the challenge with WSN virus, displayed no detectable pulmonary virus titers. These results suggest that both humoral and cellular responses contribute to the protection induced by pHA immunization of newborn or adult mice.

After the challenge with LD_{100} of WSN virus, all adult mice immunized with WSN live virus and 60% of the mice immunized with pHA survived ($p<0.05$), whereas all mice non-immunized or injected with CP died between 8 and 11 days after the infection (Fig. 15A). A statistically significant cross-protection conferred by pHA, was observed when similar groups of animals were challenged with LD_{100} of PR8 virus (Fig. 15B).

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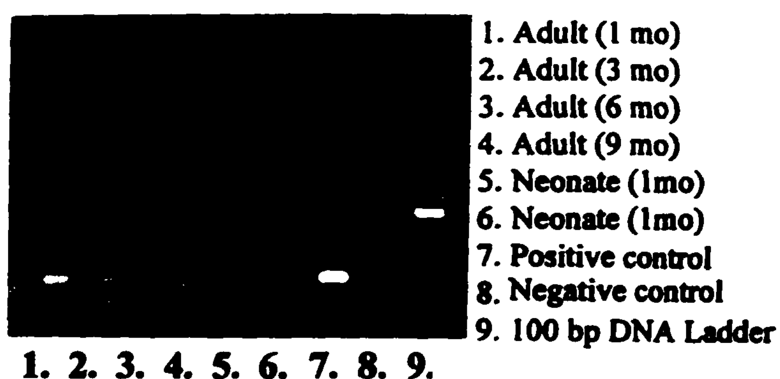

Fig.14. Persistence of the pHA plasmid at the site of injection. The plasmid was detected by PCR of DNA isolated from the anterior tibial muscles (adult mice) or gluteal muscles (neonates), harvested at various intervals after the completion of immunization. At least three mice in each group were tested and representative results are shown. As positive control we used pHA and as negative control pRc/CMV plasmid (CP). Lane 9: 100bp DNA ladder.

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Table 7. Effect of immunization with pHA plasmid on pulmonary virus titer measured after challenge with lethal doses of WSN or PR8 virus

Mice immunized as	Immunization with	Challenge with 3×10^7 $TCID_{50}$ WSN			Challenge with 1.5×10^4 $TCID_{50}$ PR8		
		3 day	7 day	16 day	3 day	7 day	16 day
Adults	nil	5.4 ± 0.7[a]	3.7 ± 0.3	+[b]	6.4 ± 0.7	5.7 ± 0.3	+
	WSN virus	0	0	ND[c]	0	0	ND
	CP	4.9 ± 0.5	2.8 ± 0.5	+	6.8 ± 0.1	5.7	+
	pHA	2.0 ± 2.2	1.4 ± 0.8	0	2.7	0	ND
Newborns	CP	4.2 ± 0.5	+	+	ND	ND	ND
	pHA	4.0 ± 0.6	0	0	4.7 ± 0.9	0.8 ± 1.4	ND

[a]Data expressed as means ± SD of $\log_{10}$ viral titer in $TCID_{50}$ units. Lungs were removed from three or four mice in each group at various intervals after infection and virus pulmonary titers were measured in a standard MDCK assay.
[b]No survivors on day 16 after infection.
[c]ND, not done.

Approximately fifty percent of the 1-month-old mice immunized with pHA as newborns, survived after aerosol challenge with LD_{100} of WSN virus and 30% survived after cross-challenge with PR8 virus. In contrast, 1 month-old non-immunized or CP injected mice died by day 9 after the challenge with LD_{100} of Influenza virus (Figure 15 C,D). One month-old mice immunized with WSN virus one week previously, survived the infection with PR8 or WSN virus (Fig. 15 C,D).

The mice challenged via aerosols with lethal doses of live Influenza virus die subsequent to the pneumonia due to the spread of virus in the lung tissue followed by the associated DTH reaction. These mice also display weight loss (Ulmer *et al.* 1993). Whereas the pHA-immunized mice that survived the lethal challenge displayed no significant or transient weight loss, the mice that did not survive progressively lost approximately one third of their weight and died (data not shown). Thus, the pHA immunization of adult and newborn mice conferred comparable protection in terms of survival following lethal challenge with the homologous virus or the PR8 drift variant.

2.6. Conclusion

To study the humoral and Th response of the neonates to DNA vaccines, we injected

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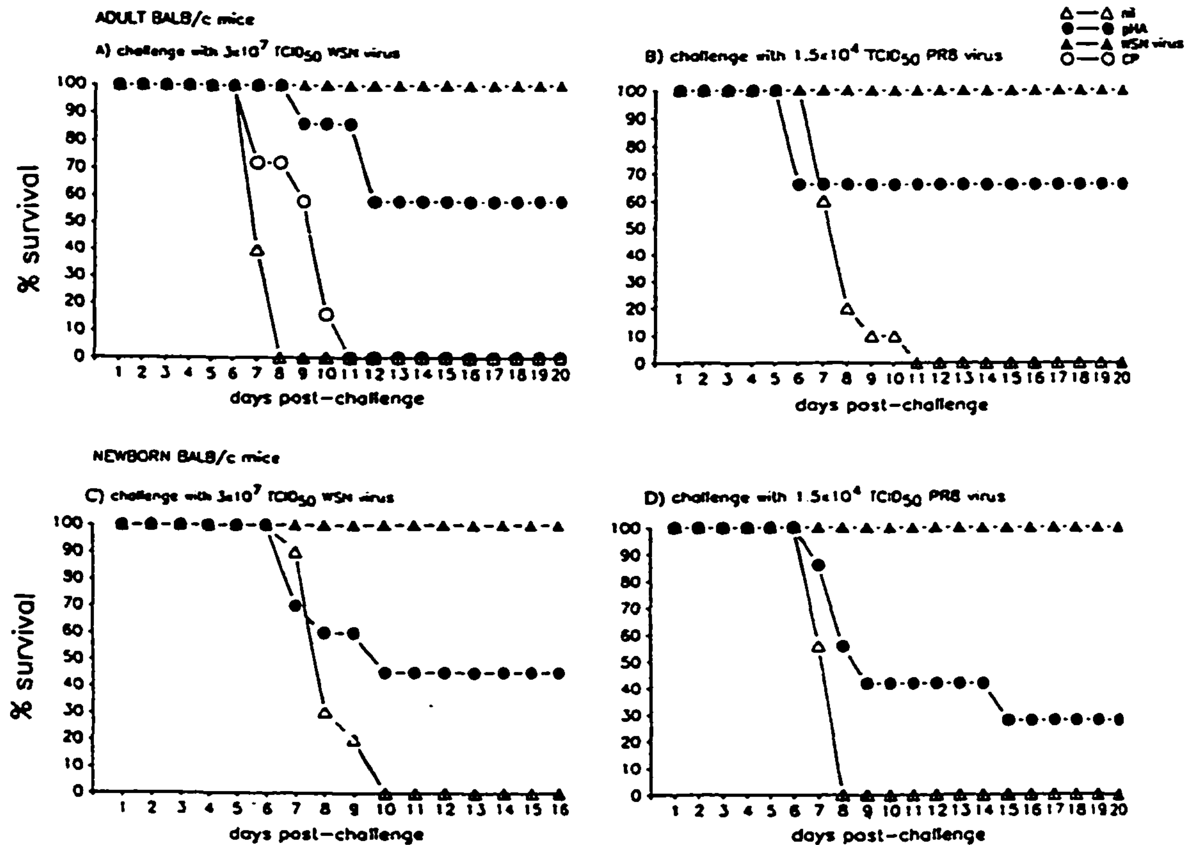

Fig.15. Survival of mice immunized with pHA as adults (A,B) or neonates (C,D) and challenged with lethal doses of WSN (A,C) or PR8 (B,D) viruses. As controls, we used mice inoculated with CP, naïve mice and adult mice immunized with live-WSN virus one week previous to the challenge. Each group consisted of at least six mice.

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newborn mice with a plasmid expressing the HA from A/WSN/33 virus, driven by the initial-early CMV promoter. The data showed that like the adult mice, the neonates are primed rather than tolerized following inoculation of pHA. The neonatal immunization with pHA induced:

a) specific B cells secreting neutralizing antibodies (Table 5);

b) Th1 cells, as indicated by the isotype pattern of the specific antibodies (Fig. 12) and Th2 precursors, that were relevant subsequently to the virus boost (Fig. 12 and Table 6). Furthermore, whereas pHA immunization of neonates did not impair a subsequent CTL response to the live-virus, the injection of WSN virus into the neonates was followed by decreased cytotoxicity (Fig. 13).

The humoral and cellular immune responses triggered by the neonatal immunization with pHA exhibited protective abilities similar to those of adult mice, manifested by clearance of the pulmonary virus (Table 7) and significant survival following lethal challenge with the homologous virus or the drift variant, PR8 virus (Fig. 15).

Studies carried out in our laboratory aimed at characterizing the reactivity pattern of the B cell clonotypes showed that the pHA immunization of neonates expanded WSN-specific as well as cross-reactive clonotypes, like in the case of adult mice immunized with WSN virus or pHA plasmid (Antohi *et al.* submitted). Thus, the DNA immunization circumvented the poor ability of the neonates to mount cross-reactive humoral responses to the Influenza virus, due to the restricted B cell repertoire at this stage of development (Cancro *et al.* 1979). However, the data

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regarding the humoral response (Table 5) as well as the pattern of virus clearance after the infection (Table 7), suggest that the T cell immunity plays an important role in the protection of mice immunized as newborns with pHA.

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3. Neonatal immunization with a mixture of plasmids expressing HA and NP

Previous studies showed that the neonatal immunization of BALB/c mice with plasmids expressing NP or HA of Influenza virus was followed by the priming of B, Th and cytotoxic cells, rather than tolerance (Bot *et al* 1996a, 1997b). However, the protection in terms of survival against lethal challenge with homologous or heterologous strains of Influenza virus, was not complete (Bot *et al* 1997a, 1997b). Further, in the case of NP expressing plasmid, the protective immunity required a longer time to develop following neonatal inoculation, as compared to adult immunization (Bot *et al* 1997c).

In an effort to improve the protection conferred by plasmid vaccines, we coinjected pHA together with pNP in newborn or adult mice. We challenged the mice at the age of 5 weeks with LD_{100} of WSN virus or the drift variant, PR8 virus.

3.1. CTL and Th induced by neonatal inoculation of pHA+pNP or UV-attenuated WSN virus

We studied the cytotoxic immunity and the cytokine profile of the T cells from mice immunized as neonates with pHA+pNP, or from mice immunized with UV-attenuated WSN virus. Splenocytes from mice immunized as neonates with UV-inactivated virus, did not exhibit cytotoxicity against a panel of type A Influenza viruses or

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against the dominant NP K^d epitope, following *in vitro* stimulation with PR8-infected APCs (Fig. 16A). In contrast, the neonatal immunization with pHA+pNP primed a significant cytotoxic response against H1N1 strains like PR8 and WSN, against HK that is an H3N2 strain and against the dominant cross-reactive CTL epitope, namely NP 147-155 (Fig. 16B). No response was detected against a type B virus or a peptide that binds to D^b instead of K^d class-I molecules.

The T helper profile was assessed following separation of $CD4^+$ T cells from 5 week-old mice immunized as neonates with pHA+pNP, UV-attenuated WSN virus or naive. The $CD4^+$ T cells were in vitro stimulated with a panel of sucrose-purified UV-attenuated viruses in the presence of exogenous IL-2 that greatly increased the ratio of signal over background. The $CD4^+$ T cells from mice immunized as newborns with pHA+pNP secreted significant amounts of IFNγ but no IL-4 when restimulated with PR8 or WSN viruses (Fig. 17A). Interestingly, the $CD4^+$ T cells from mice immunized as newborns with UV-attenuated WSN virus secreted besides IFNγ, significant amounts of IL-4 following restimulation with PR8 or WSN virus. In fact, even in the absence of specific antigen, the IL-2 added to the culture media was sufficient to trigger significant production of IL-4 by the $CD4^+$ T cells from mice immunized as neonates with UV-attenuated WSN virus. It is possible that part of the IL-4 produced by the T cells from the neonates injected with UV-inactivated WSN virus, may be accounted by a subpopulation of lymphocytes specific for bovine-serum contaminants, although we have minimized this effect by using sucrose-purified virus for immunization.

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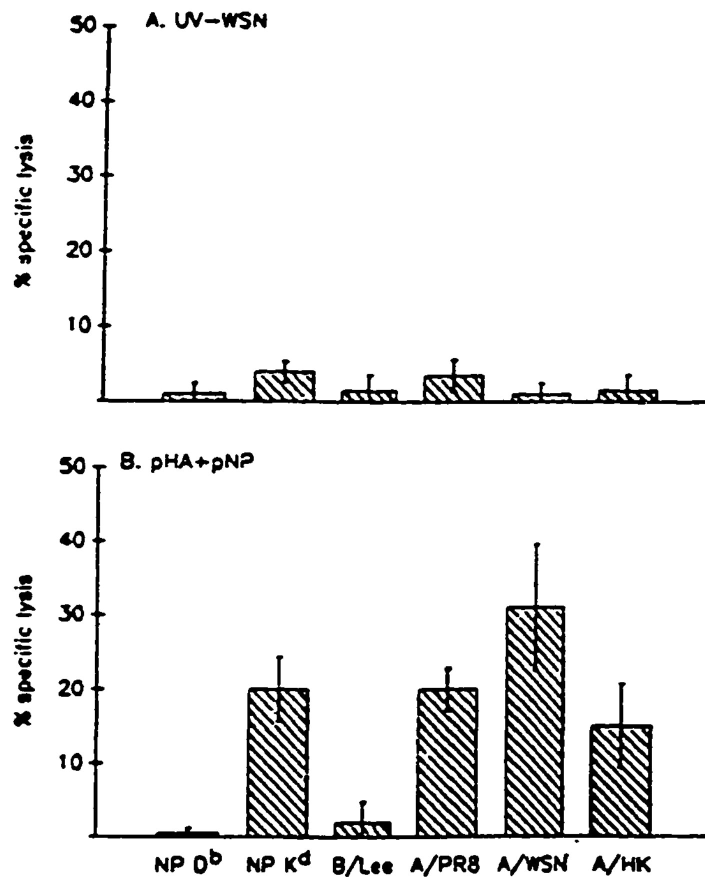

Fig.16. CTL response of mice immunized as newborns with UV-attenuated WSN virus (A) or a combination of pHA and pNP plasmids (B). Splenocytes pooled from three mice in each group were in vitro stimulated with PR8 virus-infected APC and tested against P815 cells coated with NP peptides or infected with various Influenza viruses, at E/T ratio of 10:1. The results are expressed as means of % specific lysis ± SD of triplicates.

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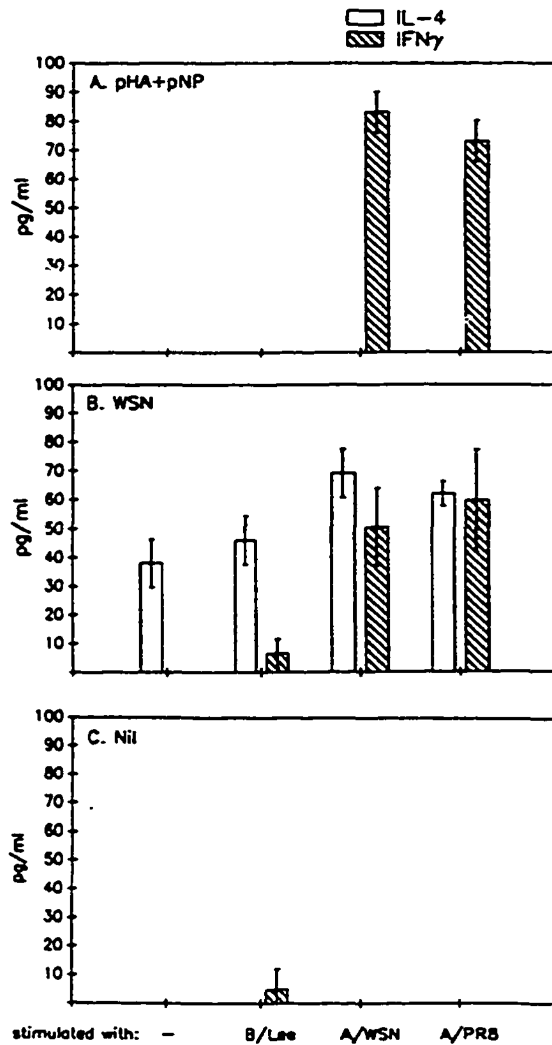

Fig.17. Cytokine secretion by $CD4^+$ T cells from mice immunized as neonates with a combination of pHA and pNP plasmids (A), UV-attenuated WSN virus (B) or nil (C). Negatively selected $CD4^+$ T cells were incubated four days in the presence of sucrose-purified UV-inactivated viruses (3μg/ml), APC and rIL-2 (6U/ml). The concentration of IFNγ and IL-4 was estimated by ELISA and the results were expressed as means of duplicates ± SD (pg/ml).

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In contrast, the $CD4^+$ T cells from non-immunized, age-matched mice did not secrete significant amounts of either IFNγ or IL-4 (Fig. 17B and C).

Thus, the neonatal immunization with pHA+pNP induced virus-specific cross-reactive CTLs and Th1 cells. In contrast, the neonatal immunization with UV-attenuated WSN virus did not prime CTLs but induced Th cells that secreted IL-4 as well as IFNγ.

3.2. Humoral response of mice immunized as neonates with pHA+pNP

In order to estimate the titer of protective antibodies generated by neonatal immunization with virus or plasmids expressing Influenza virus HA and NP, we measured the hemagglutination-inhibiting ability of the sera harvested from 5 week-old mice. As shown in Table 8, the neonatal immunization with pHA+pNP induced small but significant HI titers of antibodies specific for the homologous virus, in 5 out of 8 mice. In contrast, neonatal injection with UV-attenuated WSN virus did not prime a protective humoral response. Furthermore, studies carried out in our laboratory showed that the neonatal exposure to UV-attenuated WSN virus induced long-lasting B cell unresponsiveness (Antohi *et al.* submitted). Thus, the neonatal unresponsiveness to the neutralizing B cell epitopes of WSN virus was due to the induction of tolerance. As further detailed, we could not test the responsiveness of newborn mice to live-WSN virus, because of its lethality.

