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# Characterization of Immune Responses Following Neonatal DNA Immunization: A Dissertation

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# CHARACTERIZATION OF IMMUNE RESPONSES FOLLOWING NEONATAL DNA IMMUNIZATION

A Dissertation Presented

By

TAMERA MARIE PERTMER

Submitted to the faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester, in partial fulfillment of the requirements for the degree of:

DOCTOR OF PHILOSOPHY

April 3, 2000

IMMUNOLOGY AND VIROLOGY

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# CHARACTERIZATION OF IMMUNE RESPONSES FOLLOWING NEONATAL DNA IMMUNIZATION

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## ABSTRACT

Neonatal mice have immature immune systems with defects in several components of inflammatory, innate, and specific immune responses and develop a preferential T helper type 2 (Th2) response following immunization with many vaccine antigens. Although maternal antibody is the major form of protection from disease in early life when the neonatal immune system is still immature, the presence of maternal antibody also interferes with active immunization, placing infants at risk for severe bacterial and viral infection. Recent studies have suggested that immunizing with DNA plasmids encoding the vaccine antigen of interest is highly efficacious in a variety of adult animal models. However, similar extensive studies have not been conducted in infants. In this dissertation, we examine both the quantitative and qualitative differences between neonatal and adult humoral and cell-mediated immune responses in the presence or absence of maternal antibody.

First, we wished to determine if one-day-old neonatal mice immunized with plasmid DNA expressing influenza A/PR/8/34 hemagglutinin (HA) by either intramuscular (i.m.) or gene gun (g.g.) inoculation were capable of generating humoral responses comparable to those in mice immunized as adults. We found that newborn mice developed stable, long-lived, protective anti-HA-specific IgG responses similar in titer to those of adult DNA-immunized mice. However, unlike the adult i.m. and g.g. DNA immunizations, which develop polarized IgG2a and IgG1 responses, respectively, mice immunized as neonates developed a variety of IgG1-, IgG2a-, and mixed

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IgG1/IgG2a responses regardless of the inoculation method. Boosting increased, but did

not change these antibody profiles. We also found that, in contrast to the DNA

immunizations, inoculations of newborn mice with an A/PR/8/34 viral protein subunit

preparation failed to elicit an antibody response. Further, temporal studies revealed that

both responsiveness to protein vaccination and development of polarized patterns of T

help following DNA immunization appeared by 2 weeks of age.

To determine if the disparity of polarized IgG responses between neonatal and

adult DNA vaccinated mice was due to deficiencies in Th1 promoting cytokines, we

addressed the ability of DNA encoding Th1 cytokines to bias the isotype of antibody

raised by neonatal DNA immunization. We found that neonatal mice coimmunized with

HA and either IL-12 or IFNγ-expressing DNAs developed IgG2a-biased immune

responses, regardless of inoculation method, whereas these DNAs had no effect on IgG

subtype patterns in adult DNA immunized mice. Consistent with the Th1-promoting

effects of these cytokines, we also observed that codelivery of IL-12 or IFNγ DNAs

raised T helper responses toward Th1 in mice immunized both as neonates or adults.

Thus, codelivery of cytokine DNAs may be effective at tailoring immune responses

depending on the required correlates of protection for a given pathogen.

Finally, we addressed the effect of maternal antibody on the elicitation of humoral

and cell-mediated immune responses. We tested the ability of i.m. and g.g. immunization

with DNA expressing influenza HA and/or nucleoprotein (NP) to raise protective

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humoral and cellular responses in the presence and absence of maternal antibody. We found that neonatal mice born to influenza-immune mothers raised full antibody responses to NP but failed to generate antibody responses to HA. In contrast, the presence of maternal antibody did not affect the generation of long-lived CD4+ and CD8+ T cell responses to both HA and NP. Thus, maternal antibody did not affect cell-mediated responses, but rather it limited humoral responses, with the ability to limit the antibody response correlating with whether the DNA-expressed immunogen was localized in the plasma membrane or within the cell. We further observed that protection from influenza virus challenge was dependent on the presence of anti-HA IgG and was independent of the presence T cell responses.

Taken together with other published studies, the data presented in this dissertation help better characterize the responses elicited by DNA vaccines at birth. This dissertation presents several novel observations including the temporal development of polarized IgG subtype responses, the ability of codelivered Th1 cytokine DNA to affect both antibody and T cell responses in the neonate, and the ability to generate humoral responses to intracellular, but not plasma membrane proteins, in the presence of maternal antibody. Furthermore, the data provides rationale for further development of DNA vaccines in the neonate.