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Table 8. Hemagglutination-inhibition titers of sera from mice immunized as neonates with pHA+pNP

Age of immunization	Immunized with	Number of mice	HI titer of antibodies against[a]	
			WSN	PR8
Adult	Nil	2	0[b]	0
	UV-WSN	3	4.7±0.6	0
	live WSN	3	7.0±1.0	0
	pHA+pNP	3	3.3±1.1	0
Neonatal	Nil	2	0	0
	UV-WSN	3	0	0
	pHA+pNP	5[c]	2.2±0.8	0

[a] Results were expressed as means of $\log_2$ individual HI titers ± SE

[b] Titers less than 1/40 were considered 0.

[c] Results shown for the five responder mice out of the eight mice tested

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In sharp contrast, live-virus immunization of adult mice with WSN virus induced high titers of HI antibodies against the homologous virus. Immunization of adult mice with UV-attenuated virus or pHA+pNP induced smaller HI titers against the homologous virus (Table 8). In all cases, the HI titers against the drift variant PR8 virus, were not significant.

Thus, the neonatal immunization with pHA+pNP induced significant titers of HI antibodies in a subset of animals. In contrast, the neonatal inoculation with UV-attenuated WSN virus was not effective in inducing detectable titers of protective antibodies.

3.3. Enhanced protection against lethal challenge with Influenza virus by neonatal inoculation with pHA+pNP

The newborn mice immunized with a dose of 25μg+25μg of pHA+pNP/inoculation and subsequently challenged with WSN virus, displayed 100% survival, in spite of the fact that mice immunized only with pHA showed approximately 50% mortality, or that mice immunized with pNP did not survive (Fig. 18A). Newborn and adult mice injected with control plasmid or non-immunized, displayed no survival when challenged with either WSN or PR8 virus, four weeks after the completion of immunization (Fig. 18). Similarly, the neonates immunized with a mixture of pHA and pNP displayed approximately 80% survival following lethal challenge with PR8

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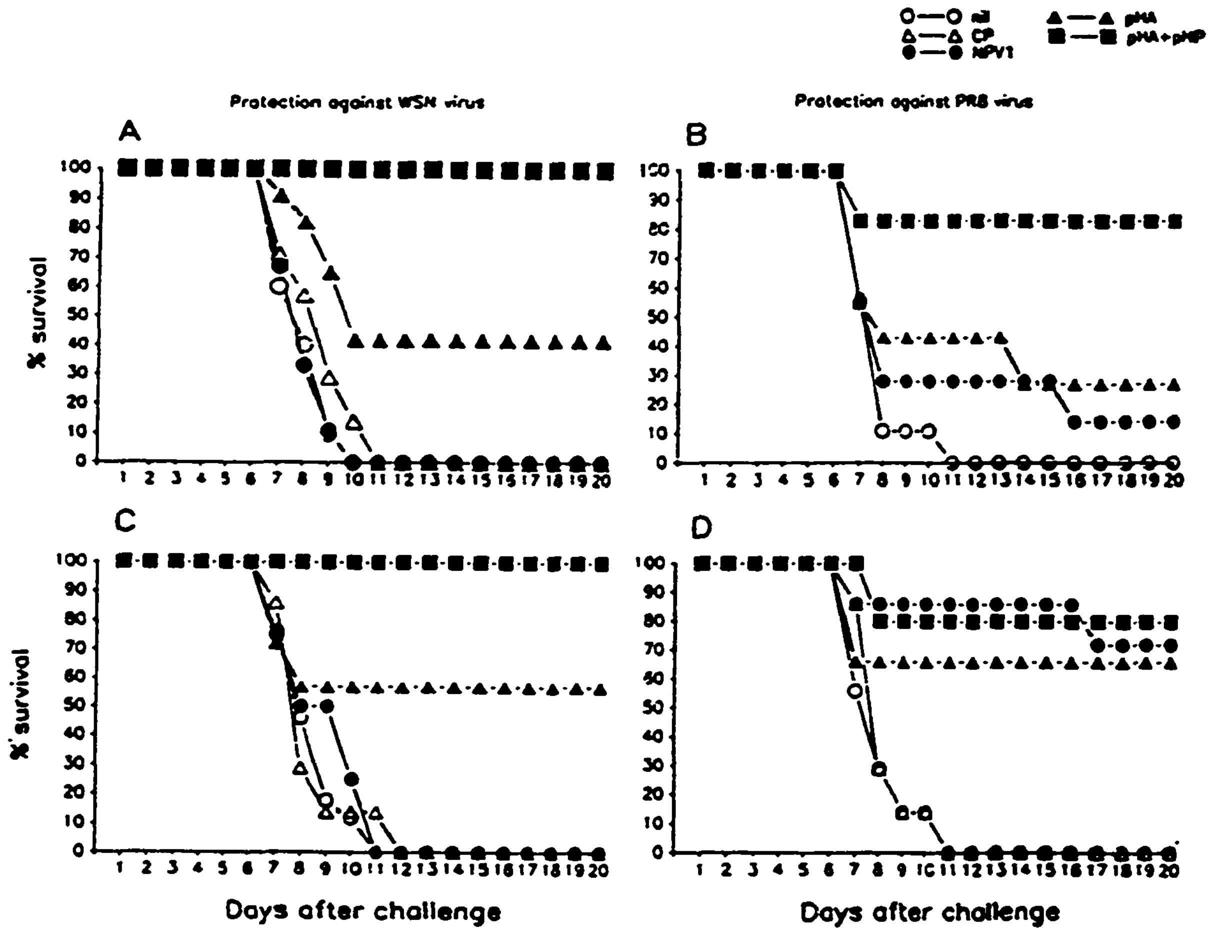

Fig.18. Protection against lethal challenge with WSN (A,C) or PR8 (B,D) virus of mice immunized as newborns (A,B) or adults (C,D) with a combination of pHA and pNP plasmids. As controls, we used naïve mice, mice inoculated with a control plasmid (pRc/CMV) and mice immunized with pHA or pNP, separately. The mice were challenged with lethal doses of virus at four weeks following the completion of immunization.

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virus, compared to the mice immunized with pHA or pNP alone, that showed approximately 25% and 15% survival, respectively (Fig. 18B). The adult mice immunized with both pHA and pNP were significantly more protected against WSN virus than the adult mice immunized with either pHA or pNP (Fig. 18C).

In contrast, the mice immunized as adults with pNP+pHA displayed similar survival rates as compared to those immunized with pHA or pNP alone, following lethal challenge with PR8 virus (Fig. 18D).

Together, these survival data showed that the coinjection of plasmids expressing HA and NP of Influenza virus type-A into newborn mice, greatly enhanced the protection against lethal infection with two distinct strains. This is more consistent with a synergistic rather than an additive relationship between HA and NP, due to the distinct nature of the immune effectors generated by the two components of the vaccine.

3.4. Dose dependency of protection following neonatal immunization with naked DNA

We carried out further experiments in order to estimate the dose requirements for significant protection following neonatal immunization with plasmids expressing HA and NP of Influenza virus type A. Different groups of mice were inoculated with various doses of pHA, pNP or pHA+pNP. Control groups were inoculated with CP,

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representing the plasmid pRc/CMV lacking Influenza virus insert. Four weeks after the completion of immunization, the mice were challenged with LD_{100} of WSN virus. The number of mice that survived the challenge was recorded (Table 9) and the recovery of the surviving mice was demonstrated by the lack of pulmonary virus,16 days after the challenge. The mice were inoculated three times with plasmid, according to a previously established protocol that was followed by immunity in a reproducible manner (Bot *et al* 1997c). Administration of 25µg of pHA together with 25 µg of pNP/dose resulted in complete protection, whereas inoculation of 50µg of pHA or pNP was followed by approximately 50% and no protection, respectively (Table 9). In order to rule out the possibility of high zone tolerance in neonates, we immunized newborn mice with decreasing doses of pHA or pNP, separately. As shown in Table 9, the percentage of surviving mice decreased in the case of pHA and did not increase in the case of pNP. In contrast, adult or neonatal immunization with doses as small as 7.5µg of each plasmid / injection was still followed by statistically significant protection after lethal challenge with WSN virus. The immunization of neonates with similar quantities of either pHA or pNP (15µg/dose) induced no significant protection, further underlining the tremendous beneficial effect of associating the two plasmids in the same vaccine formulation.

We studied the relationship between the number of inoculations and the protection conferred by neonatal immunization with pHA+pNP. As shown in Fig. 19, one or two inoculations with 25µg of each plasmid/dose, failed to induce significant protection.

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Table 9. Enhanced protection conferred by neonatal or adult immunization with a combination of HA and NP expressing plasmids

Age of immunization	Quantity (μg) / dose[a]			No. survivors / total infected	Percentage survival (%)[b]	p value[c]
	pHA	pNP	CP			
Adult	-	-	-	0/17	0	-
	-	-	50	0/7	0	>0.1
	50	-	-	4/7	57	0.0003
	-	50	-	0/4	0	>0.1
	25	25	-	5/5	100	<0.0001
	15	15		6/6	100	<0.0001
	7.5	7.5	-	6/7	86	0.0002
Newborn	-	-	-	0/10	0	-
	-	-	50	0/7	0	>0.1
	50	-	-	5/12	42	0.01
	30	-	-	2/7	29	>0.1
	15	-	-	1/6	17	>0.1
	-	50	-	0/9	0	>0.1
	-	15	-	0/4	0	>0.1
	25	25	-	10/10	100	<0.0001
	15	15		5/6	83	0.0026
	7.5	7.5		4/7	57	0.029
	3	3		1/4	25	>0.1

[a] Mice were inoculated three times and challenged with WSN virus at 4 weeks after the completion of immunization

[b] Survival was followed until day 20 after the challenge

[c] Statistical significance of survival as compared to the nil group was estimated by Fisher's exact test

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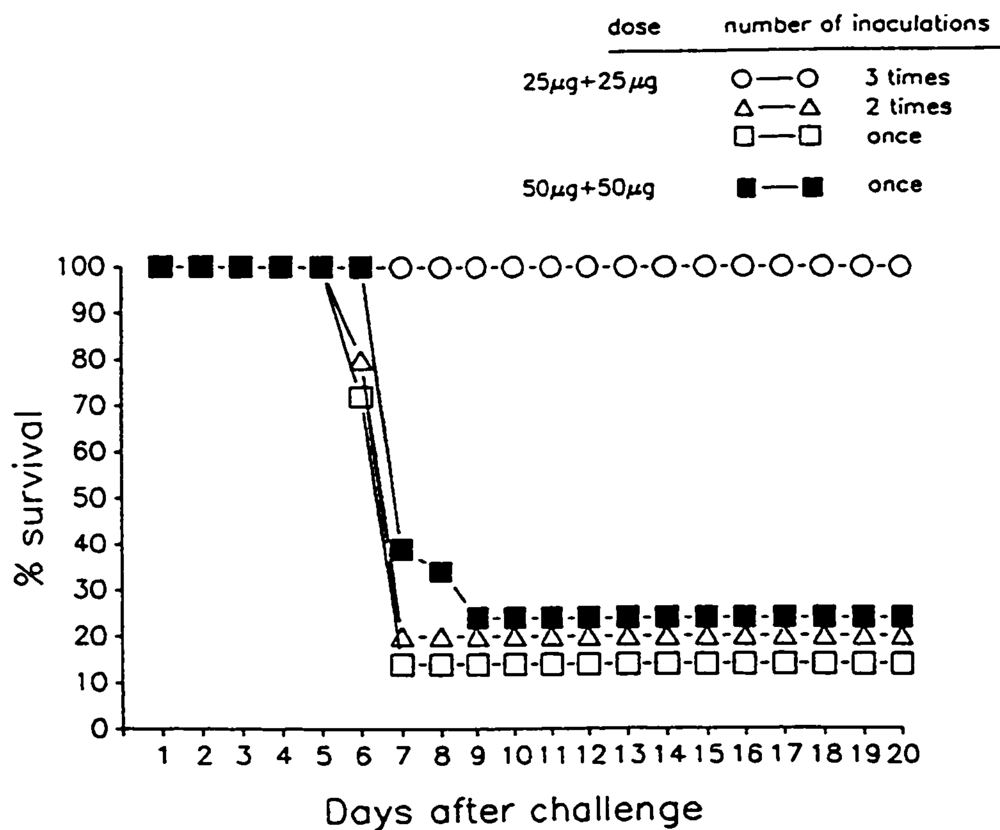

Fig.19. Dependence of the protection on the number of inoculations. The newborn mice were inoculated at day 1, 1 and 3, or 1,3 and 6 with a mixture of pHA and pNP plasmids. At four weeks after the completion of immunization, the mice were challenged with a lethal dose of WSN virus.

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Even single inoculation of a larger dose of pHA together with pNP, did not result in significant protection. This was not due to the lack of immunogenicity of the plasmid inoculated into 1 day-old mice, since a single injection of 20μg of pNP resulted in significant CTL priming (data not shown). Thus, distribution of the naked DNA vaccine into multiple inoculations has beneficial effects in terms of protection.

3.5. Lack of protection by neonatal immunization with UV-inactivated WSN virus

Previous studies showed that live virus immunization of adult mice with WSN virus induced complete protection against homologous and heterologous challenge (Bot *et al.* 1997b). This correlated with the priming of a broad T and B cell response specific for the homologous strain as well as cross-reactive epitopes. We could not test the ability of the WSN live-virus to induce protective immunity when inoculated into newborn mice, since the injection of this neurovirulent strain of Influenza virus into neonates was lethal at doses between less than 1μg to 25μg of sucrose-purified virus. The injection of live WSN virus in the gluteal region of 1 day old BALB/c mice was followed by impaired thriving beginning with 24-48 hours after inoculation and culminating with dehydration and death at 3 to 5 days postinjection (Fig. 20). Distinct batches of WSN virus displayed less pronounced but significant and reproducible lethality in terms of percentage survivors (data not shown).

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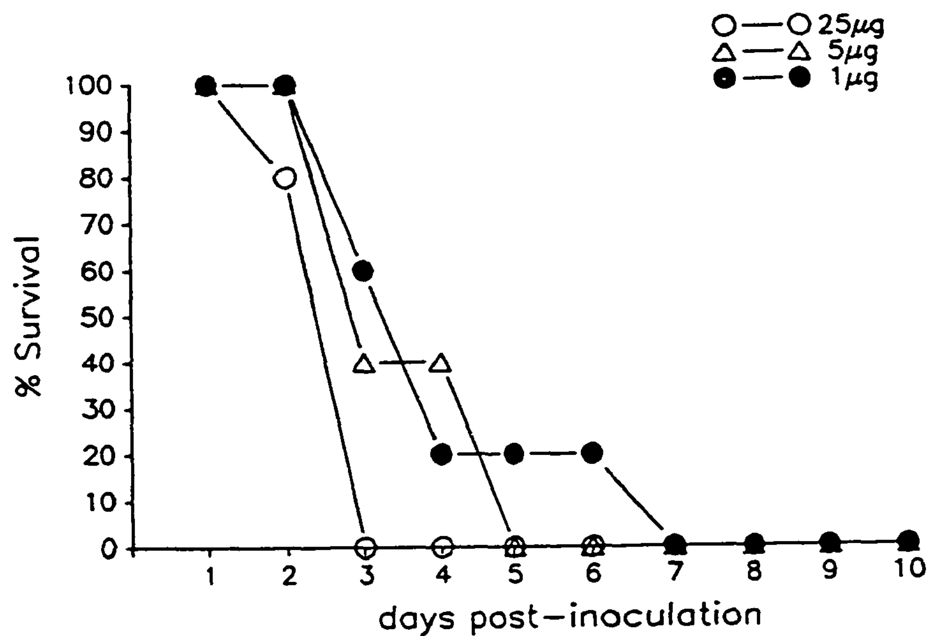

Fig.20. Lethality of live WSN virus following neonatal inoculation of mice. Various doses of live WSN virus were injected in the gluteal muscle of 1 day-old BALB/c mice. The survival of the neonates was followed for one week after the injection.

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Consequently, we carried out further experiments with UV-attenuated WSN virus, that is similar to the conventional killed Influenza virus vaccine. In sharp contrast to the adult mice immunized with UV-attenuated WSN virus, the neonates although surviving the immunization, were not protected against the challenge with LD_{100} of WSN virus (Fig. 21). This is consistent with the lack of CTL response, the deviated Th response and the B cell tolerance, following the neonatal inoculation of UV-attenuated WSN virus, as shown above.

3.6. Clearance of the pulmonary virus in mice immunized as newborns with pHA+pNP

Immunization of adult mice with live WSN virus leads to generation of optimal titers of protective antibodies specific for the homologous strain (Table 8). A subsequent exposure to the same strain of virus does not lead to infection due to the presence of hemagglutination inhibiting antibodies, that prevent the virus binding to the sialoreceptors on the epithelial cells of the respiratory tract. Indeed, no pulmonary virus could be detected as early as three days after the homologous challenge of mice immunized with live WSN virus (Table 10). In contrast, non-immunized mice or mice injected with CP as adults or neonates displayed significant pulmonary virus titers at day 3 and 7 after infection.