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## ABBREVIATIONS

| | |
|---|---|
| Ab | antibody |
| AP | alkaline phosphatase |
| AP-1 | activation protein-1 |
| APC | antigen presenting cell |
| ASC | antibody secreting cell |
| C' | complement |
| CD | cluster of differentiation |
| cDNA | complementary DNA |
| CpG | 5'-purine-purine-C-G-pyrimidine-pyrimidine-3' |
| CpG-N | CpG-neutralizing |
| CpG-S | CpG-stimulatory |
| CTL | cytotoxic T lymphocyte |
| DNA | deoxyribonucleic acid |
| ELISA | enzyme-linked immunosorbant assay |
| EPI | Expanded Programme of Immunization |
| EtOH | ethanol |
| F | fusion protein |
| FACS | fluorescence-activated cell sorter |
| FDC | follicular dendritic cell |
| FITC | fluorescein isothiocyanate |
| g.g. | gene gun |

| gB | glycoprotein B |
| gD | glycoprotein D |
| GFP | green fluorescent protein |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| GMT | geometric mean titer |
| gp | glycoprotein |
| H | hemagglutinin |
| HA | hemagglutinin |
| HBV | hepatitis B virus |
| HRP | horseradish peroxidase |
| i.m. | intramuscular |
| i.n. | intranasal |
| i.p. | intraperitoneal |
| ICAM | intercellular adhesion molecule |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |
| LCMV | lymphocytic choriomeningitis virus |
| LDA | limiting dilution assay |
| LFA | lymphocyte function-associated antigen |
| MHC I | major histocompatibility complex class I |
| MHC II | major histocompatibility complex class II |

| | |
|---|---|
| N | neuraminidase |
| NA | neuraminidase |
| NFκB | nuclear factor kappa B |
| NK | natural killer |
| NP | nucleoprotein |
| PBS-T | phosphate-buffered saline/Tween-20 |
| PE | phycoerythrin |
| RNA | ribonucleic acid |
| SCID | severe combined immunodeficiency |
| SDB | sample dilution buffer |
| TCR | T cell receptor |
| Th | T helper cells |
| Th1 | T helper type 1 |
| Th2 | T helper type 2 |
| TNF | tumor necrosis factor |

# INTRODUCTION

## INTRODUCTION

Vaccines have profound and long lasting effects on world health. Small pox has been eradicated, polio is near elimination, and diseases such as diphtheria, measles, mumps, pertusis, and tetanus are contained. Nonetheless, microbes remain major killers, with current vaccines addressing only a handful of the infections of man. Common infectious diseases for which there are no vaccines cost the United States $120 billion per year (Robinson *et al.*, 1997b). In first world countries, emerging infections such as immunodeficiency viruses, as well as reemerging diseases like drug-resistant forms of tuberculosis, pose new threats and challenges for vaccine development. The need for both new and improved vaccines is even more pronounced in third world countries where effective vaccines are often unavailable or cost-prohibitive.

The need for new and improved vaccines is highlighted by statistics of infant mortality rates. There have been overall significant improvements in rates of child survival over the past 20-30 years. This is due to several factors including improvements in living conditions, such as water supplies, sanitation and nutrition, as well as the healthcare improvements such as the Expanded Programme of Immunization (EPI). The EPI has had a profound effect on infant mortality rates due to the effects of both measles and pertussis vaccines. Nevertheless, infant mortality remains high. For instance, of the approximately 126 million live births in 1995, 8 million died within the first year (Mulholland, 1998). The development of safe and effective immunization protocols is particularly challenging due to the unique profile of the immature neonatal immune

system. Recently, promise for the development of new vaccines has been realized with the discovery that direct injections of antigen-expressing DNAs can initiate protective immune responses. However, the efficacy of these novel vaccines in infants remains to be characterized.

DNA-based vaccines use bacterial plasmids to express protein immunogens in vaccinated hosts. Recombinant DNA technology is used to clone cDNAs encoding immunogens of interest into eukaryotic expression vectors. Vaccine plasmids are then amplified in bacteria, purified, and directly inoculated into the hosts being vaccinated. DNA can be inoculated by a needle injection of DNA in saline, or by a gene gun device which delivers DNA-coated gold beads into the skin (Figure 1). Plasmid DNA is taken up by host cells, the vaccine protein is expressed, processed and presented in the context of self-MHC class I (MHC I) and MHC class II (MHC II) molecules, and an immune response against the DNA-encoded immunogen is generated.

## PLASMID VECTORS AND DELIVERY

At least part of the efficacy of DNA-based vaccines can be attributed to the ability of plasmid DNA to serve as an adjuvant as well as the source of the encoded immunogen (Sato *et al.*, 1996). This adjuvant activity appears to be a general property of CpG motifs (5'-purine-purine-C-G-pyrimidine-pyrimidine-3') in bacterial-derived DNA (for reviews, see (Krieg *et al.*, 1998b; Pisetsky, 1996). CpG motifs in bacterial DNA distinguish themselves from those in eukaryotic DNAs both by being unmethylated and more

# DNA Vaccine



gene

Gene for an immunogen

promoter
gene
poly A

plasmid

Insert gene into an expression plasmid

Transform bacterial cells, grow bacteria, purify plasmid DNA

Immunize with immunogen-expressing plasmid
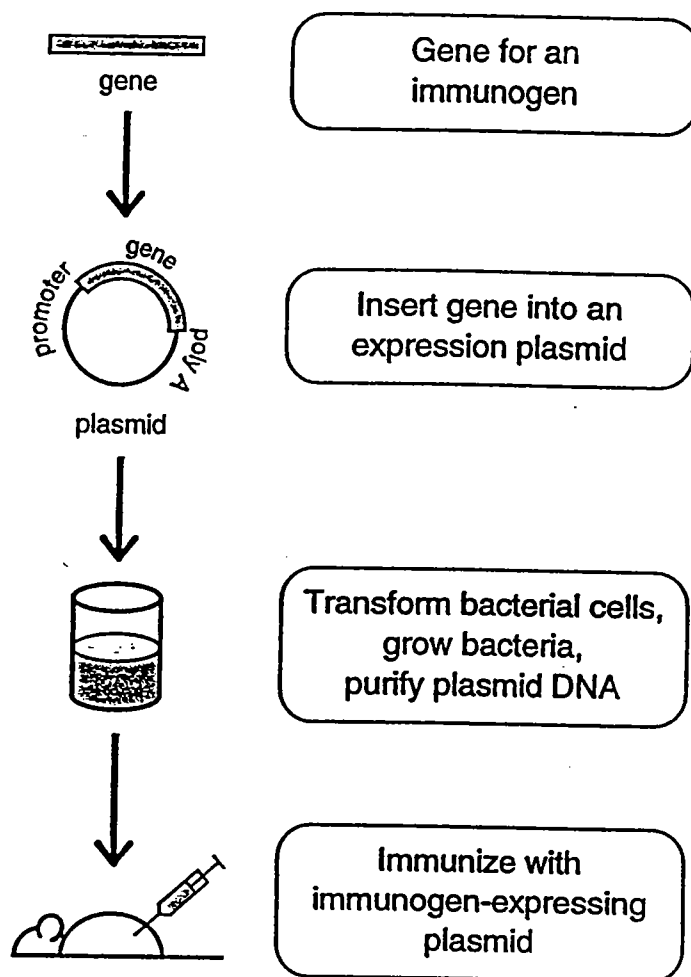
**Figure 1.** Schematic for the construction, purification and inoculation of a DNA vaccine.

frequent. In certain nucleotide sequence contexts, termed CpG-stimulatory (CpG-S),

CpG sequences trigger innate immune defenses, the production of Th1 cytokines, and the

activation of dendritic cells (Jakob *et al.*, 1998; Krieg *et al.*, 1998a; Krieg *et al.*, 1998b).

These innate responses synergize with acquired immune responses against the DNA-

expressed protein. In other base contexts, termed CpG-neutralizing (CpG-N), CpG

sequences inhibit activation of innate immune responses, potentially providing a

mechanism for a microbe to evade host immune defenses. CpG-N sequences are over-

represented in the human genome, where they are 2-5 times more frequent than CpG-S

motifs (Krieg *et al.*, 1998a).

The manipulation of CpG-S and CpG-N sequences in vaccine vectors can

improve the efficacy of immunization (Krieg *et al.*, 1998a). For example, in a vector that

contained 254 CpG dinucleotides, of which 134 are CpG-N, the mutation of 56 of the

CpG-N motifs increased the ability to raise antibody ~2-fold and cytotoxic T lymphocyte

(CTL) lysis from 30- to 40%. Adding CpG-S sequences to the CpG-N mutated vector

further increased antibody responses ~6-fold, but did not affect CTL activity.

In vitro studies on the immunostimulatory activity of CpG DNA reveal the CpG-S

motifs activate cells through both the NF$\kappa$B and AP-1 signaling pathways (Yi *et al.*,

1996; Yi and Krieg, 1998). Activation of both NF$\kappa$B and AP1 are required for CpG-

mediated cytokine induction. Chloroquine, an inhibitor of endosomal acidification,

selectively abolishes the activation of both pathways and appears to block a very early event in the signaling triggered by CpG DNA (Yi *et al.*, 1996).

The two most broadly used approaches to DNA delivery are injection of DNA in saline using a hypodermic needle or gene gun delivery of DNA-coated gold beads. Saline injections deliver DNA into extracellular spaces, whereas gene gun deliveries bombard DNA directly into cells. The saline injections require much larger amount of DNA (100-1000 times more) than the gene gun (Fynan *et al.*, 1993). These two types of delivery also differ in that saline injections bias responses towards type 1 T cell help, whereas gene gun deliveries bias responses to type 2 T cell help (Feltquate *et al.*, 1997; Pertmer, Roberts, and Haynes, 1996).

## ADVANTAGES OF USING DNA VACCINES AS COMPARED WITH CONVENTIONAL METHODS OF VACCINATION

DNA-based immunizations have a number of advantages, as well as disadvantages, over more classical methods of immunization. The major immunological advantage is the ability of the immunogen to be presented by both MHC class I and class II molecules. Endogenously synthesized proteins readily enter the processing pathway for the loading of peptide epitopes onto both MHC I and MHC II molecules. MHC I-presented epitopes raise CTL responses whereas MHC II-presented epitopes raise helper T cells (Th). When compared with live attenuated vaccines or recombinant viral vectors that produce immunogens in cells that raise both Th and CTL, DNA vaccines have the

advantages of not being infectious and of focusing the immune response on only those antigens desired for immunization. DNA vaccines also have the advantage that they can be manipulated relatively easily to raise type 1 or type 2 T cell help. This allows a vaccine to be tailored for the type of immune response that will be mobilized to combat infection. DNA vaccines are cost effective due to the ease with which plasmids can be constructed using recombinant DNA technology, the ability to use a generic method for vaccine production (growth and purification of plasmid DNA), and the stability of DNA over a wide range of temperatures. Overall, disadvantages of DNA vaccines include the limitation of immunizations to protein immunogens, the potential for atypical processing of bacterial and parasitic proteins by eukaryotic cells, the production of anti-DNA antibodies, and the possibility of vaccine vector integration into chromosomal DNA. Infant DNA immunization may cause additional problems. Integration could pose a particular threat when introduced into the genome of rapidly dividing cells in infants. Additionally, deliberate alterations in the Th1/Th2 balance in infants may eventually lead to immunopathological responses (e.g., such as those seen following the initial trials of inactivated respiratory syncytial virus) (Siegrist *et al.*, 1997). The induction of tolerance to the vaccine antigen may also be possible (Mor *et al.*, 1996).