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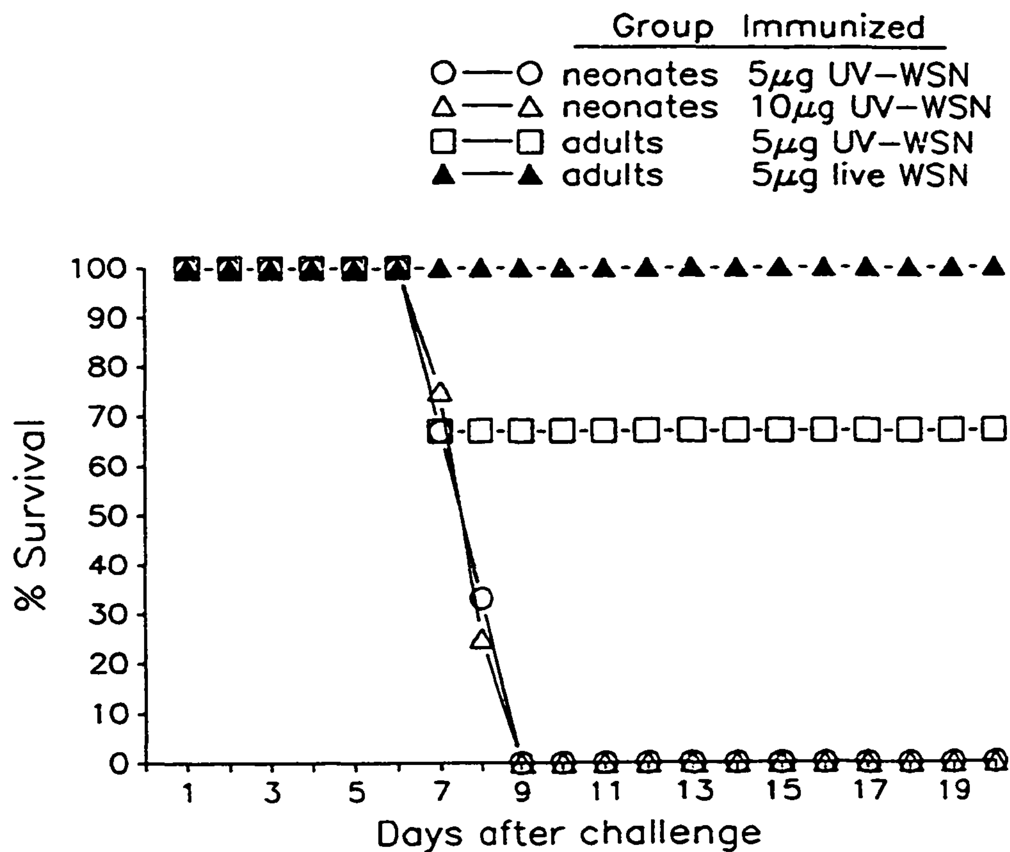

Fig.21. Protection against the homologous challenge of mice immunized as adults or newborns with WSN virus. Mice were inoculated i.m. with UV-inactivated or live-WSN virus, in the case of adult mice and challenged four weeks later with a lethal dose of WSN virus.

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Table 10. Clearance of the pulmonary virus by mice immunized as neonates or adults with a combination of plasmids expressing HA and NP

Age of immunization	Log_{10} of $TCID_{50}$ (mean±SE)[a]		
	day 3	day 7	day 20
Adult mice injected with:			
Nil	5.4±0.7	3.7±0.3	+[b]
CP	4.9±0.5	2.8±0.5	+
WSN virus	<1.0[c]	<1.0	<1.0
NPV1	4.8±0.1	+	+
pHA	2.0±2.2	1.4±0.8	<1.0
NPV1+pHA	4.4±1.1	<1.0	<1.0
Newborn mice injected with:			
CP	4.2±0.5	+	+
NPV1	4.7	+	+
pHA	4.0±0.6	<1.0	<1.0
NPV1+pHA	3.4±1.2	<1.0	<1.0

[a] At day 3 and 7 after the lethal challenge with WSN virus, the pulmonary virus titers were estimated. At day 20, all the surviving mice were sacrificed and the lung titers measured.

[b] No surviving mice.

[c] Titers were considered lower than 1 if infectious virus was not detected.

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All of the mice immunized with pHA+pNP as adults or newborns, although displaying significant pulmonary virus at day 3, showed no virus at day 7 following infection with WSN virus (Table 10). Furthermore, the mice immunized with pHA successfully cleared the virus by day 7. However, not all the mice immunized with pHA survived the challenge (Fig. 18), probably because of the extensive DTH reaction due to delayed clearance of the virus. Together, these data suggest that, while the plasmid immunization did not induce optimal titers of neutralizing antibodies capable to prevent the homologous infection, the memory response led to effective clearance of the virus, in mice immunized either as adults or as neonates with pHA+pNP.

3.7. Conclusion

Thus, we show that following the neonatal co-administration of two plasmids expressing NP (pNP) and HA (pHA), the protection against lethal challenge with the homologous virus and a drift variant is significantly increased. Furthermore, the neonatal inoculation of UV-attenuated WSN virus, that is similar to the killed vaccine, does not trigger a protective immune response.

Whereas HA bears dominant B and Th epitopes that are mostly strain or subtype specific, NP carries major cross-reactive CTL epitopes. The neonatal inoculation of pHA+pNP was followed by induction of CTLs that displayed cross-

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reactivity against various type A strains (Fig. 16). Furthermore, neonatal DNA immunization induced $CD4^+$ Th1 cells specific for epitopes shared by WSN virus and the drift variant, PR8 virus (Fig. 17). Finally, DNA immunization of newborn mice elicited protective antibodies against the homologous strain of virus, that was used for cloning the HA cDNA insert of pHA (Table 8). However, only 5 out of 8 mice were responders and the HI titers were significantly reduced as compared to the adult mice immunized with live or UV-attenuated virus. Together, the virus-specific CTL, Th and B cells mediated a significantly increased protection against lethal challenge with WSN, in mice immunized as neonates or as adults (Fig. 18). In the case of the drift variant PR8 virus, the enhanced protection was probably due to the induction of PR8 specific Th and CTL, since no PR8 specific HI antibodies were measured (Table 8). The dose-protection relationship shown in Table 9, suggests strong synergism between the main immune effectors since lower doses of pHA+pNP were sufficient to induce levels of protection that could not be obtained with either pHA or pNP. In particular, although pNP elicited CTLs against the major epitope NP 147-155 shared by the PR8 and WSN viruses, it failed to induce significant protection in terms of survival against the lethal challenge with WSN virus. The most reasonable explanation is the enhanced virulence associated with increased replication of the WSN strain due to a mutation in neuraminidase (Li *et al* 1993), so that CTLs alone are not sufficient for significant protection against this particular strain.

In stark contrast with the neonatal immunization with pHA+pNP, the inoculation of WSN virus was not followed by protection. First, injection of live

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WSN virus in newborn mice was lethal (Fig. 20). Since the inoculation of live WSN virus in adult mice was not lethal and induced complete protection against homologous challenge, this result confirms that neonatal immaturity of the immune system may facilitate serious side effects in the case of live vaccines. Secondly, the neonatal inoculation with UV-attenuated WSN virus, although not lethal because of the impaired virus replication, did not elicit protection (Fig. 21). No CTLs or B cells secreting protective antibodies were primed by the UV-attenuated virus inoculated into newborn mice (Fig. 16 and Table 8). Whereas the absence of cytotoxicity may be easily explained by the lack of synthesis of viral proteins, the absence of an humoral response is most probably due to the immaturity of the neonatal immune system since adults mounted HI antibodies to UV-attenuated WSN virus. Indeed, recent data suggest that the neonatal exposure to UV-attenuated WSN virus induces B cell tolerance (Antohi *et al,* submitted). Further, the neonatal inoculation with UV-attenuated virus induced $CD4^+$ Th cells that secreted IFNγ and IL-4 (Fig. 17). It is not clear at this point how much of the IL-4 is due to the Th cells specific for bovine proteins, although we used for immunization virus purified by sucrose-gradient ultracentrifugation. It is noteworthy to mention that the immunization of adult mice with UV-attenuated WSN virus, in contrast to neonatal immunization, resulted in significant but not complete protection to lethal homologous challenge (Fig. 21). Thus, neonatal and adult immunization with UV-attenuated Influenza virus, that is similar to the conventional vaccine, appears to be less effective as compared to DNA immunization with mixtures of plasmids encoding multiple Influenza antigens.

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Consequently, the present study extends the above mentioned conclusion from adults (Donnelly *et al* 1995) to neonates.

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4. Immune responsiveness following neonatal inoculation with a plasmid expressing an Ig chimera bearing T and B epitopes of hemagglutinin

Previous studies showed that self immunoglobulin molecules are effective vehicles for delivering foreign epitopes to MHC class-II molecules in the endosomal compartment of professional APC (Zaghouani *et al.* 1993, Brumeanu *et al.* 1993). We have engineered a chimeric gene by replacing the CDR3 and CDR2 segments of the VH fragment from an anti-arsonate mAb with the gene segments encoding major HA epitopes: HA 110-120 that is recognized by $CD4^{+}$ T cells in the context of $I\text{-}E^{d}$ class-II molecules and HA 150-159 respectively, that is a B cell epitope (Brumeanu *et al.* 1996). Subsequently, the VH-TB chimeric gene was inserted into a mammalian expression vector bearing the CMV initial-early promoter and the BGH poly-adenylation signal (Casares *et al.* 1997). Further studies showed that myoblast cells transfected with the VH-TB plasmid secrete the chimeric protein in the supernatant (Casares *et al.* unpublished data).

Recent studies showed that neonatal inoculation with plasmids expressing the circumsporozoite antigen of Plasmodium yoelii induced tolerance to certain, but not all the epitopes (Mor *et al.* 1996). We took advantage of the VH-TB chimera that bears defined T and B cell epitopes of Influenza virus, to address the question if a

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plasmid that expresses a secreted protein induces neonatal tolerance. Consequently, we tested the priming effect of the VH-TB plasmid, subsequently to the inoculation in adult or newborn mice.

4.1. The immune response generated by adult immunization with VH-TB plasmid

Adult BALB/c mice immunized with VH-TB plasmid develop both T and B cell immunity (Table 11). The $CD4^+$ T cells separated from adult mice immunized with VH-TB at day 7 after the completion of immunization, secreted significant amounts of IFNγ but no IL-4 when restimulated with PR8 virus or an antigen bearing the HA 110-120 peptide. In contrast, the $CD4^+$ T cells from PR8 immunized mice secreted both IFNγ and IL-4. Adult mice immunized with VH-TB mounted HA 150-159 specific antibodies at 4 weeks after the completion of immunization (Table 11). The titers of HA 150-159 and PR8-specific antibodies measured in VH-TB immunized adult mice, were significantly lower than those of the mice immunized with live PR8 virus. Thus, VH-TB immunization of adult mice induced immune responses to the Th, as well as the B cell epitope encoded by the chimeric gene.

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Table 11. The immune response of adult mice to VH-TB plasmid

Mice immunized with:	Cytokine production by $CD4^+$ T cells, *In vitro* stimulation with[a]:								Antibody response[b]	
	Nil		IgG2b		IgG-gal-HA		PR8 virus		anti-PR8	anti-HA 150-159
	IFNγ	IL-4	IFNγ	IL-4	IFNγ	IL-4	IFNγ	IL-4		
Nil	0	0	0	0	0	0	0	0	0	0
B/Lee/40 virus	0	0	0	0	0	0	0	0	0	0
PR/8/34 virus	0	0	ND[c]	ND	ND	ND	74±3	24±3	42±9	12±4
VH-TB plasmid	0	0	0	0	39±6	0	56±3	0	4±2	5±3

[a] Negatively selected $CD4^+$ T cells were restimulated for four days in the presence of 5μg/ml of antigen. The concentration of cytokines in the supernatant was determined by ELISA and expressed as mean±SD of duplicates in pg/ml.

[b] The binding of antibodies to PR8 or HA 150-159 coupled to BSA was estimated by sandwich RIA using 1/100 dilutions of sera and iodinated rat anti-mouse k light chain antibodies. The standard curve was constructed using B2H1 HA-specific antibodies. Results were expressed as mean±SD of triplicates (μg/ml).

[c] ND - not done.

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4.2. Cellular responsiveness subsequent to the neonatal inoculation of VH-TB plasmid

We separated $CD4^+$ T cells from 4 week-old mice immunized as neonates with VH-TB and we tested their proliferation upon *in vitro* stimulation with HA 110-120 peptide or NP 147-155 peptide. Some of the mice were boosted with live PR8 virus at the age of 3 weeks, in order to address the question of tolerance induction. As shown in Fig. 22B, the $CD4^+$ T cells from mice immunized as neonates with VH-TB and boosted with PR8 virus, proliferated to a similar extent as the $CD4^+$ T cells from mice immunized with live-virus at the age of 3 weeks. In contrast, the $CD4^+$ T cells from non-immunized mice or mice immunized as newborns with VH-TB did not proliferate when restimulated with HA 110-120 peptide. No significant proliferation was measured when the $CD4^+$ T cells where stimulated with NP 147-155 peptide, that is a major CTL epitope (Fig. 22A).

We tested the ability of nylon-wool purified T cells to produce cytokines following *in vitro* stimulation with NP 147-155 or HA 110-120 peptide. The T cells from mice that received an inoculation with live PR8 virus, previously immunized or not with VH-TB, secreted significant IFNγ but no IL-4 when restimulated with NP 147-155 peptide (Fig. 23A,C). Furthermore, significant amounts of IFNγ and IL-4 were produced after the stimulation with HA 110-120 peptide,

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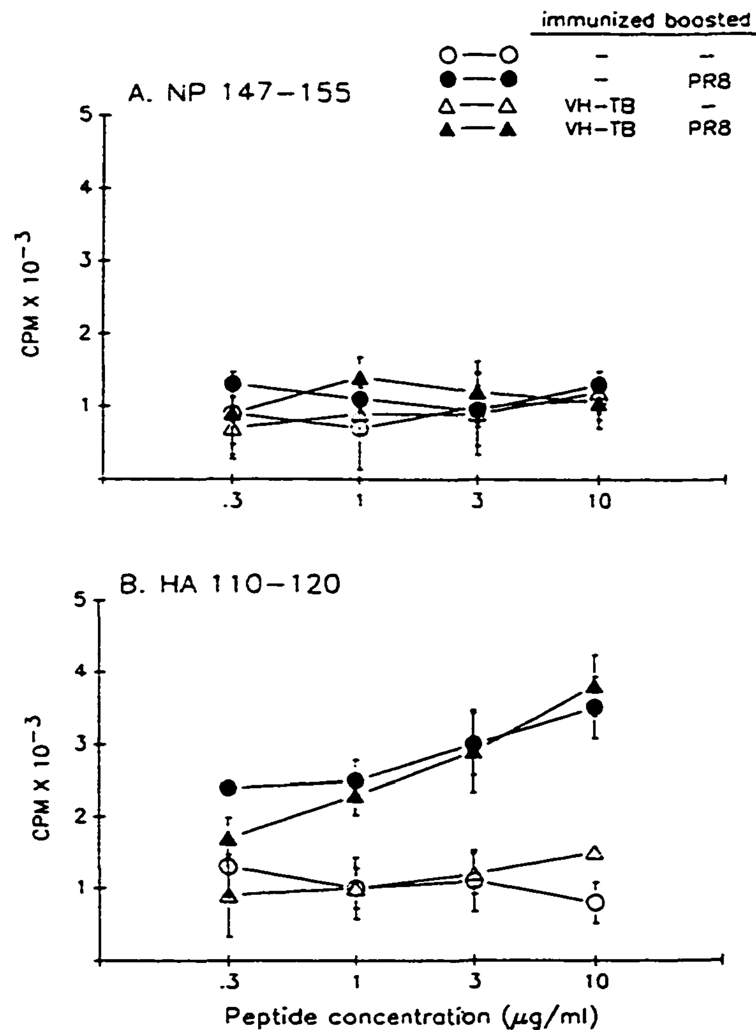

Fig.22. Proliferation of the CD4$^+$ T cells from mice immunized as newborns with VH-TB plasmid. Negatively selected CD4$^+$ T cells from mice immunized with VH-TB as neonates, were incubated with APC in the presence of various concentrations of NP 147-155 (A) or HA 110-120 (B) synthetic peptides. ^{3}H-Thymidine was added after 72 hours and the radioactivity incorporated was measured after other 14 hours. The results are expressed as means of triplicates ± SD of proliferation indexes. Part of the mice immunized with VH-TB were boosted with PR8 virus. As controls, we used naïve age-matched mice and mice immunized with live PR8 virus one week previous to the sacrification.