## MECHANISTIC BASIS FOR DNA-RAISED IMMUNE RESPONSES

DNA vaccines generate immune responses with nanogram levels of expressed protein. DNA vaccines are likely efficacious due to the high efficiencies of antigen presentation that are achieved by the direct transfection of professional antigen presenting

cells (Banchereau and Steinman, 1998; Casares *et al.*, 1997; Condon *et al.*, 1996; Porgador *et al.*, 1998). The adjuvant activity of unmethylated CpG sequences in plasmid DNA also may contribute to the efficacy of DNA vaccines (Klinman, Yamshchikov, and Ishigatsubo, 1997) (see above).

*Antigen Presentation by Bone Marrow-Derived Cells.* The firmest knowledge as to how DNA vaccines work comes from studies using chimeric mice. These studies demonstrate that antigen presentation is by bone marrow-derived cells. Bone marrow-derived cells include the dendritic cells, macrophages, and B cells that are specialized for antigen presentation and termed professional antigen presenting cells (APC). For studies on antigen presentation, F1 mice were bred and their bone marrows destroyed by irradiation. The irradiated mice were reconstituted with the marrow of one parent, immunized, and tested for MHC-restriction of the raised CTL response. Following both gene gun and i.m. immunizations, the haplotype specificity of the raised CTL response was restricted to that of the reconstituting marrow (Corr *et al.*, 1996; Fu *et al.*, 1997; Iwasaki *et al.*, 1997b). This restriction held in the presence of cotransfected DNAs expressing the B7 costimulatory molecule or GM-CSF plus IL-12 (Iwasaki *et al.*, 1997). Thus, the addition of molecules that are associated with antigen presentation to keratinocytes or muscle cells was not sufficient to support antigen presentation.

The site of antigen presentation and the nature of the antigen presenting cell have been tracked further using reporter plasmids and sorted cells. Following gene gun

immunizations, reporter plasmid-bearing epidermal Langerhans cells migrate to the draining lymph node (Porgador *et al.*, 1998; Condon *et al.*, 1996), where they present antigen (Porgador *et al.*, 1998). Approximately 60 directly transfected epidermal Langerhans-derived dendritic cells were observed in the draining lymph node following delivery of four gene gun shots to the abdominal skin (Porgador *et al.*, 1998). Epidermal Langerhans cells constitute bout 8% of the cells in the skin. Thus, direct transfection of these cells by gene gun bombardment of DNA would be expected. Following i.m. saline injections of DNA, dendritic cells in the draining lymph node also have been found to carry vaccine DNA and present antigen (Casares *et al.*, 1997). Approximately 0.4% of the dendritic cells in the draining node were estimated to be presenting antigen following i.m. DNA immunizations (Casares *et al.*, 1997). Given that the number of dendritic cells in a lymph node range from 15,000-40,000, this would suggest that between 60-160 directly transfected APC are present in the draining nodes of i.m.-inoculated mice. Studies using GFP-expressing plasmids have localized GFP-expressing cells in the peripheral blood (Chattergoon *et al.*, 1998). These cells, which were identified as macrophages, coexpressed CD80 and CD86 and were hypothesized to be able to induce T cell responses. In this last study, GFP-expressing cells in the draining node were identified as macrophages rather than dendritic cells. In all of the studies on the localization of antigen presenting cells in the lymph node, transfected cells have been clustered. This clustering has been hypothesized to reflect the drainage pattern of the inoculated site into the node.

Cross-priming of dendritic cells or macrophages also contributes to antigen presentation. Cross-priming occurs when a bone marrow-derived cell (such as a dendritic cell) presents peptides derived from proteins synthesized in another cell (Carbone and Bevan, 1990). Cross-priming has been demonstrated to prime CD8+ CTL responses following injection of transfected myoblasts (Ulmer, Donnelly, and Liu, 1996). Cross-priming has been demonstrated in i.m. immunization of SCID mice subsequently infused with a competent bone marrow (Doe *et al.*, 1996). In these experiments, the addition of bone marrow as late as 21 days after i.m. immunization resulted in the raising of CTL. This implies that factories of antigen last for several weeks. Following gene gun immunization, the temporal dependence of CTL responses on the skin target site suggest that cross-priming is important for the realization of a full primary response (Torres *et al.*, 1997). However, in another study, cross-priming did not appear to play a major role in the elicitation of CD8+ CTL following gene gun DNA immunization (Porgador *et al.*, 1998).

*Role of the Target Site.* The skin and muscle targets of DNA delivery play different roles in the initiation of DNA-raised immune responses. The highest density of transfected cells are found at the sites of DNA delivery, with the most frequent expressing cells in the skin being keratinocytes (Eisenbraun, Fuller, and Haynes, 1993; Hengge *et al.*, 1995; Raz *et al.*, 1994), and in muscle, striated muscle cells (Davis, Whalen, and Demeneix, 1993; Wolff *et al.*, 1990). Temporal ablation studies of gene gun-bombarded skin revealed that the skin plays an essential role in the mounting of both

antibody and CTL responses (Klinman *et al.*, 1998; Torres *et al.*, 1997). By contrast, ablation of the muscle target revealed both antibody and CTL responses being independent of the injected muscle within minutes of DNA delivery (Torres *et al.*, 1997). These two different patterns of target site dependence held for DNAs that expressed plasma membrane, secreted, or intracellular antigen (Torres *et al.*, 1997). Transplantation of the gene gun target site revealed that cells capable of inducing a primary antibody response moved out of the skin within 12 hours, but that sedentary as well as the mobile cells were required for the full antibody response (Klinman *et al.*, 1998). Secondary gene gun-raised antibody and Th responses appeared to be entirely mediated by cells that moved out of the target site within 12 hours (Klinman *et al.*, 1998). These results suggest that following gene gun inoculations, both directly transfected epidermal Langerhans cells and antigen expression by keratinocytes contribute to the primary responses, whereas directly transfected Langerhans cells are sufficient for the stimulation of the secondary responses. They also suggest that following i.m. immunization, the immunologically important transfection events occur at distal sites or in trafficking lymphoid cells. The rapid spread of i.m. inoculated DNA throughout the body would facilitate distal transfections, especially in lymphoid organs that filter blood and lymph. Why skin and not muscle serves as a factory for antigen is not clear. However, the skin is much more heavily populated by dendritic cells than muscle, with localized dendritic cells potentially supporting the uptake and presentation of locally produced antigen.

*Maintenance of the Immune Response.* A characteristic of DNA-raised immune responses is the generation of effective memory. Normal mechanisms for the development of immunological memory as well as unique aspects of DNA-based immunization are likely to contribute to this phenomenon. An immunological mechanism for sustaining the viability and differentiation of activated B cells is the display of antigen-antibody complexes by follicular dendritic cells (FDC). FDC are non-bone marrow-derived cells that reside in the germinal centers of lymph nodes and display both Fc and complement receptors (Banchereau and Steinman, 1998; Liu *et al.*, 1996). These cells are potent stimulators of B cells. A second type of germinal center dendritic cell can serve as a potent stimulator of T cells (Grouard *et al.*, 1996). Following both gene gun and i.m. deliveries of DNA, antigen presentation occurs for several weeks (Doe *et al.*, 1996; Boyle and Robinson, 2000). This length of time for antigen presentation overlaps the appearance of generated antibody. Thus, there is the potential for the generation of FDC-displayed immune-complexes between the newly appearing antibody and the still expressed antigen. This phenomenon could account for the ability of single DNA immunizations to raise long lasting memory responses. In mice, these long lasting memory responses are frequently accompanied by the persistence of antibody.

## IMMUNE RESPONSES RAISED BY DNA VACCINES

### T cell Help

***Background.*** One of the unique features of DNA vaccines is the relative ease with which

immunizations can be manipulated to bias the type of raised T cell help towards type 1 or

type 2 (Feltquate *et al.*, 1997). The type of T cell help that supports an immune response

determines whether the raised antibody will be C'-dependent and phagocyte-mediated

defenses will be mobilized (a type 1 response), or whether the raised antibody will be

C'-independent and phagocyte-independent defenses will be mobilized (a type 2

response) (Figure 2) (for reviews, see Fearon and Locksley, 1996; Seder and Paul, 1994;

Ward, Bacon, and Westwick, 1998). Each of these types of responses are associated with

Th and CTL cells with distinctive patterns of lymphokine and chemokine expression that

support specific rearrangements of Ig genes, patterns of lymphocyte trafficking, and types

of innate immune responses. Signature lymphokines and chemokines for Th1 cells

include IFN-$\gamma$ and RANTES, and for Th2 cells, IL-4 and eotaxin. Historically type 1

responses have been associated with the raising of cytotoxic T cells and type 2 responses

with the raising of antibody. These associations arose at least in part from the historical

use of infections, which undergo endogenous synthesis and the loading of MHC I, to

raise type 1 responses; and the use of inactivated viruses or protein subunits, which do

not undergo endogenous synthesis and do not load MHC I, to raise type 2 responses.

With the advent of DNA vaccines, endogenous expression of antigens and the loading of

MHC I antigens became possible under conditions that raise type 2 as well as type 1

responses. Thus, the ability to study type 2 responses that raise CTL as well as antibody

became possible. At present, relatively little is understood about the frequency and types

of CTL raised by DNA immunizations (see below). However, it is clear that both

| Type of T-help | Associated Antibody | Associated Innate Immunity |
|---|---|---|
| Th 1 | C' dependent | Phagocyte-mediated |
| IFNγ | FcγR-binding | → activated macrophages |
| IL-2 | IgG2a, IgG2b | → opsonization |
| rantes | | |
| Th2 | C' independent | Phagocyte-independent |
| IL-4 | FcεR-binding | → activated eosinophils, mast cells |
| IL-5 | IgG1, IgE | → anti-phagocytic controls |
| IL-6 | | → mucosal responses |
| IL-10 | | |
| eotaxin | | |

**Figure 2.** Role of the target site and lymphoid tissue in DNA-based immunizations following different methods of DNA delivery. The predominant Th-types of responses raised by different methods of DNA delivery and forms of DNA-expressed antigen are summarized.

antibody and CTL are supported by type 1 (Ulmer *et al.*, 1993) as well as type 2 (Pertmer, Roberts, and Haynes, 1996) T cell help.