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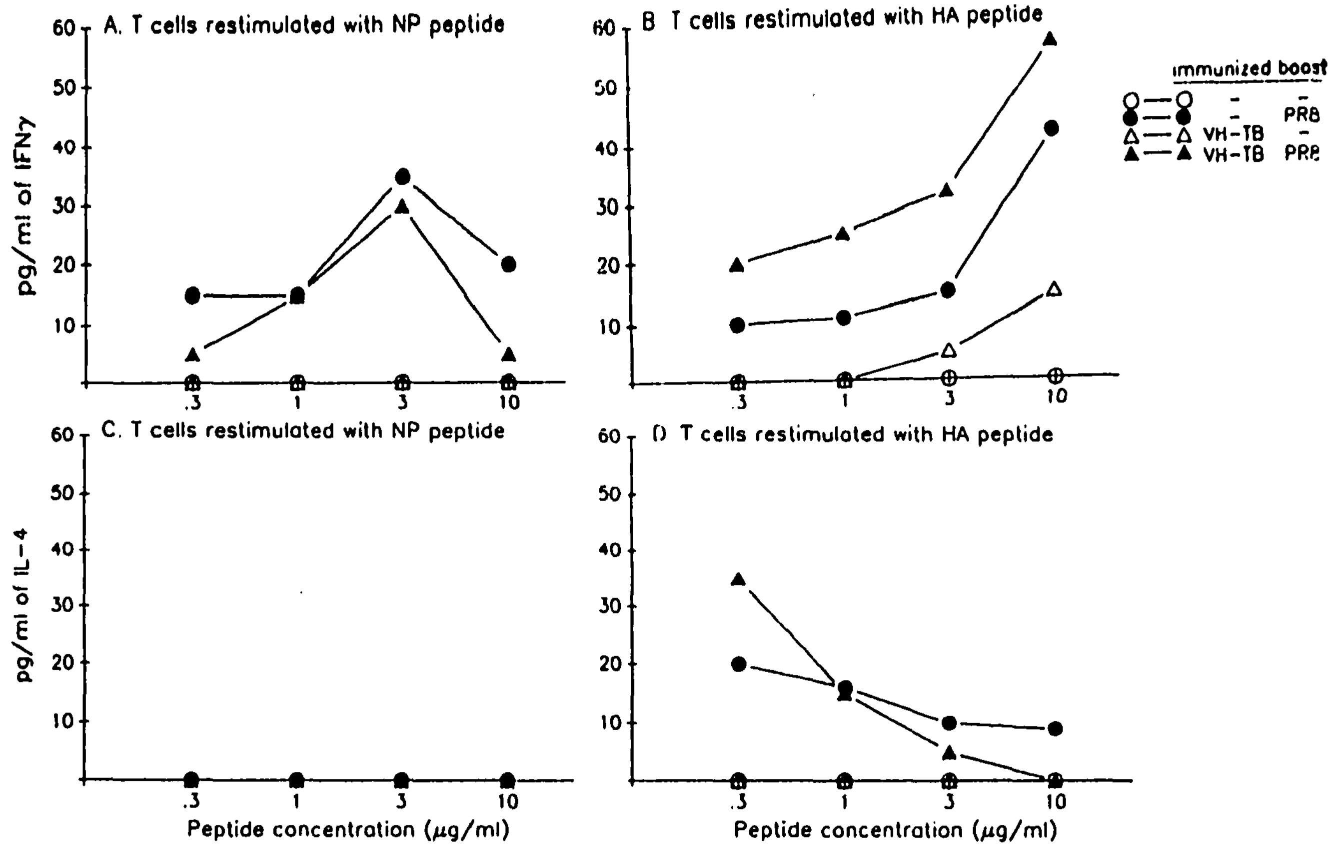

Fig.23. Cytokine production of the T cells from mice immunized as newborns with VH-TB plasmid. Nylon wool-purified T cells from spleens of mice immunized as neonates with VH-TB were incubated with various concentrations of NP 147-155 (A,C) or HA 110-120 (B,D) synthetic peptides in the presence of APC and 6U/ml rIL-2. IFNγ (A,B) and IL-4 (C,D) were measured three days later by ELISA and the results were expressed as means of duplicates (pg/ml). SE was less than 25% of the mean, in each case. As controls, we used naïve mice and mice immunized with PR8 virus one week previous to the sacrification. Part of the mice immunized with VH-TB were boosted with PR8 virus one week previous to the study.

by the T cells from mice injected with live-virus, that were previously immunized or not with VH-TB as neonates (Fig. 23 B,D). The T cells from mice immunized as neonates with VH-TB and not boosted with PR8 virus, secreted low but measurable amounts of IFNγ when *in vitro* stimulated with HA 110-120 peptide (Fig. 23B). Interestingly, the T cells from mice immunized with live-virus displayed dissimilar profiles of IFNγ and IL-4 secretion, depending on the concentration of the HA 110-120 peptide: whereas at lower concentrations IL-4 dominated, at higher concentrations the T cells produced more IFNγ and less IL-4.

The mice inoculated as neonates with VH-TB mounted significant cytotoxicity subsequently to the live-PR8 virus boost (Fig. 24). The splenocytes harvested from mice injected with live virus and previously immunized or not with VH-TB, after *in vitro* stimulation with PR8 virus, lysed the target cells infected with PR8 or HK virus as well as those coated with NP 147-155 peptide. Thus, neonatal inoculation with VH-TB did not impair a subsequent T cell response to the live PR8 virus.

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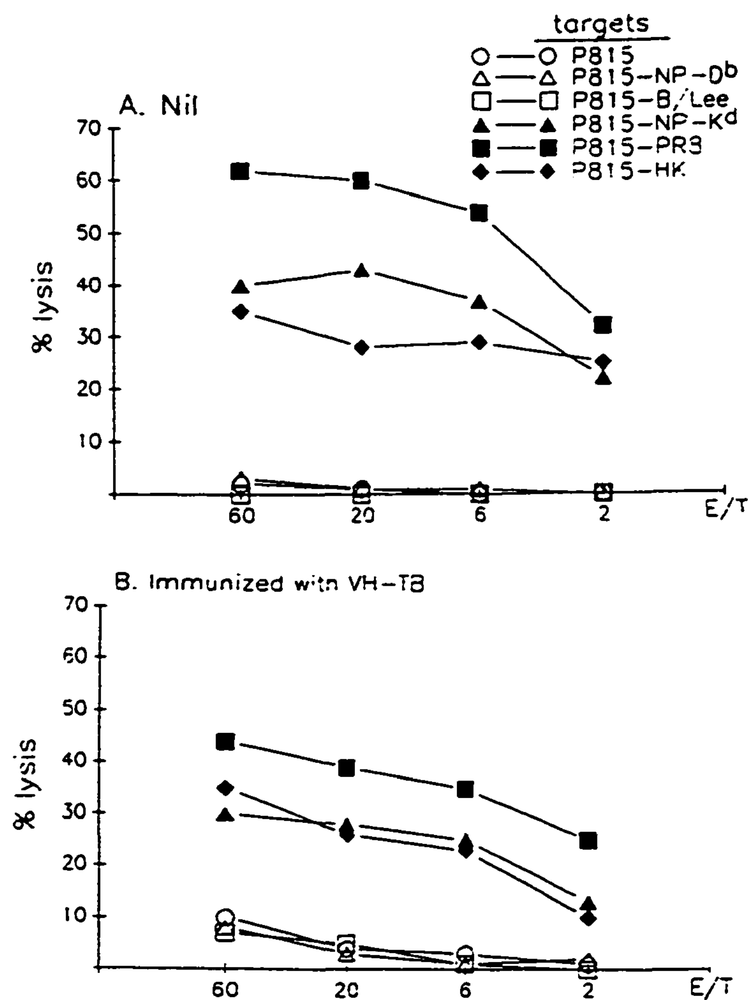

Fig.24. The CTL response to PR8 virus of mice immunized as neonates with VH-TB plasmid. Mice immunized with VH-TB as newborns were boosted three weeks later with live PR8 virus. The splenocytes from three mice in each group (injected only with PR8 virus - (A) and immunized with VH-TB and boosted with PR8 virus - (B)) were harvested and pooled one week later and in vitro stimulated with PR8 infected APC. The cytotoxicity was measured against P815 target cells infected with various strains of Influenza or coated with NP synthetic peptides. The results are expressed as means of % specific lysis of duplicates.

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4.3. Humoral responsiveness following the neonatal inoculation of VH-TB plasmid

Neonatal inoculation of the VH-TB plasmid was not followed by the induction of humoral responses, as revealed by the lack of PR8-neutralizing antibodies (Table 12). The binding activity for HA 150-159 peptide or PR8 virus of the sera harvested from mice immunized as newborns with VH-TB, was similar to that of naïve mice (Table 12). Neonatal injection of VH-TB plasmid did not induce unresponsiveness to PR8 virus, since the mice boosted with live-virus showed unaffected humoral responses. Furthermore, the response to the HA 150-159 peptide, that is a major B cell epitope expressed by VH-TB, was not impaired by the inoculation of the plasmid, as revealed by our results (Table 12). Thus, the neonates inoculated with VH-TB, although did not mount humoral immunity, were not impaired in their response to the live virus.

4.4. Conclusion

We addressed the question if neonatal inoculation of a plasmid that encodes a secreted protein bearing defined T and B cell epitopes induces immune tolerance. Inoculation of the VH-TB plasmid into adult mice, generated immune responses consisting in HA 110-120 specific Th1 cells and B cells secreting antibodies specific for the dominant HA 150-159 epitope (Table 11).

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Table 12. The humoral responsiveness of mice immunized as neonates with VH-TB plasmid

Group		HI titer against PR8 virus[a]	Binding to[b]:	
Immunized	Boost		HA 150-159	PR8 virus
-	-	0	177±33	163±20
VH-TB	-	0	175±61	183±17
-	PR8	7.0±1.0	352±48	337±79
VH-TB	PR8	6.0±0.7	308±39	354±26

[a] Results were expressed as mean±SE of log_2 HI titers. HI titers less than 40 were considered 0.

[b] The binding of antibodies to the B epitope and PR8 virus was estimated by sandwich ELISA using sera at a dilution of 1/200 and biotin-conjugated goat anti-mouse IgG antibody. Results were expressed as mean±SEM of OD_{450}.

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In contrast, the neonatal inoculation of VH-TB did not lead to an overt humoral response (Table 12). Nevertheless, neonatal injection of VH-TB induced a low but detectable Th1 response to the HA 110-120 peptide (Fig. 23). The mice injected as newborns with VH-TB and boosted with PR8 virus developed:

1) antibodies that are neutralizing for PR8 virus and bind the HA 150-159 peptide (Table 12) ;

2) T cells that secreted IFNγ and IL-4 following in vitro stimulation with HA 110-120 peptide (Fig. 23);

3) $CD4^+$ T cells that proliferated upon in vitro stimulation with HA 110-120 peptide (Fig. 22);

4) CTLs that lysed target cells infected with type A Influenza viruses or coated with NP 147-155 peptide (Fig. 24).

This demonstrated that the neonatal inoculation of VH-TB did not impair the humoral, Th or CTL response in the case of subsequent exposure to the live-virus. Two non-exclusive factors may account for this: the secreted protein that reaches the central lymphoid organs is insufficient to delete most of the specific precursors and secondly, the adjuvant effect of the plasmid may preclude the induction of tolerance at the cellular level. However, the neonates responded less effective than the adults to VH-TB. The lack of antibody response to the HA 150-159 peptide may be explained by the stepwise acquisition of the B cell repertoire. Indirectly, two observations support this assumption: first, the neonatal inoculation of plasmids encoding whole

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Influenza virus proteins is immunogenic (Bot *et al.* 1996a, 1997b) and secondly, the reactivity pattern of the B cell clonotype repertoire differ in adults versus neonates immunized with pHA (Antohi *et al.* submitted).

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5. Neonatal responsiveness and maternal immunity

It has been previously shown that the inoculation of mammalian expression vectors is followed by continuous production of antigens (reviewed by Pardoll and Beckerleg, 1995). Furthermore, the plasmid may leak from the site of injection into the circulation and be expressed in various tissues (Torres *et al.* 1997). Since the presence of foreign antigens during the fetal development may induce central tolerance (Zinkernagel *et al.* 1991), we tested the responsiveness to Influenza virus of the offspring born from dams immunized with plasmids expressing viral antigens.

Secondly, we tested the immune responsiveness to the DNA vaccine of offspring born from mothers immunized with PR8 virus, in order to address the question of negative interference by maternal antibodies. Antibodies of γ isotype are transmitted across the placenta to the offspring and they confer passive immunity, together with the IgA antibodies transmitted via collostrum. The passive immunity acquired by the offspring can negatively interfere with the active immune responses against microbes as well as conventional vaccines (Albrecht et al. 1977; Francis and Black, 1986; Harte et al. 1982; Xiang and Ertl, 1992). Previous studies showed that antibodies against antigens bearing MHC class-II restricted epitopes prevented the generation of immunogenic peptides in professional APCs (Bot *et al.* 1996c). For example, neutralizing antibodies against the PR8 strain of Influenza virus or other

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antigens bearing the HA 110-120 peptide, inhibited the generation of class-II / peptide complexes on the surface of professional APC (Fig. 25).

Thus, our *in vitro* data argue against the hypothesis that maternal inhibition is mediated by suppressor cells (Harte and Playfair, 1983).

Third, we tested the protection of the offspring conferred by maternal immunization with pHA, since we previously demonstrated that this plasmid induced the production of WSN-specific HI antibodies.

5.1. Immune responsiveness of offsprings from dams immunized with a plasmid expressing NP

Three month-old female BALB/c mice were immunized with pNP plasmid three times, 30µg/dose at three weeks interval. One week after the completion of immunization, the females were crossed with BALB/c males. The offspring were born three weeks later and at the age of four weeks, they were immunized with live PR8 virus. After one week, the splenocytes were harvested and *in vitro* stimulated with PR8 virus-infected APCs. As control, we used splenocytes from age-matched PR8-immunized offsprings, born from naïve dams. The CTL activity was tested against P815 target cells coated with NP 147-155 peptide. As shown in Fig. 26, the offspring born from mothers immunized with pNP mounted significant NP-specific CTL activity, subsequently to the live virus immunization, like the offspring born

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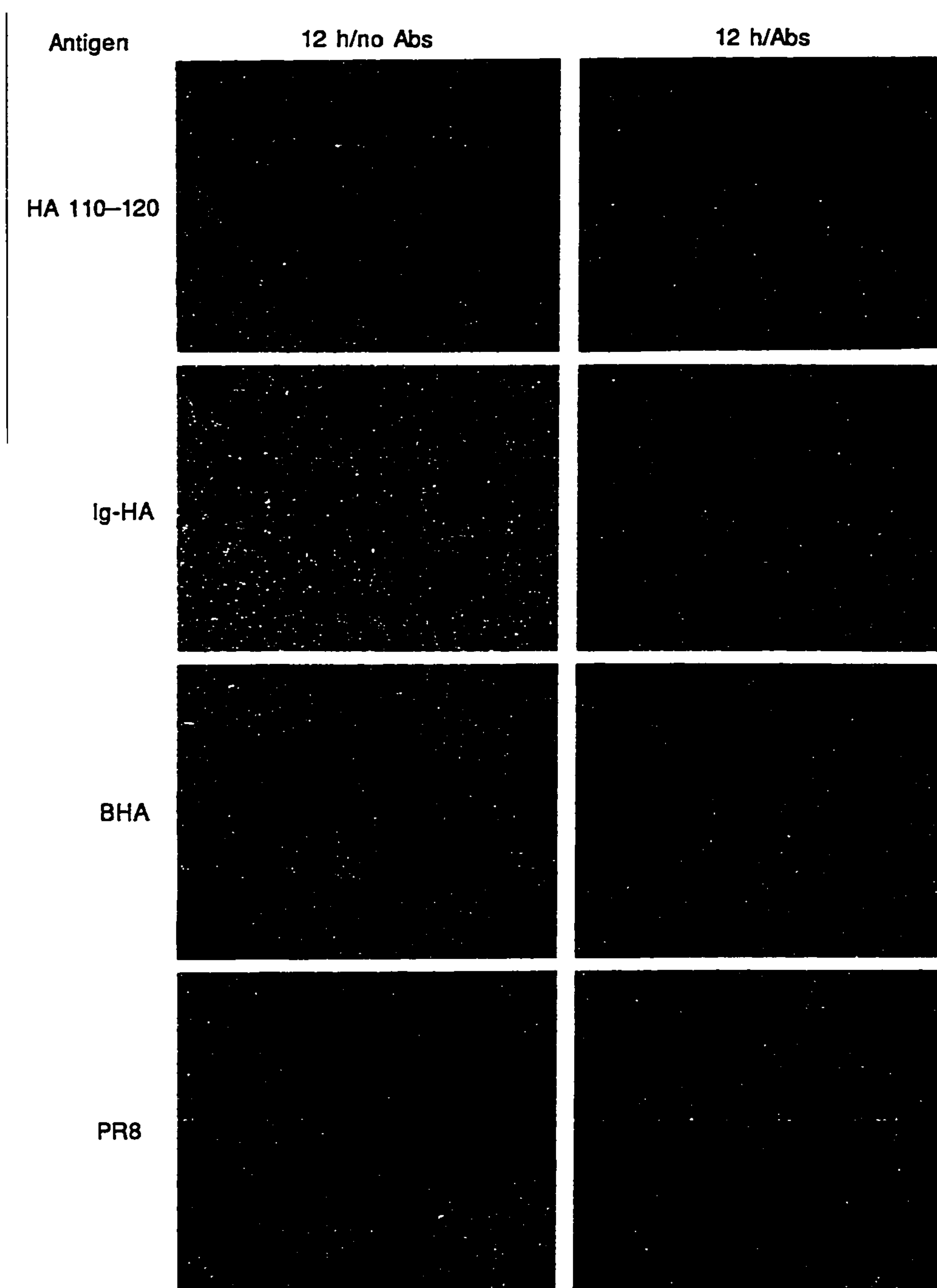

Fig.25. Inhibition of the antigen processing and presentation by carrier-specific antibodies. Mouse B lymphoma 2PK3 cells were incubated with 10μg/ml of various antigens bearing the HA 110-120 I-E^d restricted epitope. The antigens were preincubated or not with antibodies as follows: HA 110-120 and Ig-HA with anti-HA 110-120 polyclonal Abs; BHA and PR8 with PY201 anti-PR8 virus mAbs. The subsequent incubation of 2PK3 with antigen or antigen-antibody complex was carried out in the presence of a TcH (14-3-1) that is specific for HA 110-120 peptide + I-E^d and has a β-gal reporter gene driven by IL-2 promoter. After 12 hours of incubation, the cells were stained for β-gal.