***Raising of different types of T cell help.*** Both the method of DNA delivery and the expressed immunogen affect the predominant type of T cell help that is raised by a DNA immunization (Figure 3). In general, saline injections of DNA raise immune responses that are biased towards type 1 help, whereas gene gun deliveries raise immune responses that are biased towards type 2 help (Feltquate *et al.*, 1997; Pertmer, Roberts, and Haynes, 1996). These patterns of raised help hold for intracellular and plasma membrane-bound forms of DNA-expressed antigens and are determined by the method and not the route of inoculations (Feltquate *et al.*, 1997). However, for secreted antigens, both i.m. and gene gun inoculations tend to raise type 2 help (Torres *et al.*, 1999; Boyle, Koniaras, and Lew, 1997; Haddad *et al.*, 1997; Lewis *et al.*, 1997; Sallberg *et al.*, 1997). The Th2-biased response raised by secreted antigens is the same type of response observed for inoculated proteins. Some exceptions to these generalizations have been reported. For example, Th1-biased responses are raised by a secreted form of the hepatitis C nucleocapsid protein (Inchauspe, 1997). Also, some MHC backgrounds give IgG1 predominant, Th2-biased responses for all methods of antigen delivery (Sallberg *et al.*, 1997).

**A**

Free DNA, transfected cells in lymph and blood

Response mounted in draining node, spleen

Muscle irrelevant to response within 1 min

I.m. saline injection

**Th1-biased response**
• Cell-associated Ag
**Th2, mixed responses**
• Secreted Ag

**B**

Transfected ELC or Ag-bearing ELC in lymph

Skin essential to response for ≥ 3 days

Response mounted mainly in draining nodes

Gene gun bombardment of epidermis

**Th2-biased response**
• Cell-associated Ag
• Secreted Ag

**Figure 3.** Two different types of T cell help and the associated antibody and innate immune responses in mice

For most DNA-raised responses, the type of raised T cell help is stable over time. Furthermore, the type of the DNA-primed help is maintained in the face of challenge infections or subsequent immunizations that would raise the opposite type of help in a naive animal (Feltquate *et al.*, 1997; Pertmer, Roberts, and Haynes, 1996). For example, when a Th2-primed response undergoes challenge with a Th1 viral infection, the anamnestic response retains the Th2-type of the prime (Feltquate *et al.*, 1997). An example where the type of help has changed with time is the malaria circumsporozoite protein, where an initial type 1-biased response changed to type 2 over the first month of immunization (Mor *et al.*, 1995).

***Mechanistic basis for different types of T cell help.*** The mechanism by which different methods of DNA immunization, or forms of DNA-expressed antigens, raise different types of T cell help is not understood. Our bias is that differences in the nature, or differentiated state of antigen presenting cells determine the type of T cell help. Dendritic cells can differentiate to become either IL-12 secreting, which would support the development of Th1 cells, or IL-4 secreting, which would support the differentiation of Th2 cells (Banchereau and Steinman, 1998). Saline injections result in DNA undergoing endocytosis, a process that stimulates dendritic cells to differentiate for the production of Th1 cytokines (Jakob *et al.*, 1998; Yi and Krieg, 1998) (see above). Gene gun deliveries bombard DNA directly into cells, thus bypassing this Th1-stimulating endocytic step. An influence of APC on the type of raised response is suggested in neonatal immunizations in mice, where the polarization of responses into the patterns

observed for adult DNA immunizations occurs over the same developmental time course as the appearance of markers characteristic of mature APC (Pertmer and Robinson, 1999).

A popular hypothesis as to why i.m. immunizations raise Th1-biased immune responses is that the large amounts of DNA used for these immunizations trigger type 1 help (see above). Tests of this hypothesis for a membrane associated antigen have revealed no difference in the type of response raised by different doses of DNA (Feltquate *et al.*, 1997). However, for a secreted antigen, larger doses of DNA can move a Th2-biased response toward a Th1-bias (S. Johnston, personal communication). This is consistent with the Th1 adjuvant activity of CpG sequences for protein immunizations DNA (for reviews, see Krieg *et al.*, 1998b; Pisetsky, 1996).

*Practical uses of polarized T cell help.* The ability to polarize T cell help by DNA-based immunizations has been applied to preclinical models for allergy and autoimmune diseases. In allergy, the goal has been to shift the allergic Th2 response (and the associated production of IgE) to non-allergic Th1 responses. In autoimmune disease, the goal has been to shift the response from a self-destructive Th1 response to a non-destructive Th2 response. In both models, pre-disease priming of responses for the desired Th type has prevented or ameliorated the induction of disease (Waisman *et al.*, 1996). Shifting the pattern of T cell help for established disease has also had some success (Raz *et al.*, 1996). Finally, using DNA vaccines to circumvent Th2-biased

immune responses in neonates is currently being explored (Kovarik *et al.*, 1999); (see below). The long term importance of the ability to tailor the type of T cell help for microbial vaccines remains to be explored.

### Cytotoxic T cells

A forte of DNA vaccines is the ability to raise CTL. Most analyses for DNA-raised CTL have been done in bulk assays following in vivo or in vitro restimulation. However, more recently, limiting dilution analyses have been undertaken to more rigorously define precursor frequencies. These analyses reveal similar frequencies of CTL raised by i.n. immunizations with Sendai virus and gene gun DNA immunizations (Chen, Webster, and Woodland, 1998). Similar frequencies of CTL were also observed following i.m. immunization with a ubiquitinated minigene for LCMV and intraperitoneal (i.p.) immunizations with the Armstrong strain of LCMV (Rodriguez, Zhang, and Whitton, 1997). In the Sendai model, similar frequencies of responses were mounted to dominant and subdominant epitopes by the DNA immunization and the natural infection (Chen, Webster, and Woodland, 1998). In the influenza model, LDA showed similar NP-specific CTL precursor frequencies in mice i.m. immunized with NP DNA or i.n. infection with influenza virus (Fu *et al.*, 1999). In the LCMV model, the T cell receptors on the DNA-raised and infection-raised CTL had similar affinities as measured by the ability to lyse targets coated with from 0.1 nm to 1000 nm peptide (Rodriguez, Zhang, and Whitton, 1997).

Studies in the LCMV model have revealed DNA-raised CTL causing immunopathology as well as protection (Zarozinski *et al.*, 1995). In these studies, a suboptimal nucleoprotein-expressing DNA vaccine raised precursor frequencies of ~1 in 90,000. Upon challenge with the Armstrong strain of virus, some mice had accelerated disease, whereas others were protected. The Armstrong strain of LCMV does not kill infected cells. Thus, disease enhancement presumably reflected the CTL not controlling the LCMV infection sufficiently to prevent spread of virus and consequent widespread destruction of cells by CTL.

## Interferons

Cytotoxic as well as helper T cells also control viral infection by virtue of secreted interferons. The clearance of non-cytopathic viruses by an immune response is widely perceived as being mediated by the destruction of infected cells by MHC I-restricted cytolytic T cells. However, upon antigen recognition, CTL secrete antiviral cytokines such as IFN-$\gamma$ or TNF-$\alpha$. These cytokines can severely limit viral infections without killing the infected cell. For DNA vaccines, a non-destructive IFN-$\gamma$-mediated control of an infection has been reported for hepatitis B virus (HBV). Immunization of transgenic mice that replicate the HBV genome and sustain the HBV life cycle with a DNA expressing the HBV surface antigen raised CD8+ cells that abolished HBV gene expression and viral replication while killing only a small fraction of the hepatocytes (Mancini *et al.*, 1996). Studies of this phenomenon using transfer of HBV surface antigen-specific clones of CTL into the transgenic mice revealed that the antiviral effect

was mediated by IFN-γ and TNF-α (Guidotti *et al.*, 1996; Heise *et al.*, 1999).

Administration of antibody to IFN-γ and TNF-α completely abolished the protective

effect, whereas administration of antibody to one, but not the other, was only partially

effective. This suggests that the limitation of virus expression was likely due to IFN-γ

and TNF-α activating different but synergistic pathways for HBV control. IFNα/β can

also contribute to the down regulation of HBV expression. The phenomenon can be

mediated by HBV-specific Th1 cells (Franco *et al.*, 1997). Interestingly, these

cytokine-mediated effects have not been able to be replicated in vitro and require in vivo

experiments (Heise *et al.*, 1999).


## Antibody

DNA-raised antibody responses differ in a number of ways from those raised by

protein inoculations or viral infections. DNA-raised antibody responses rise much more

slowly than protein- or infection-raised antibody. In mice, the maximum titer that will be

achieved by a single immunization is sometimes not reached until as late as 12 weeks

after the immunization. Titers at 12 weeks can be 5-10 times higher than titers at 4 weeks

post immunization (Robinson *et al.*, 1997a). Boosts increase the rate of the rise in

responses (Robinson *et al.*, 1997a). The appearance of antibody secreting cells (ASC) in

the bone marrow parallels the slow rise in antibody but with an approximately 4-week

delay (Boyle *et al.*, 1996). The very slow kinetics for antibody responses following DNA

immunizations presumably reflect the raising of responses by low levels of antigen that

are expressed over several weeks. The prolonged presence of antigen serves to support

both primary and secondary phases of antibody responses. Raised antibody undergoes avidity maturation (Boyle *et al.*, 1997) and ASC migrate to the bone marrow for long term production of antibody (Boyle *et al.*, 1996; Slifka *et al.*, 1998). In murine models, the plateau titer for a DNA-expressed antigen is typically similar to that raised by the same antigen expressed in a viral infection (Deck *et al.*, 1997; Michel *et al.*, 1995; Robinson *et al.*, 1997a; Xiang *et al.*, 1994).

DNA-raised antibody responses also differ from those raised by protein immunizations or viral infections in that the method of DNA inoculation can be used to bias the isotype of the raised antibody (Feltquate *et al.*, 1997). In mouse studies, most protein immunizations raise C'-independent antibodies and most viral infections raise C'-dependent antibodies. Depending on the method of delivery or the form of an antigen, DNA immunizations can be used to raise either predominantly C'-dependent or C'-independent antibodies for the same protein (Figure 2, section on T cell help).