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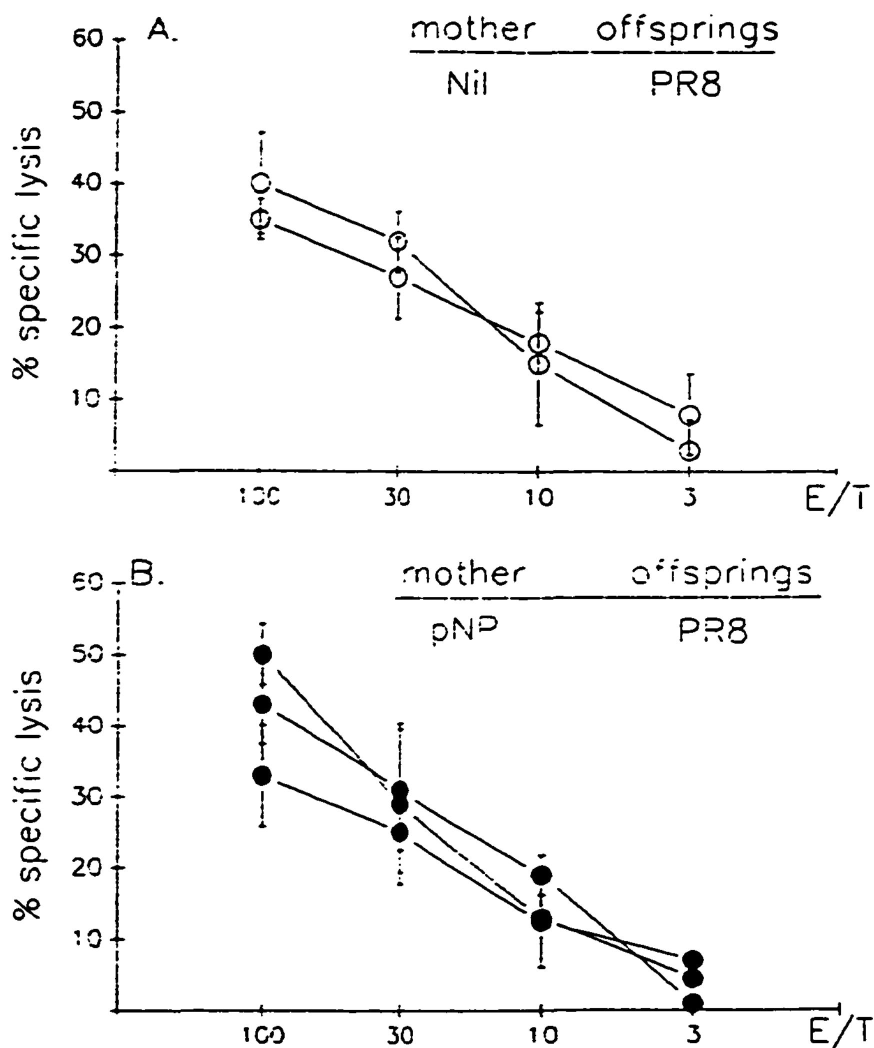

Fig.26. CTL responsiveness of the offsprings born from mothers immunized with pNP (B). Splenocytes from PR8 immunized offsprings born from dams inoculated with pNP were individually harvested and in vitro stimulated with PR8-infected APC. The cytotoxicity was tested against P815 cells coated with NP 147-155 peptide. The results were expressed as means of %specific lysis ± SD of duplicates, for each mouse. As control, we used PR8 immunized offsprings born from naïve dams (A).

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from naïve dams. Thus, the pNP immunization of the mothers did not impair the CTL response of their offspring to the NP of Influenza virus.

5.2. The CTL response to a plasmid expressing NP, of offspring from dams immunized with Influenza virus

Female BALB/c mice were immunized with live PR8 virus and were crossed with BALB/c males in the same day. During the time of birth, the maternal reciprocal titer of PR8-specific HI antibodies was between 1280 and 2560. Some of the offspring were inoculated with pcNP plasmid that expresses NP of the PR8 virus at day 1, 3 and 6 after birth. Other offspring were immunized with 5μg of PR8 virus i.p., at day 4 after birth. As controls, we used offspring from naïve dams. Whereas the inoculation of live PR8 virus into offspring of naïve dams earlier than day 4 after birth was lethal, the offspring of PR8 immunized dams were completely protected (data not shown). This observation demonstrated the acquisition of passive immunity by the offspring of PR8-immunized dams.

At the age of four months when the antibodies of maternal origin were not detectable any more, the offspring were sacrificed and the splenocytes were harvested and *in vitro* stimulated with PR8 virus-infected APCs. The CTL activity was tested against P815 target cells infected with PR8 virus, in an individual manner. The data in Fig. 27 show that two out of three pcNP immunized offspring from mothers

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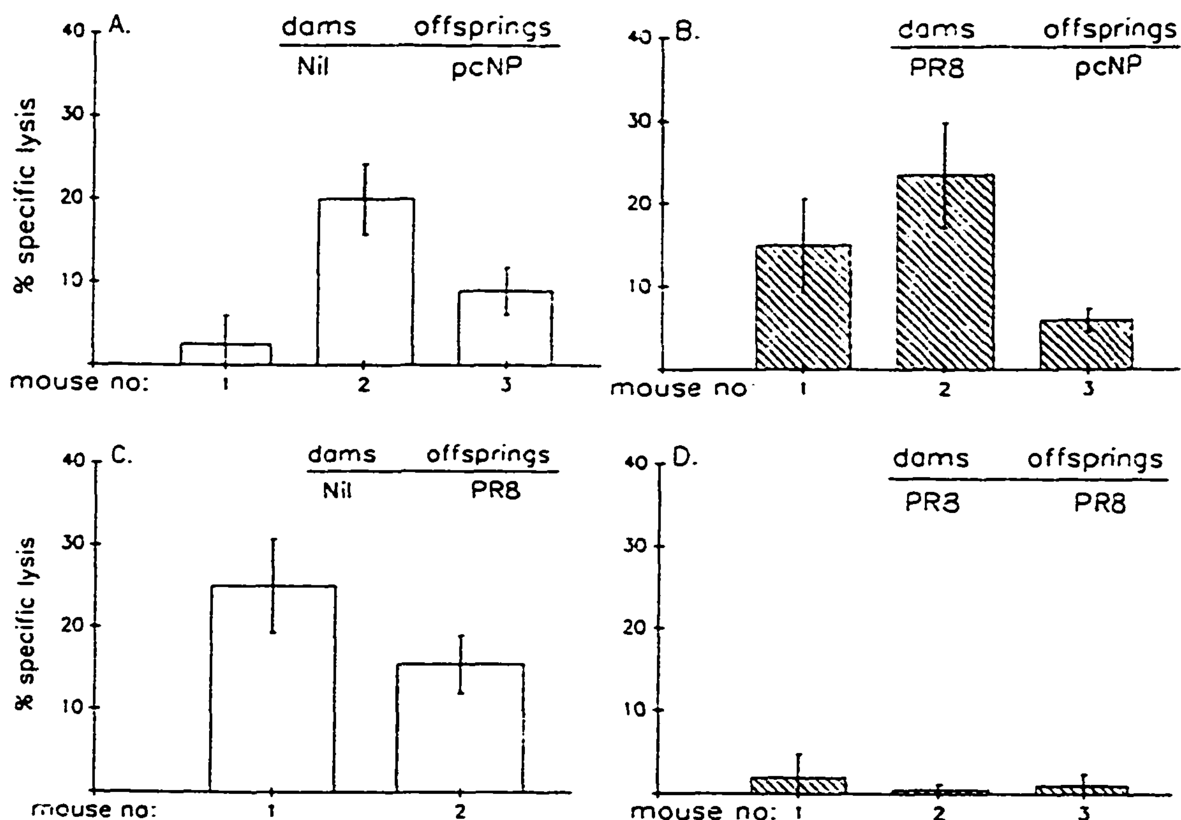

Fig. 27. CTL induction by neonatal inoculation of pcNP into offsprings born from mothers immunized with PR8 virus (B). The splenocytes were harvested and in vitro stimulated with PR8-infected APC in an individual manner. The cytotoxicity was tested against P815 cells infected with PR8, at E/T ratio of 3:1. The results were expressed as mean of %specific lysis ± SE of duplicates. As controls we used: pcNP immunized offsprings from naïve dams (A); PR8 immunized offsprings from naïve (C) or PR8 immunized dams (D).

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inoculated with live virus, exhibited significant CTL activity. In contrast, none of the PR8-immunized offspring from dams inoculated with PR8 virus displayed CTL activity (Fig. 27).

The offspring born from naïve dams and immunized with pcNP or PR8 virus, exhibited CTL activity with a large degree of individual variation (Fig. 27). However, these data suggest that the maternal antibodies specific for Influenza virus did not prevent the priming of CTL response to NP expressed by the pcNP plasmid.

5.3. Protection of offspring from dams immunized with a plasmid expressing HA

We immunized two month-old BALB/c females with pHA (100μg/dose), three times at three weeks intervals. At one week after the completion of immunization, the females were bred to BALB/c males. Two week-old, as well as 1 and 3 month-old offspring were challenged with lethal doses of WSN virus. Experiments carried out in our laboratory in collaboration with Antohi *et al.* showed that the 2 week-old offsprings born from pHA-immunized mothers, were significantly protected as assessed by the survival and pulmonary virus clearance, subsequently to the lethal challenge with WSN virus (Table 13). The protection of the offspring born from pHA-immunized dams, against WSN virus, correlated with the presence of WSN specific HI antibodies, most probably transmitted via the placenta and to an unknown extent, via the milk. In fact, the unexpectedly increased protection of the 2 week-old

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Table 13. Homologous protection of the offsprings born from dams immunized with pHA plasmid

Dams injected with:	2 weeks[a]					1 month					3 months				
	Survival	Lung titer[c]			HI titer	Survival	Lung titer			HI titer	Survival	Lung titer			HI titer
		d3	d7	d16			d3	d7	d16			d3	d7	d16	
WSN virus	5/5[b]	<1	ND	<1	5.2±0.9[d]	5/7	3.9	0	ND	3.8±1.3	0/7	4.0	3.9	NS	0
Control plasmid	ND	ND	ND	ND	0	0/6	5.4	3.7	NS	0	ND	ND	ND	ND	ND
pHA plasmid	8/9	3.7	1.6	<1	1.8±0.9	0/8	4.6	4.2	NS	0	1/7	5.4	4.7	NS	0

[a] The offsprings were studied at the age of 2 weeks, 1 month and 3 months.

[b] Survival expressed as number of survivors / total number of mice infected with WSN virus.

[c] Pulmonary lung titers were estimated at day 3, 7 and 16 after the challenge. The results were expressed as geometric means of $\log_{10}TCID_{50}$ for at least 3 mice in each group.

[d] HI titers of WSN specific antibodies were expressed as means±SD of individual $\log_2$HI values.

ND-not done; NS-no survivors.

offspring born from mothers immunized with pHA, might be due to the secretory IgA acquired via the collostrum. The offspring born from mothers immunized with live WSN virus exhibited a more pronounced protection associated with detectable HI titers lasting at least 1 month, but not more than 3 months after birth (Table 13). As expected, no protection was noted in the case of offspring born from dams injected with control plasmid (CP).

5.4. Conclusion

The ability of the offspring from dams immunized i.m. with pNP to mount CTL responses to live-PR8 virus, indicates the lack of tolerance induction to NP during the fetal life. It indicates that the quantities of antigen or plasmid transmitted through the maternal-fetal circulation are insufficient to induce specific tolerance. This result is significant since it was previously shown that myoblasts transfected with pNP release the protein in the supernatant (Donnelly, J.J., communication), although the NP is initially targeted to the nucleus (Wang *et al.* 1997b). Thus, our results indicate the lack of immunological tolerance in the offspring, as potential side effect of maternal immunization with naked DNA vaccines.

In contrast, studies carried out in our laboratory showed that the maternal immunization with pHA expressing HA of the WSN virus, induced significant protection in the offspring (Table 13 and Antohi *et al*, manuscript in preparation). As

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expected, the protection correlated with the maternal-fetal transmission of neutralizing antibodies.

Secondly, the ability of the offspring born from PR8-immunized dams to mount CTL responses subsequent to the neonatal inoculation with pcNP plasmid, indicate that the DNA immunization may circumvent the inhibitory effect of the specific maternal antibodies on the immune response of the offspring to conventional vaccines. This is an important point since most of the population was exposed to Influenza virus and consequently display antibodies to one or more strains.

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6. Mechanisms of protection following DNA immunization

Previous studies showed that the profile of Th response is closely related to the type of immune effectors generated as response to an antigenic stimulation (reviewed by Brown and Hural, 1997; A. Billiau, 1996). Whereas Th1 responses are associated with delayed type of hypersensitivity and cytotoxicity, as well as the generation of complement-fixing and opsonizing antibodies, the Th2 responses are associated with the induction of humoral responses consisting of non-complement fixing antibodies as well as the recruitment of eosinophils and basophils. To a certain extent, the signals that mediate the differentiation of a particular subtype of Th cells, inhibit the generation of the other subtype. For example, in the mouse model, whereas IL-4 inhibits the generation of Th1 cells (Brown and Hural, 1997), IFNγ impairs the induction of Th2 cells (reviewed by A. Billiau, 1996). In the particular case of DNA immunization, the adjuvant effect of the bacterial plasmid consisting in the rapid production of IFNγ, IL-12 and IL-6 (Klinman *et al.* 1996), is thought to play an important role in the generation of Th1 responses (Roman *et al.* 1997). We tested the requirement for IFNγ and IL-4 in the CTL induction and protection against lethal challenge with PR8 virus, of BALB/c mice immunized with a plasmid expressing NP.

Secondly, we addressed the role of *in vivo* transfected APCs in the generation of CTL responses following the inoculation of DNA-based expression vectors. In particular, we assessed if the APCs with the ability to migrate from the site of

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inoculation can uptake the pNP plasmid, express the NP protein and prime PR8 virus-specific CTLs.

6.1. DNA immunization of IFNγ and IL-4 deficient mice with a plasmid expressing NP

Three month-old BALB/c mice, as well as IFNγ-/- and IL-4-/- mice of the same age and genetic background, were immunized with pNP and challenged with LD_{100} of PR8 virus at one month after the completion of immunization. At seven days after the infection, the mice were sacrificed and the pulmonary virus titers were estimated in an individual manner. In contrast to the pNP immunized wild-type mice that completely cleared the virus by day 7 after the challenge (all five mice with titers less than 1.0), the IFNγ-/- as well as the IL-4-/- mice exhibited an impaired clearance of the virus (geometric means±SD of 1.7±1.5 and 1.9±0.4, respectively; Table 14). Whereas two out of three IFNγ-/- did not clear the pulmonary virus, all four IL-4-/- mice failed to clear the virus by day 7. Various numbers of splenocytes harvested at day 7 after the challenge, were incubated with stimulator cells infected with PR8 virus and tested after four days against PR8-infected and non-infected M12 target cells. Interestingly, the IFNγ-/- mice mounted secondary CTL responses similar to the wild-type mice (Table 14). In contrast, the IL-4-/- mice displayed reduced CTL responses against the PR8 virus.

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Table 14. The cellular immune response of IFNγ and IL-4 deficient mice to pNP plasmid

		BALB/c	IFNγ-/-	IL-4-/-
Lung titer at day 7[a]		<1.0	2.7	1.4
		<1.0	2.4	2.4
		<1.0	<1.0	1.9
		<1.0		1.9
		<1.0		
Cytotoxicity[b]				
R/S:	1:1	24±1	29±2	14±4
	1:2	21±3	24±1	11±3
	1:4	19±3	8±1	5±3
	1:8	1±1	0	1±1
Cytokine secretion[c]				
	IFNγ	155±21	0	185±32
Splenocytes	IL-4	6±3	12±4	0
	IL-2	42±12	60±5	44±15
	IFNγ	27±7	0	17±12
BAL	IL-4	8±3	6±5	0
	IL-2	34±10	36±5	14±10

[a] The mice were infected with LD_{100} of PR8 virus at one month following the completion of immunization with pNP. The pulmonary virus titers were measured at seven days after infection and expressed as $\log_{10}TCID_{50}$ in an individual manner.

[b] The cytotoxicity was measured after incubation of responder cells, namely splenocytes harvested at seven days since challenge, with stimulator cells infected with PR8 virus, at various responder/stimulator ratios. The responder cells were tested after 4 days of stimulation, against M12 target cells infected with PR8 virus. The results are expressed as means of triplicates ± SD of % specific lysis values.

[c] $2x10^5$ splenocytes or $5x10^4$ cells obtained by bronchial lavage (BAL) were in vitro stimulated for four and two days respectively, with $2x10^5$ PR8-infected splenocytes. The cytokine concentrations measured by ELISA were expressed as means±SD of duplicates.

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The estimation of cytokine production by T cells harvested from spleen, or by bronchoalveolar lavage (BAL) cells, showed only subtle differences among the various groups, except the absence of IFNγ and IL-4 respectively, in the knock-out mice. Whereas the splenocytes from the IFNγ-/- mice secreted slightly higher amounts of IL-2, those from the IL-4-/- mice produced slightly higher levels of IFNγ, as compared to the wild-type mice. The BAL cells from the IL-4-/- mice produced lower amounts of IL-2, as compared to the BALB/c mice (Table 14).

Thus, the IFNγ-/- and the IL-4-/- mice immunized with pNP, showed a relatively impaired protective response against the lethal challenge with PR8 virus, manifested by a failure to completely clear the pulmonary virus by day 7 subsequent to the infection. Whereas the cytotoxic response of the IL-4-/- mice was reduced, that of the IFNγ-/- mice was comparable to the one of wild-type mice.

6.2. The role of *in vivo* transfected APCs in the induction of CTL responses

The immune response to foreign antigens encountered at the level of skin or mucosal barriers is initiated by a specialized subset of professional APCs, namely the Langerhans cells (reviewed by Steinman *et al.* 1993). The Langerhans cells, that constitutively express co-stimulatory molecules, uptake the antigens, process them and subsequently to their migration in the local lymph nodes, present the resulting peptides in the context of MHC molecules to specific T cells. We addressed the

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question if upon intradermal inoculation, the cells with the ability to migrate are also able to uptake the plasmid and to express the foreign protein.

In the first experiment, we carried out PCR using primers specific for the NP of PR8 virus and the DNA extracted from cells that migrated out of epidermal layers, obtained from mice inoculated with pNP plasmid. As shown in the Fig. 28A, strong specific signals were obtained from the DNA extracted from $2x10^5$ migratory cells. The titration of the number of migratory cells showed a significant decrease in the intensity of the PCR product when the number of cells used for the DNA extraction was below 10^3. However, weak but detectable signals were still obtained from $8x10^2$ migratory cells, indicating that approximately 1 in 100-1000 migratory cells were transfected subsequent to the *in vivo* exposure to the pNP plasmid. The specificity of the PCR products was certified by the digestion with two different restriction enzymes, *Mva* and *Acs1*. As shown in the Fig. 28B, similar digestion fragments were obtained from the PCR products of pNP and the DNA extracted from migratory cells.

Besides the ability to take up the plasmid, some migratory cells express the NP protein, as indicated by the staining with anti-NP antibody after the fixation and permeabilization of the cells (Fig. 29). The cells were counterstained with rat anti-mouse MHC-II specific antibody, as well as a fluorocrome that binds to the genomial DNA. This allowed us to quantify the percentage of the cells that expressed the NP and are MHC-II$^+$ or MHC-II$^-$. Out of 409 migratory cells examined, 205 were MHC-II$^+$, a result that is concordant with previous studies showing a high percentage of MHC-II$^+$ Langerhans cells among the crawl-out cells (Ortner *et al.* 1996).

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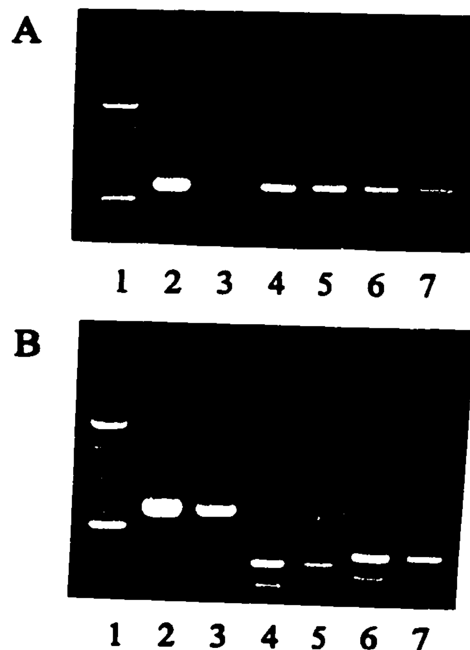

Fig. 28. Uptake of the pNP plasmid by the crawl-out APCs following intradermal inoculation. (A): PCR of DNA extracted from 10^5 (lane 4), $2x10^4$ (lane 5), $4x10^3$ (lane 6) and $8x10^2$ (lane 7) crawl-out cells obtained by tissue culture of epidermal layers *in vivo* inoculated with pNP. The specificity of the primers amplifying NP was tested with positive control (pcNP, lane 2) and negative control (CP, lane 3). Lane 1: 100bp ladder. (B): The characterization by digestion with restriction enzymes, of the PCR products obtained from crawl-out cells. The PCR products were obtained by amplification with NP specific primers of DNA extracted from crawl-out cells and non-digested (lane 3), or digested with *Mva* (lane 5) or *AcsI* (lane 7). As control, we used the PCR product of pNP, non-digested (lane 2), or digested with *Mva* (lane 4) or *AcsI* (lane 6). Lane 1: 100bp ladder.

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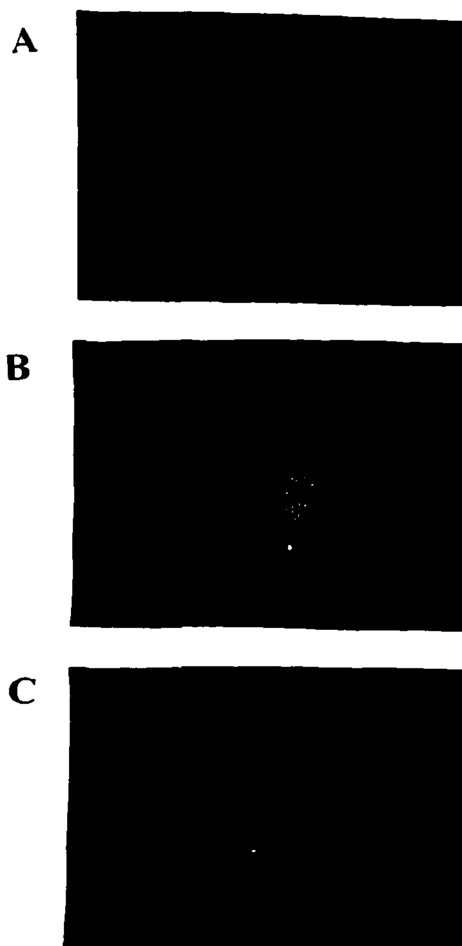

Fig.29. Expression of NP by MHC-II⁺ and MHC-II⁻ crawl-out cells, subsequent to the intradermal inoculation of pNP plasmid. (A) Staining of the cells with DAPI that binds to the nuclear DNA. (B) The same cells stained with polyclonal anti-NP antibody and (C) monoclonal anti-MHC class-II antibody.

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Six out of the 205 MHC-II$^+$ migratory cells expressed the NP with a predominant nuclear-granular pattern. Furthermore, 4 out of the 204 MHC-II$^-$ migratory cells expressed the NP protein. Thus, these results showed that approximately 2-3% of the migratory cells express the NP protein following the *in vivo* transfection with pNP.

We assessed the ability of the migratory cells to prime virus-specific CTL responses when adoptively transferred into naïve BALB/c mice. We chose this experimental protocol rather than the *in vitro* stimulation of NP-specific CTL lines or clones, since it is known that the ability to restimulate previously activated cells, does not necessarily reflect the priming ability of naïve cells, that generally requires co-stimulation (Dubey *et al.* 1996). At seven days after the intrasplenic adoptive transfer of migratory cells from mice inoculated with pNP, the splenocytes from recipient mice displayed small but significant virus-specific cytotoxicity, after *in vitro* expansion with PR8-infected stimulator cells (Table 15). The estimation of frequency of the virus-specific pCTLs by limiting dilution analysis, showed that the migratory cells harvested at day 3 after initiating the epidermal tissue cultures, displayed higher ability to prime CTLs, since 10 times less APCs were required to induce a similar response with that of the crawl-out cells harvested at day 1 (Table 15). No significant responses were noted in the case of migratory cells from animals inoculated with control plasmid, that lacks the NP open reading frame. Surprisingly, the frequency of pCTLs was below $1/10^6$ splenocytes after the adoptive transfer of migratory cells

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Table 15. CTL response of mice injected with crawl-out cells obtained from mice inoculated with plasmids expressing NP of PR8 virus

Group			%Lysis±SEM against:[d]		PR8 specific pCTL[e]	
Donors inoculated with:[a]	Day of harvest:[b]	Number of cells transferred:[c]	P815	PR8 infected P815	pCTL frequency[f]	Total pCTL number
pNP	1	$1x10^6$	9±3	23±4	$1.79x10^5$	558
	2	$2x10^5$	4±4	13±3	$2.50x10^5$	400
	3	$1x10^5$	19±6	32±3	$1.72x10^5$	581
Control plasmid	1	$5x10^5$	9±5	7±2	$>1x10^6$	<100
	2	$1x10^5$	0	6±4	$>1x10^6$	<100
	3	$5x10^4$	0	0	$>1x10^6$	<100

[a] The donor mice were inoculated into the ears (i.d.) with 30μg in 10μl saline of plasmids, five times: day-2, day-1 and three times in the day of sacrification, 6, 3 and 1 hour before ear harvest.

[b] The ears were harvested at day 0 and flaps were prepared and incubated. The crawl-out cells were daily harvested for three days.

[c] Recipient mice were surgically opened and inoculated with various numbers of crawl-out cells into the spleen. A total number of nine mice were inoculated.

[d] The cytotoxicity was estimated at seven days after inoculation, using effectors restimulated with PR8 infected APC.

[e] The frequency of virus-specific pCTLs in the spleen of recipients was estimated by limiting dilution analysis at seven days after inoculation. The total number of splenic pCTLs was estimated based on the total number of splenocytes (mean=10^8 cells).

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from epidermal layers *in vivo* inoculated with pcNP74 (data not shown), that expresses a truncated form of NP (Wang *et al.* 1997b). The reason for the lack of activity for pcNP74 in this assay, may be the lower transcription efficiency of the pcDNA I vector that contains an SV40 instead of a BGH polyadenylation signal (Montgomery *et al.* 1993), rather than the absence of the dominant K^d - restricted CTL epitope.

Thus, a small percentage of both MHC-II$^+$ and MHC-II$^-$ cells with the ability to migrate from the inoculation site, are *in vivo* transfected and express the foreign antigen. Furthermore, they are able to prime specific CTL responses when adoptively transferred into naïve recipients.

6.3. Conclusions

The study of the protective ability of the immune response in IFNγ-/- and IL-4-/- mice immunized with pNP and infected with PR8 virus, showed that neither IFNγ nor IL-4 were crucial for the induction of virus-specific cytotoxicity. However, the magnitude of the CTL response in the IL-4-/- mice was decreased as compared to the wild-type controls, most probably accounting for their impaired ability to clear the pulmonary virus by day 7 subsequently to the challenge. Interestingly, in spite of their ability to mount significant CTL responses, the clearance of the pulmonary virus by the IFNγ-/- mice immunized with pNP and challenged with lethal doses of PR8 virus, was significantly affected. Based on the previous literature as well as ongoing research in

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our laboratory, this result may be explained taking into consideration the role of IFNγ in the recruitment of effector cells at the site of infection and/or in the up-regulation of MHC and adhesion molecules on the infected cells.

The study of the migratory cells after the intradermal inoculation of the pNP plasmid, showed that approximately 2-3% of the cells express the NP protein following the uptake of the plasmid. Roughly, 50% of the transfected cells are MHC-II^+, namely Langerhans cells known for their role as professional APC. When adoptively transferred into naïve recipients, the migratory cells primed virus-specific CTL responses. Together, these results suggest that subsequently to the inoculation of plasmids expressing foreign antigens, a few APCs are transfected and express the antigen that is processed by the endogenous pathway. Following the migration into the local lymphoid organs, these transfected APCs might directly turn-on the naïve CTL precursors.

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DISCUSSION

Our results using plasmids expressing various antigens of influenza virus, showed that naked DNA immunization of newborns was immunogenic rather than tolerogenic. Thus, neonatal inoculation of BALB/c mice with plasmids expressing NP or HA of type A Influenza viruses was followed by the priming of NP-specific CTLs or HA-specific B and $CD4^+$ T cells, respectively.

The neonatal immunization with pNP plasmid, that expresses NP of the A/PR/8 virus, led to the priming of type-A influenza virus cross-reactive CTLs (Fig. 4). The CTLs generated by the neonatal inoculation of pNP, lysed target cells coated with the NP 147-155 peptide that is a major NP epitope, or target cells infected with various subtypes of type A viruses but not with a type B virus (Fig. 2,3 and 4). The CTL response was significantly increased after the boost with live-PR8 virus, as demonstrated by the primary and secondary cytotoxicity data (Fig. 2 and 3), as well as by the pCTL-frequency estimation (Fig. 7). Furthermore, the infection of mice immunized as newborns with pNP resulted in faster and increased pulmonary recruitment of virus-specific CTLs (Table 4). The ability of the T cells from mice immunized with pNP as neonates and boosted with live-PR8 virus to secrete IFNγ and to proliferate upon antigen stimulation, was significantly enhanced as compared to that of the T cells harvested from mice immunized only with virus (Fig. 5 and 6). Cell separation experiments demonstrated that the NP 147-155 peptide-specific $CD8^+$

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T cells induced by the pNP immunization of neonates exhibited a Tc1 profile (Fig. 5), like the NP-specific cells elicited in adults immunized with live-virus (Fig. 23). Further experiments aimed at characterizing the expansion requirements for the virus-specific CTLs primed by pNP, showed that an optimal T help tremendously increased the cytotoxic activity (Fig. 10). This result has direct implications in the design of vaccines, meaning that optimal CTL responses are obtained when strong $CD4^+$ T cell epitopes are co-delivered. Thus, our results showed that the neonatal inoculation of pNP primed CTL precursors as in adults, endowed with the ability to proliferate, differentiate into effector cells, migrate into the lungs, produce IFNγ and lyse type-A Influenza virus infected cells, subsequently to antigen-specific stimulation.

The estimation of PR8-virus specific pCTL frequency showed that the immunization of adult BALB/c mice with pNP plasmid increased the precursor frequency from less than $1/10^6$ splenocytes to approximately $1/2x10^4$ splenocytes, at 1 month after the completion of immunization (Fig. 7). Live-virus immunization of adult mice led to a higher frequency of specific pCTLs ($1/1x10^4$ splenocytes), but the virus boost of mice immunized as adults or neonates with pNP was followed by even higher increase of the pCTL frequency ($1/2.5x10^3$ and $1/5x10^3$ splenocytes, respectively; Fig. 7). Significantly, the pCTL frequency in mice immunized with pNP and boosted with live PR8 virus, was of the same order of magnitude with that of mice immunized with an H3N2 virus (A/HK/68) and boosted with an H1N1 virus (A/WSN/33), indicating that pNP is a potent priming vehicle. The estimation of pCTL frequency and total number of pCTLs/spleen showed that the pNP

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immunization of adult mice was more effective than the neonatal immunization (Fig. 7 and 8). The total number of pCTLs in mice immunized as neonates with pNP, although lower at the age of 1 month, increased until 3 months attaining a peak of approximately 10^3 pCTLs/spleen, comparable to the number of pCTLs in pNP immunized adult mice (Fig. 8). The different kinetics of the pCTL number in mice immunized with pNP as newborns or adults, was most likely due to the immaturity of the immune system in young mice, namely the reduced number of mature T cells in neonates. Significantly, the total number of virus-specific pCTLs correlated with the degree of protection against lethal challenge with PR8 virus (Fig. 11), suggesting that at least 10^3 pCTLs/spleen are associated with significant protection. At three months after the completion of immunization, the number of virus-specific pCTLs declined in both categories of mice, immunized with pNP as newborns or adults (Fig. 8). This was due to the decay of the memory pool rather than tolerance induction, since the subsequent CTL response to live PR8 virus was not impaired (data not shown). These results are in striking contrast with the long-lasting persistence of the plasmid at the site of inoculation in adult mice (Fig. 8), suggesting a lack of correlation between the presence of the plasmid in the muscle and the persistence of the immunological memory. Furthermore, the plasmid was not detectable any more in the gluteal muscles of 3 month-old mice immunized as newborns (Fig. 9), although they exhibited a relatively high pCTL frequency as well as protection (Fig. 8 and 11). Together, these data showed that pNP induced a qualitatively similar CTL response in

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newborn as well as adult mice, although the kinetics of the pCTL induction was slower in the mice immunized as neonates.

The neonatal inoculation with a plasmid (pHA) expressing HA of the A/WSN/33 strain of Influenza virus was followed by the induction of humoral and cellular immune responses. In terms of WSN-specific HI antibodies, both adult and neonatal immunization with pHA led to detectable responses in more than 50% of the mice, at one and three months after the completion of immunization (Table 5). The HI titers in adult mice peaked at three months after the completion of immunization with pHA and were comparable to those elicited by the immunization with live-WSN virus. The HI titers in the responder mice immunized as adults with pHA declined after three months and were down to almost undetectable levels at six months after the completion of immunization. However, the live-virus boost was followed by a strong humoral response, indicating that the decay of the HI titers was due to the decrease of the B memory pool rather than tolerance induction. Again, there was a discrepancy between the long-lasting persistence of the plasmid at the site of inoculation and the decay of the antibody titers (Fig. 14). In contrast with adult mice, the mice immunized with pHA as neonates although mounted comparable humoral responses at one month, displayed significantly lower HI titers at three months after the immunization (Table 5). No detectable HI titers against the drift variant PR8 virus, were detected in pHA immunized mice. After the boost with live virus, significant PR8 specific cross-reactive titers were measured, that might contribute to the heterologous protection of the pHA-immunized mice (Table 5). Previous studies

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showed that antibodies may participate not only in the prevention of infection, but to certain extent, in the clearance of the pulmonary virus too (Scherle *et al.* 1992).

The assessment of the isotype profiles of WSN-specific antibodies induced by pHA immunization showed a similarity of patterns between the mice immunized as adults or neonates, that were dominated by γ2a+γ2b antibodies (Fig. 12). In striking contrast, after the virus boost, whereas the isotype profile of the adults remained unchanged, the γ2b titers dramatically decreased in the mice immunized as newborns with pHA. Furthermore, the γ1 and γ3 antibodies were increased and dominated the isotype profile of the mice immunized with pHA as neonates and boosted with live-WSN virus. However, the concentration of γ2a antibodies were comparable in the mice immunized as neonates or adults with pHA and boosted with live virus (Fig. 12). Both neonatal and adult immunization with pHA elicited significant titers of WSN-specific IgA antibodies, comparable to the titers obtained in mice immunized as adults with live WSN virus (Fig. 12). The cytokine profiles of the WSN-specific $CD4^+$ T cells (Table 6) correlated well with the isotype patterns of the WSN-specific antibodies: whereas the IFNγ dominated before and after the virus boost in the group of pHA-immunized adult mice, the $CD4^+$ T cells from the mice immunized with pHA as neonates displayed a mixed cytokine pattern, with significantly enhanced IL-4 levels (Table 6). The Th1 profile of the immune response in the mice immunized as adults with pHA, was more pronounced that the one showed by adult mice immunized with live WSN virus, both in terms of isotypes as well as cytokines secreted by $CD4^+$ T cells (Table 6 and Fig. 12). In contrast, the mice inoculated as

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neonates with WSN virus exhibited a strongly-biased Th2 cytokine profile that accentuated following the virus boost (Fig. 12). This illustrates the different immune responsiveness to Influenza virus of newborns and adults. This point is strengthened by the observation that neonatal inoculation with live-WSN virus, besides exhibiting increased lethality, induced decreased CTL responses in the secondary cytotoxic assays, probably due to the inactivation of virus-specific pCTLs (Fig. 13). This was not due to the induction of neutralizing antibodies that interfered with CTL priming, since the adult mice mounted significant secondary cytotoxicity. In contrast, the strong Th2 profile may play a role since it has been previously shown that high concentrations of IL-4 inhibited the CTL response to Influenza virus (Moran *et al.* 1996). Notably, the pHA immunization of newborns as well as adult mice did not prevent the induction of CTL responses against live-WSN virus (Fig. 13). In the aggregate, these results demonstrate that the neonatal pHA inoculation is immunogenic rather than tolerogenic and it induces protective antibodies as well as $CD4^+$ Th cells. The neonatal immunization with pHA elicited slightly lower titers of HI antibodies and primed $CD4^+$ T cells of a mixed Th1/Th2 profile, compared to the pHA immunization of adult mice, that led to a strongly biased Th1 response.

The inoculation of newborn as well as adult BALB/c mice with a plasmid (VH-TB) expressing a VH construct carrying $CD4^+$ T and B cell epitopes from the HA of PR8 virus, induced Th cells that secreted IFNγ upon *in vitro* stimulation with HA 110-120 synthetic peptide (Fig. 23 and Table 11). The IFNγ response of the T cells from mice immunized as neonates with VH-TB, was enhanced subsequently to

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the virus boost, above the levels of control mice immunized only with PR8 virus, strongly suggesting a Th priming effect of the neonatal inoculation with VH-TB (Fig. 23). Besides IFNγ, the T cells from mice immunized as newborns with VH-TB and boosted with live-PR8 virus secreted significant amounts of IL-4 (Fig. 23). This was not due to the reactivity against host proteins from the virus inoculum, since the T cells were restimulated with HA 110-120 synthetic peptide. The priming effect of the neonatal inoculation with VH-TB, was not apparent at the level of $CD4^+$ T cell proliferation subsequent to *in vitro* stimulation with synthetic peptides (Fig. 22). This is most probably due to the lower sensitivity of the proliferation compared to the cytokine assay, rather than due to tolerance induction, since the proliferation of the HA 110-120 specific T cells from mice immunized as newborns with VH-TB and boosted with live PR8 virus was not impaired (Fig. 22). Similarly, the neonatal inoculation of VH-TB did not significantly affect the CTL response to live-PR8 virus (Fig. 24).

In terms of humoral response, in contrast to the adult mice (Table 11), no significant titers of specific antibodies were measured in the mice immunized as neonates with VH-TB (Table 12). Again, this was not due to the B cell tolerance induction, since the virus boost was followed by both significant PR8-specific HI titers as well as increased levels of antibodies specific for the B cell epitope expressed by VH-TB, indistinguishable from those of controls not primed with VH-TB (Table 12). Together, these results showed that the neonatal inoculation of BALB/c mice with the VH-TB plasmid that encodes a secreted protein bearing immunodominant

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epitopes recognized by $CD4^+$ T and B cells, did not induce specific tolerance to the defined HA epitopes.

The experimental model of Influenza virus allowed us to assess the protection ability of the neonatal DNA immunization with plasmids expressing HA and NP. Adult or newborn immunization with pNP resulted in reduced but significant protection against lethal challenge with LD_{100} of PR8 or HK virus. This protection was mirrored into the ability of the survivor mice to clear the pulmonary virus and to completely recover from the acute infectious disease elicited by aerosol infection with either PR8 or HK virus (Table 3). The percentage of the survivor mice was statistically significant (p of Fisher's exact test < 0.05) for the group of mice immunized as newborns with pNP and challenged with PR8 virus three months later (Fig. 11). In contrast, the adults immunized with pNP displayed statistically significant survival rates subsequent to the PR8 infection, slightly higher than 50%, both at one as well as three months following the completion of immunization (Fig. 11). This correlated with the frequency and total number of PR8 virus-specific pCTLs in the spleens of mice immunized as newborns or adults with pNP (Fig. 8). The survival rates of the mice immunized with pNP after lethal challenge with HK were reduced (Fig. 11), in spite of their ability to clear the pulmonary virus (Table 3). Furthermore, although pNP induced a significant CTL response against the WSN strain of influenza virus (Fig. 16), no significant protection in terms of survival, was noted after lethal challenge with this neurovirulent strain (Fig. 18). These discrepancies between the ability of pNP to induce cross-reactive CTLs and the

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protection against lethal challenge with various strains of type-A influenza virus, may be due to differences regarding the strain virulence, rather than due to the involvement of distinct immune mechanisms. A prompt recruitment of effector CTLs into the lungs of infected mice is critical for the early clearance of the virus (Table 4) and limitation of the DTH-mediated inflammatory reaction that may otherwise lead to morbidity and mortality, in spite of the virus titer reduction.

Adult and neonatal inoculation of pHA conferred significant protection against lethal challenge at one month after the completion of immunization, with the homologous virus (A/WSN/33) or the drift variant (A/PR/8/34). The percentage of the surviving mice varied between 25% and 60% depending on the group of mice (Fig. 15), but the p value was always <0.05. The mice immunized as newborns with pHA displayed slightly less survival rates than the pHA immunized adult mice. Interestingly, like the surviving mice immunized with pNP, the protected mice inoculated with pHA displayed significant pulmonary virus titers at day three after the homologous or heterologous challenge, but cleared the virus until day 7 (Table 7). Thus, both pNP and pHA induced protective mechanisms that did not prevent the infection but helped the recovery. In contrast to the case of pNP where the virus-specific $CD8^+$ CTLs played the most important role, in the case of pHA, both the $CD4^+$ Th cells as well as the recall antibody response might have contributed to the clearance of the virus.

In stark contrast with the plasmid immunization that induced suboptimal protective responses manifested by limited survival rates subsequent to lethal

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challenge, the immunization of adult mice with live virus conferred complete protection (Fig. 11 and 15). In an attempt to increase the protection ability of the neonatal immunization with naked DNA, we co-inoculated pNP and pHA and challenged the mice with WSN or PR8 virus at four weeks after the completion of immunization. The neonatal as well as the adult immunization with pHA+pNP, elicited increased protection against challenge with WSN (survival rate 100%, with $p<0.0001$) or PR8 virus (survival rate approximately 80%, with $p<0.001$) (Fig. 18). The neonatal inoculation with pHA+pNP elicited type-A cross-reactive CTLs (Fig. 16), $CD4^+$ T cells secreting IFNγ (Fig. 17), as well as small but significant WSN-specific HI antibody titers (Table 8). However, the immunization with pHA+pNP did not prevent the infection but elicited immune effectors able to effectively clear the pulmonary virus by day 7 following the lethal challenge with WSN virus (Table 10). The cellular immunity played a major role in the protection since the neonatal inoculation of pHA+pNP elicited smaller titers of HI antibodies, compared to adult immunization with UV-inactivated WSN virus (Table 8), that was associated with suboptimal protection in terms of survival, following lethal homologous challenge (Fig. 21). The study of the relationship between the dose of plasmid and the protection in terms of survival, showed that doses as small as 7.5μg/plasmid/inoculation led to significant protection, both in the case of mice immunized as newborns or as adults (Table 9). From a practical point of view, the coinjection of synergistic plasmids may allow the induction of protective immune responses with lower doses of naked DNA.

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As compared to the neonatal immunization with live or UV-inactivated virus, DNA immunization with pHA+pNP was clearly superior from a protective point of view. First, the neonatal inoculation with various doses of live-WSN virus was lethal, inducing impairment of motility associated with feeding failure, cyanosis, dehydration and hypotermia followed by death of the offsprings in an interval of two to six days since the inoculation (Fig. 19). Secondly, although the UV-inactivated WSN virus was not lethal when inoculated into the neonates, it did not elicit protective responses at various doses of immunization (Fig. 21). This was in sharp contrast to the protective response elicited by UV-inactivated WSN virus in adult mice (Fig. 21), underlining again the differences in the immune responsiveness of the neonates versus the adults. The UV-inactivated WSN virus did not elicit specific CTLs (Fig. 16) or HI antibodies (Table 8), but it induced $CD4^+$ Th cells secreting IL-4 as well as IFNγ (Fig. 17). Further studies carried out in our laboratory showed that the neonatal inoculation of UV-inactivated WSN virus induced specific B cell unresponsiveness that lasted at least 3 months, but could be reversed by adoptive transfer of the B cells followed by *in vitro* antigen stimulation together with T cell help (Antohi *et al.* submitted). The neonatal inoculation of pHA, in contrast to the UV-inactivated WSN virus, expanded WSN-specific as well as cross-reactive B cell clonotypes (Antohi *et al.* submitted), circumventing the poor ability of young mice to mount cross-reactive humoral responses (Cancro *et al.* 1979) to Influenza virus.

Since our first report showing the induction of protective immunity subsequently to neonatal DNA immunization (Bot *et al.* 1996a), other studies

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confirmed the immunogenicity and some of them the protective ability of newborn immunization with naked DNA (Table 16).

The immunization of newborn piglets with a plasmid encoding the pseudorabies virus gD glycoprotein, induced a significant level of specific antibodies (Monteil *et al.* 1996), like the neonatal immunization of mice with plasmids expressing the rabies virus glycoprotein (Wang *et al.* 1997a), the Measles virus hemagglutinin, the Sendai virus nucleoprotein and the C fragment of tetanus toxin (Martinez *et al.* 1997). Similarly, the neonatal inoculation of chimpanzees with plasmids expressing the HBV surface antigen (Prince *et al.* 1997), the gp120 or the gag/pol of HIV-1 (Bagarazzi *et al.* 1997), was followed by the induction of specific humoral responses. Significantly, the antibodies induced by plasmid immunization of newborn mice with the glycoprotein of rabies virus, or of infant monkeys with the HBV surface antigen, conferred protection against subsequent challenge (Wang *et al.* 1997a; Prince *et al.* 1997). In contrast, the humoral response induced by the neonatal immunization of pigs with the pseudorabies gp antigen, was not protective (Monteil *et al.* 1996). In terms of isotype profile, the neonatal inoculation with plasmids expressing the Measles virus hemagglutinin, the Sendai virus nucleoprotein or the C fragment of tetanus toxin, elicited antibodies of γ2a rather than γ1 subclass (Martinez *et al.* 1997). This correlated with the observed Th1 or Th1/Th2 cytokine pattern (Martinez *et al.* 1997) and supported our observations indicating that, in contrast to the conventional immunization, the naked DNA immunization of newborns induced an adult-like Th profile (Bot *et al.* 1997c). Finally, the neonatal inoculation of mice

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Table 16. The immune response and protection conferred by neonatal inoculation of plasmids expressing various microbial antigens

Microbe / Reference	Antigen	Species	Immunogenicity	Protection
Influenza virus				
Bot *et al.* 1996a	NP	mouse	CTL	yes
Bot *et al.* 1997b	HA	mouse	Abs and Th cells	yes
Pseudorabies virus				
Monteil *et al.* 1996	gD glycoprotein	pig	Abs	no
Rabies virus				
Wang *et al.* 1997a	glycoprotein	mouse	Abs and Th cells	yes
Hepatitis B virus				
Prince *et al.* 1997	HBs antigen	chimpanzee	Abs	yes
Murine retrovirus				
Sarzotti *et al.* 1997	Cas-Br-M Ag	mouse	CTL	yes
HIV				
Bagarazzi *et al.* 1997	env	chimpanzee	Abs	not known
	gag/pol	chimpanzee	Abs	not known
Measles virus				
Martinez *et al.* 1997	Hemagglutinin	mouse	Abs and Th cells	not known
Sendai virus				
Martinez *et al.* 1997	Nucleoprotein	mouse	Abs and CTL	not known
Clostridium tetanii				
Martinez *et al.* 1997	C fragment of TT	mouse	Abs and Th cells	not known

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with plasmids expressing murine leukemia virus antigens (Sarzotti *et al.* 1997) or the Sendai virus nucleoprotein (Martinez *et al.* 1997), was followed by the induction of specific CTLs that mediated significant protection against challenge, in the case of murine leukemia virus. These reports extended our previous observation in the influenza virus model (Bot *et al.* 1996).

In stark contrast with the reports describing immunogenicity and protection ability of the neonatal DNA inoculation, there is one study showing tolerance elicited in newborn mice subsequent to the inoculation of a plasmid expressing the circumsporozoite protein of the *Plasmodium yoelii* (Mor *et al.* 1996). The plasmid induced tolerance to certain CTL, Th and B cell epitopes of the circumsporozoite protein, while preserving the responsiveness to other epitopes. It is not clear yet what is the role of the vector or the antigen in the induction of tolerance as opposed to immunity, although the same group reported other plasmids expressing malaria antigens that were not tolerogenic when inoculated into newborn mice (D.M. Klinman, personal communication).

In conclusion, our data as well as most of the evidence coming from other laboratories support the notion that plasmids expressing foreign antigens are immunogenic rather than tolerogenic when administered to newborns. In spite of the fact that the newborns possess functional T and B cells in the periphery that are able to differentiate to memory and effector cells upon antigenic stimulation, they are more susceptible to high-dose tolerance and strongly biased Th2 responses (reviewed

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by Bona and Bot, 1997). The lack of tolerance as a rule in DNA immunization of neonates, may be due to two mechanisms that can act in a complementary manner: first, the adjuvant effect of the bacterial plasmid (Klinman *et al.* 1996) may have a role in the recruitment and activation of professional APC as well as direct co-stimulation of the naïve lymphocytes. Recently, it has been suggested that bacterial DNA containing CpG motifs may bind to certain receptors expressed on APCs leading to the activation of NF-kB, that results in the transcription of genes expressing cytokines and co-stimulatory molecules (C. Millan, communication). Previous studies suggested that whereas in the absence of co-stimulation the naïve cells are readily tolerized, strong co-stimulation precludes the tolerance induction at the level of naïve but not memory cells (Fuchs and Matzinger, 1992; Bluestone, 1997). Secondly, the continuous production of very low amounts of antigen may be incompatible with the requirements for high-zone tolerance, even in the neonates. Thus, the exposure of lymphocytes to low amounts of antigen in a continuous manner and in the presence of co-stimulation, may explain the immunogenicity rather than the tolerance subsequent to the inoculation of neonates with DNA-based vectors expressing foreign antigens.

In our studies, we also addressed the effect of the maternal immunity on the offspring responsiveness to virus antigens. It was previously demonstrated that the maternal antibodies transmitted to the progenies, while mediating protection against infectious challenge, inhibited the generation of active immunity by conventional antigens

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(reviewed by C.-A. Siegrist, 1997). Our studies carried out in the experimental model of HA-derived MHC class-II restricted epitopes, showed that antibodies against the carrier affected the rate of generation of the epitope - MHC class-II complex on professional APCs (Bot *et al.* 1996; reviewed by Bona *et al.*, 1997), pinpointing to a possible mechanism responsible for the lack of response to conventional vaccines in the presence of maternal antibodies. A more recent study showed that while antibodies of maternal origin against carriers interfered with the generation of active immunity, replacing the carrier would prevent the inhibitory effect of the maternal antibodies (Wang *et al.* 1997). Thus, one may expect that the neonatal inoculation of naked DNA would by-pass to certain extent the negative effect of the passive immunity. This seems to be indeed the case in our experimental model that addressed the generation of CTLs by pNP (Fig. 27). Our results together with emerging data from other laboratories (Rouse, B.T., communication; Whitton, J.L., communication), suggest that in the case of T cell epitopes, the DNA immunization may circumvent the inhibitory effect of the maternal antibodies. This could be due to either the fact that professional APCs are directly transfected and/or to the continuous production of antigen by the plasmid, that persists after the decay of the maternal antibodies to non-inhibitory titers. In contrast, the priming of B cells by neonatal DNA immunization was affected to a certain extent by the maternal antibodies (Le Potier *et al.* 1997; H.C.J. Ertl, communication; H.L. Robinson, communication), which is consistent with the requirement of protein liberation by transfected cells in order to stimulate the specific B cells. Presumably, the low amount of antigen secreted or released by the *in*

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vivo transfected cells is bound by the maternal antibodies and cleared by the scavenger cells, precluding an optimal exposure of the B cells to soluble antigens.

Maternal immunization with pNP that expresses the NP of PR8 virus, did not impair the immune responsiveness of the offsprings (Fig. 26). It results that the amount of antigen and/or plasmid transmitted from the mother to the progenies via the placenta was to small to induce central tolerance of the specific CTL precursors in the developing thymus. This result was extended to the B cells, since experiments carried out in our laboratory by Antohi *et al.* showed that the maternal inoculation of pHA did not impair the humoral response of the 3 months-old offsprings to WSN virus (data not shown). These data indicate the lack of tolerance induction in progenies, as a potential side effect of maternal immunization with naked DNA. In contrast, the maternal immunization with pHA was followed by the transmission of significant amounts of virus-specific HI antibodies to the offsprings (Table 13). The two week-old progenies born from dams immunized with pHA, exhibited an unexpectedly high survival rate after the challenge with LD_{100} of WSN virus, relative to the low titer of serum HI antibodies, indicating a potential role for the IgA-specific antibodies transmitted via collostrum. The progenies of WSN-immunized dams exhibited higher HI titers that lasted longer time than those of the offsprings born from dams immunized with pHA (Table 13). Together, these results suggest that the maternal immunization with naked DNA may be a potential means for the passive immunization of neonates.

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The role of IFNγ and IL-4 in the protective response of plasmid-immunized mice to PR8 virus, were investigated using the gene targeted mice. Previous studies showed that the protection of pNP immunized mice is dependent on virus-specific $CD8^+$ CTL (Fu *et al.* 1997a; Bot *et al.* 1997a). Thus, the model of gene targeted mice allowed us to assess the requirement for IFNγ and IL-4 in the induction of protective CTL responses following naked DNA immunization. The mice lacking a functional IFNγ gene display multiple defects regarding the anti-microbial response, due to an impaired activity of macrophages as well as a reduced NK function (Dalton *et al.* 1993). The IFNγ-/- mice were found to be more susceptible to infections with intracellular pathogens like Listeria monocytogenes (Harty and Bevan, 1995). IFNγ-/- mice immunized with pNP and challenged with a lethal dose of PR8 virus, exhibited an impaired clearance of the pulmonary virus, as compared to the pNP-immunized wild-type counterparts, that completely cleared the virus (Table 14). However, a significant reduction of the pulmonary virus titers in comparison to naïve or CP injected mice was noted (Table 3), indicating that the lack of IFNγ did not completely block an immune response against the virus. This notion is supported by the fact that the IFNγ-/- mice immunized with pNP and infected with PR8 virus mounted a CTL activity comparable with the wild-type counterparts (Table 14). This result is concordant with a previous study that showed unaffected CTL responses of the IFNγ-/- mice against the A/Jap/57 strain of influenza virus (Graham *et al.* 1993). However, this cannot explain the impaired ability of the IFNγ-/- to clear the pulmonary virus

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during secondary responses against PR8 (Table 14) or WSN virus (Bot *et al.*, in preparation). The lack of complete clearance of the pulmonary virus by IFNγ-/- mice immunized with pNP and challenged with PR8 virus, might be explained by an impaired local recruitment of effector cells and/or by a defective generation of MHC class-I - peptide complexes on the infected cells that are non-professional APC. Indirect evidence support this hypothesis: previous studies showed that IFNγ up-regulates the expression of MHC class-I on virus infected cells (Yang *et al.* 1995), as well as the generation of viral peptides to be presented in the context of class-I molecules (Geginat *et al.* 1997). Furthermore, the study of β2-/- mice showed an impaired local recruitment of the $CD4^+$ T cells in the absence of IFNγ (Sarawar *et al.* 1994), but no data are yet available regarding the $CD8^+$ T cells. The estimation of cytokine production by the T cells from spleen and the bronchoalveolar lavage, showed only a slight shift of the cytokine profile toward a Th2 pattern (Table 14), a result that is concordant with previous studies (Graham *et al.* 1993; Sarawar *et al.* 1994). Furthermore, the ability of the mice to mount HI antibodies against Influenza virus was not impaired by the lack of IFNγ (Bot *et al.* in preparation). Thus, the absence of IFNγ did not prevent the induction of CTLs by pNP plasmid and did not dramatically change the Th pattern of the T cell response to PR8 virus, but indirectly affected the ability of the effector mechanisms to clear the pulmonary virus.

The role of IL-4 in the defense against Influenza virus infection is still uncertain, although previous studies reported that Th2 clones as well as high doses of IL-4 negatively interfered with the ability of the effector T cells to clear the

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pulmonary virus (Graham *et al.* 1994; Moran *et al.* 1996). We studied the protectiveness of the cellular immune response induced by pNP immunization of IL-4-/- mice. Previous studies showed that the disruption of the IL-4 gene leads to an impairment of the Th2 response, leading to strongly biased Th1 responses against foreign antigens (Kopf *et al.* 1993). Since Th1 responses are beneficial for the defense against influenza virus (Graham *et al.* 1994), one can hypothesize that in the absence of IL-4 the protective response of pNP immunized mice would not be affected. Surprisingly, none of the IL-4-/- mice immunized with pNP and infected with lethal doses of PR8 virus cleared the pulmonary virus, in stark contrast with the wild-type counterparts (Table 14). The activation and expansion of the virus-specific CTLs was impaired in the absence of IL-4 (Table 14), suggesting a possible reason for the ineffective protection of the pNP immunized IL-4-/- mice. Previous studies showed that whereas simultaneous exposure of the pCTLs to IL-4 and antigen was beneficial for their activation and expansion, pulsing the CTLs with IL-4 before or after the antigen stimulation, significantly inhibited their proliferation (Horohov *et al.* 1988; reviewed by Brown and Hural, 1997). However, in view of our results, the IL-4 was not crucial for the generation of CTL immunity, since a small but significant CTL response could be detected in the IL-4-/- mice (Table 14). Thus, IL-4 seems to exert complex effects on the $CD8^+$ T cells: whereas low amounts are necessary for the optimal activation and expansion of the CTLs, the exposure of cells to inappropriately high levels of IL-4 may result in the inhibition of cytotoxicity.

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Finally, we have studied the potential involvement of the *in vivo* transfected APCs in the priming of CTL responses following naked DNA immunization. PCR analysis of the DNA extracted from migratory cells showed the presence of the plasmid (Fig. 28). The immunostaining with NP and MHC-class II specific reagents, showed that approximately 2-3% of the migratory cells express the foreign antigen subsequent to the intradermal inoculation. This result is concordant with previous studies showing the expression of reporter genes in comparable percentages of myocytes following the intramuscular inoculation of plasmid-based expression vectors (reviewed by Pardoll and Beckerleg, 1995). The presence of the antigen in the nucleus suggests that NP is produced rather than being simply taken up by the positively stained migratory cells. Approximately 50% of the migratory cells were MHC-II^{+}, a marker for the dermal Langerhans cells (Ortner *et al.* 1996; Steinman *et al.* 1993). This result is concordant with a recent study showing the presence of transfected dendritic cells in the local lymphoid nodes subsequent to the intradermal inoculation of plasmid-based expression vectors (Condon *et al.* 1996). Since the Langerhans cells, that are precursors of lymph node dendritic cells, express enhanced levels of co-stimulatory molecules like B7 and CD40 and secrete IL-12 upon activation, their ability to prime naïve T cells is greatly enhanced (Inaba *et al.* 1995; Steinman *et al.* 1993). We showed that the adoptive transfer of migratory cells obtained from mice injected with pNP, was followed by the priming of virus-specific CTLs (Table 15). In spite of the fact that this result strongly suggests a direct involvement of in situ transfected Langerhans cells in the priming of specific CTLs subsequent to the naked DNA

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immunization, there are two caveats of this adoptive cell transfer experiment: first, according to previous studies, non-professional APCs can prime immune responses provided their migration into lymphoid organs, due to bystander co-stimulation (Kundig *et al.* 1995) and secondly, it is not clear if the *in vivo* transfected cells directly prime or conversely, secrete the antigen that is taken up and presented by other APCs. However, recent studies indicating that the surgical removal of the inoculated derm affected the T cell response if carried out in an interval of a 1-2 days since the injection but not if carried out later (Torres *et al.* 1997), indirectly supported the notion that the Langerhans cells, with a local turn-over of two to three days, might be involved in the induction of T cell immunity after intradermal DNA immunization. Other recent studies, whereas strongly supporting the involvement of the Langerhans cells in the induction of the T cell response following the i.d. inoculation of naked DNA, estimated that approximately 500 DCs are sufficient to trigger a significant immune response (S.A. Johnston, communication), result that is concordant with our quantitative data.

In stark contrast, the intramuscular inoculation of plasmid-based expression vectors may lead to T cell immunity through distinct mechanisms. The construction of F1/parent radiation-chimera demonstrated the critical involvement of the donor bone-marrow derived APCs in the induction of cytotoxicity following intramuscular DNA immunization (Corr *et al.* 1996; Doe *et al.* 1996). This finding offered an explanation for the lack of tolerance following the expression of foreign antigens by non-professional APCs, like the *in vivo* transfected myocytes. However, surprisingly,

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two lines of evidence suggested that the transfected myocytes transfer the antigen to the migrating professional APCs, that are able to prime the CTL precursors via an exogenous processing pathway (Huang *et al.* 1996): first, adoptive cell transfer experiments with transfected myoblasts resulted in the generation of CTL immunity (Ulmer *et al.* 1996) and secondly, bone-marrow chimeric mice reconstituted long after DNA inoculation mounted a CTL response (Doe *et al.* 1996). Emerging results suggest that peptides rather than the whole antigen might be transferred between the transfected myocytes and the professional APCs (J.J. Donnelly, communication). In contrast, there is one study showing that surgical ablation of the inoculated muscle immediately after injection, did not affect the CTL response (Torres *et al.* 1997). The last study suggested that in contrast to the case of intradermal DNA immunization, the intramuscular inoculation allows significant quantities of plasmid to rapidly reach other tissues, like the local lymph nodes. The apparent contradiction generated by the evidence regarding the mechanism of CTL priming following DNA immunization, can be solved if one accepts that a few pathways simultaneously take place: (1) a reduced number of professional APCs are transfected in situ before the migration to the lymph nodes, probably the predominant mechanism subsequent to the intradermal or gene-gun DNA immunization; (2) certain amounts of plasmid reaches remote tissues or professional APCs shortly after the injection; (3) the foreign antigen is released by the transfected muscle cells and taken up and processed by professional APCs. The last two mechanisms may predominantly occur subsequent to the intramuscular inoculation of plasmids expressing CTL epitopes.

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The priming mechanism in the case of Th and B cell epitopes is generally believed to require the liberation of the foreign antigen from the transfected cells. Since the induction of Th and B cell immunity is the rule rather than the exception in the case of DNA immunization through various routes, it results that certain amounts of antigen are always liberated. However, as shown by our studies, these amounts of antigen are not sufficient for the induction of central tolerance in the offsprings born from DNA immunized dams (Fig. 26), probably because of their rapid clearance from circulation by the professional APCs and/or scavenger cells. Data coming from our laboratory showed that the dendritic cells are effectively employed in the presentation of Th epitopes to MHC class-II restricted T cells, subsequent to intramuscular or intradermal inoculation of plasmid-based vectors (Casares *et al.* 1997a).

In conclusion, our studies aimed to characterize the immune response of newborn mice to three different plasmids expressing intracellular, membrane-bound or secreted Influenza virus antigens, showed the lack of tolerance subsequent to their inoculation. Furthermore, the plasmids were immunogenic, eliciting B, Th and cross-reactive CTL responses that were protective against subsequent infectious challenges. When co-injected into neonates, plasmids expressing HA and NP induced enhanced protection, with the same order of magnitude like the protection conferred by live-virus immunization of adult mice. The plasmid immunization of neonates was clearly superior in terms of protective ability, as compared to the neonatal inoculation of UV-inactivated virus that induced immune unresponsiveness. The immune response of the

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newborn mice to plasmid-based vectors, from a qualitative point of view, resembled more the response of the adults rather than that of the neonates, to virus immunization. Our studies showed that maternal immunization with plasmid-based vaccines, rather than inducing immune unresponsiveness of the offsprings, elicited the production of protective antibodies that were transmitted to the progenies. Furthermore, the maternal antibodies did not inhibited the CTL response elicited by neonatal inoculation with naked DNA. Our studies aimed to address some aspects regarding the mechanism of protection subsequent to the naked DNA immunization, suggested that *in vivo* transfected APCs with the ability to migrate into the local lymph nodes, express the foreign antigen and prime naïve CTL precursors. Whereas the absence of IL-4 but not IFNγ affected to certain extent the induction of CTLs following naked DNA immunization, both IL-4 and IFNγ were necessary for the optimal protection of the plasmid-immunized mice, against lethal challenge with Influenza virus.

Together, our data show that naked DNA immunization may be an effective approach to vaccinate newborns and infants against microbes responsible for infectious diseases with high morbidity or mortality in the young segment of the population.

Further studies are required to address the following points: a) the induction of tolerance versus immunity by neonatal inoculation of plasmids expressing foreign genes; b) the mechanism of priming following plasmid immunization, as well as the

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impact on designing more effective and reproducible strategies of vaccine delivery; c) the influence of the maternal immunity on the effectiveness of neonatal DNA versus conventional vaccines; d) the efficacy assessment of DNA vaccination protocols as compared to licensed vaccines by performing appropriate preclinical studies in outbred species, followed by clinical trials.

The possibility of tolerance induction by neonatal inoculation of plasmids expressing influenza antigens should be investigated due to the implications for designing the vaccination protocols. Two major directions should be followed: first, the timing of inoculation and second, the methylation state of the plasmid. The exact time of inoculation might be important since the neonatal window of susceptibility for tolerance induction depends on the dose and type of antigen. Newborn mice inoculated with influenza antigens expressed by various plasmids administered in various doses during the first day of life, should be studied for their responsiveness to subsequent challenge with influenza virus. This would allow the definition of the neonatal window of susceptibility to tolerance induction in the case of various expression vectors. A second parameter that may influence the immunogenicity of a particular plasmid inoculated into newborns is the adjuvant property of unmethylated CpG motifs. Since the priming ability of an antigen depends on co-stimulatory signals that are up-regulated by adjuvants, it is reasonable to postulate that removal of the unmethylated CpG motifs by DNA methylation would increase the ability of plasmid vectors to induce neonatal tolerance. If true, this would allow the design of new classes of vaccines addressing certain allergic or autoimmune diseases. A particularly

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important point that should be further addressed is the induction of B cell anergy by neonatal exposure to UV-inactivated influenza viruses. It is not known yet if the unresponsiveness is restricted to the B cell epitopes of influenza virus and if not, what is the mechanism of bystander unresponsiveness. Secondly, one should define the circumstances that lead to immunity rather than anergy and the methods to break the unresponsiveness, in order to fully understand this phenomenon.

The requirement to understand the mechanism of DNA immunization stems from the necessity to improve the ability of plasmid vectors to induce protective responses. Indeed, there is high variation in the immune responses of genetically identical animals after a single inoculation of plasmid expressing a particular antigen. The variation tends to diminish after multiple boosts with the same vaccine. This suggests the existence of a limiting step that controls the uptake, processing and/or presentation of the antigens expressed by plasmid vectors. Efforts should be directed to identify the population of professional APC that present the antigens following DNA immunization, as well as the mechanisms by which the antigens enter these cells. We are currently addressing these issues by adoptive transfer experiments of *in vivo* transfected dendritic cells of BALB/c haplotype, into (BALB/cxB6) F1 mice. A response to this question would allow the investigation of means to deliver plasmids in a manner compatible with enhanced responses. Two directions should be pursued: the site of administration and the formulation. One candidate for the site of administration is the respiratory tract because of the increased surface as well as the developed local lymphoid system. Regarding the formulation, one should investigate

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means that effectively target the plasmid into APC and stimulate the innate immunity. For example, particulate formulations (i.e. liposome structures, slow releasing particles, etc.) were shown to be preferentially taken up by APC.

One potential advantage for the neonatal immunization with plasmid-based vectors is the lack of interference of the immune response by maternal antibodies. There is contradictory evidence regarding this property of DNA vaccines, but no data are yet available in the influenza virus model. Presently, we address this point by studying the B and Th immune responses of (BALB/cxB6) F1 progenies immunized with pHA, born from influenza virus immunized BALB/c dams. This protocol will allow us to discriminate the maternal antibodies from the antibodies induced by pHA, using allotype-specific reagents. We compare the ability of the plasmid and the conventional-inactivated vaccines to circumvent the negative interference caused by maternal antibodies.

The immunogenicity and protection ability of candidate vaccines often varies tremendously among different species. Preclinical studies in outbred species are therefore required in order to assess a vaccine previous to clinical trials. We are currently pursuing this point by studying the immune response of newborn baboons to a candidate vaccine containing HA and NP-expressing plasmids. This study will allow us the assessment of DNA vaccines in non-human primates, previous to clinical trials.

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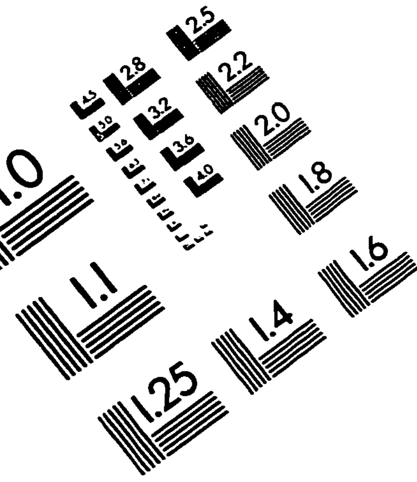
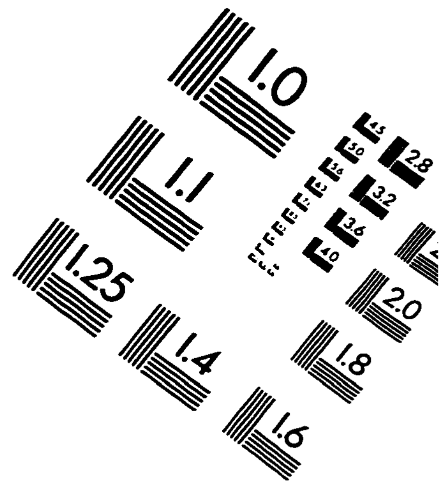
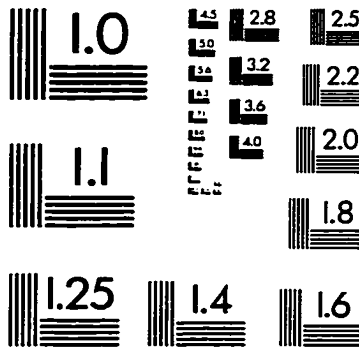
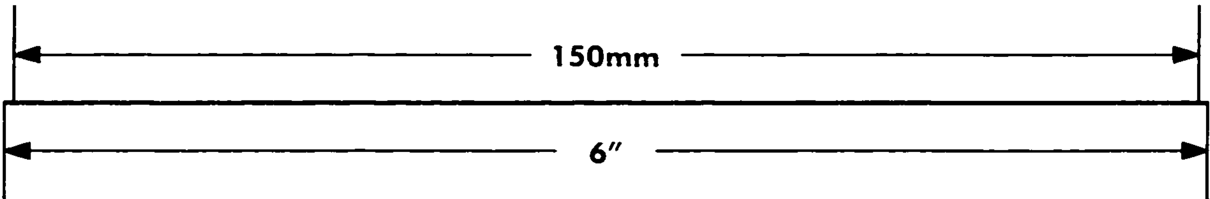
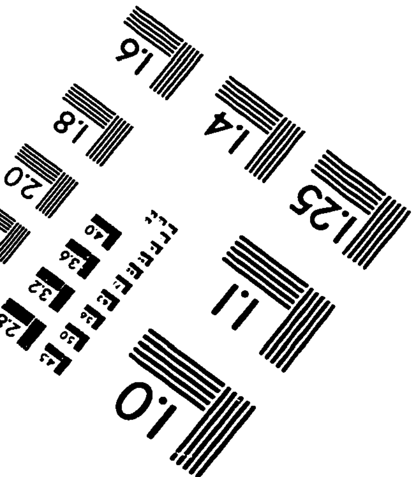
IMAGE EVALUATION TEST TARGET (QA-3)

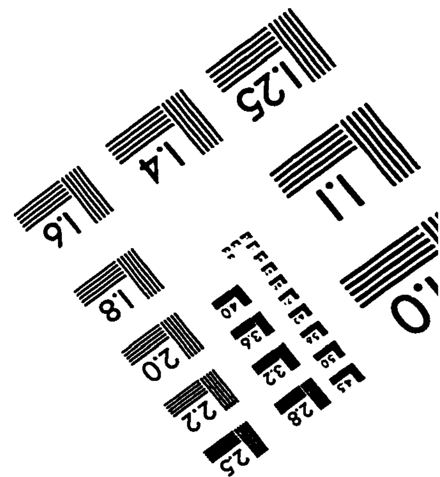












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