**INCREASING THE EFFICIENCY OF DNA-BASED IMMUNIZATIONS**

In addition to the CpG-S sequences in plasmids that act as endogenous adjuvants for DNA vaccines, both conventional as well as genetic adjuvants have been explored for their ability to increase or change the Th bias of DNA-raised immune responses. Conventional adjuvants have included gel-type adjuvants such as alum or calcium phosphate, bacterial adjuvants such as monophosphoryl lipid A (Sasaki *et al.*, 1998b; Sasaki *et al.*, 1997b) and cholera toxin (Ban *et al.*, 1997; Kuklin *et al.*, 1997), cationic

(Gregoriadis, Saffie, and de Souza, 1997; Ishii *et al.*, 1997) and mannan-coated

liposomes (Sasaki *et al.*, 1997a; Toda *et al.*, 1997), emulsifier-based adjuvants such as

QS21 (Sasaki *et al.*, 1998c), and synthetic adjuvants such as carboxymethylcellulose and

ubenimix (Sasaki *et al.*, 1998a). All of these have had some effects on the efficiency of

immunization.

Studies using genetic adjuvants have focused on the cotransfection of plasmids

expressing lymphokines, chemokines, or costimulatory molecules (for review, see Cohen,

Boyer, and Weiner, 1998). The vast majority of experiments have been conducted with

the native forms of lymphokines or costimulatory molecules by codelivery of a DNA

expressing the genetic adjuvant and a DNA expressing the immunogen. Recently, a

study comparing the temporal relationship between the delivery of IL-2 or

GM-CSF-expressing DNAs and a plasmid expressing HIV-1 gp120 (receptor binding

subunit of Env) revealed increases in immune responses when cytokine DNAs were

delivered after, but not at the time of the delivery of the DNA immunogen (Barouch *et

al.*, 1998). Pre- or codelivery of the cytokine DNA resulted in amplification of

non-specific proliferative responses and decreases in the specific response. In contrast,

delivery at two days post immunization augmented the specific immune response that had

just been primed. In this very thorough study, all effects were less than 10-fold, and

delivery of DNAs expressing IL-2, IL-4 or GM-CSF at the same time as the delivery of

the vaccine DNA uniformly resulted in reduced (not enhanced) responses. This study

also demonstrated that when the cytokine DNA was delivered 2 days after the

immunogen, Ig-fusions of IL-2 were more effective than IL-2 alone at enhancing immune responses. This was hypothesized to reflect a longer serum half life of the Ig fusion, the divalent avidity of the Ig-fusions, and the ability of the Ig-fusions to augment signaling via initiating receptor clustering.

The reported effects of genetic adjuvants have been those that would be anticipated (for review, see Robinson and Pertmer, 2000). Pro-inflammatory agents and Th2 cytokines have increased antibody responses; whereas pro-inflammatory agents and Th1 cytokines have increased CTL responses. Interestingly, the tested costimulatory agents have been more effective at inducing CTL responses than changes in Th or antibody responses. If one further breaks down the observed phenomena to Th1 or Th2 agents that have been highlighted as key inducers for the differentiation of type 1 and type 2 responses (IFN-$\gamma$ and IL-12 for Th1, IL-4 for Th2), effects are uniformly what would have been anticipated. The scattering of results on protection reveal pro-inflammatory and Th1 genetic adjuvants improving protection for viral infections more frequently than Th2 genetic adjuvants.

In general, coinoculated genetic adjuvants have had fairly minor effects on the magnitude of immune responses, with most increases being only two- to three-fold. Among the best of the increases (~8-fold) occurred in a study with cotransfected CD40 ligand (Mendoza, Cantwell, and Kipps, 1997).

## INFLUENZA

Influenza viruses are segmented RNA viruses that cause highly contagious acute respiratory infections. These viruses are endemic in man, where they are particularly devastating for the very young and the very old. The major problem associated with vaccine development against influenza is that these viruses have the ability to escape immune surveillance and remain in a host population. This escape is associated with changes in antigenic sites on the hemagglutinin (H) and neuraminidase (N) envelope glycoproteins by phenomena termed antigenic drift and antigenic shift. Antigenic drift occurs when a subtype of an influenza virus H (for example H3) is selected for antigenic determinants that are not recognized by the anti-H3 antibody present in a population. This allows the virus to superinfect individuals who have already been infected by an H3 virus. Antigenic shift occurs when the influenza virus segmented genome reassorts to acquire an H belonging to another subtype (for example H2 instead of H3). The primary correlate for protection against influenza virus is neutralizing antibody against H protein that undergoes strong selection for antigenic drift and shift. However, much more conserved antigenic cross-reactivities for different strains of influenza virus occur between internal proteins, such as the nucleoprotein (NP) (Shu, Bean, and Webster, 1993). Conventional inactivated virus or protein subunit vaccines raise antibody against H but have the limitation of being unable to generate effective CTL responses against NP. Thus, DNA vaccines that can induce both neutralizing antibody, as well as a NP-specific cell-mediated immune response, offer hope for better cross-strain protection.

Early studies with DNA vaccines were done in influenza virus models (Fynan *et al.*, 1993; Robinson, Hunt, and Webster, 1993; Ulmer *et al.*, 1993). These studies demonstrated protection in murine models following immunizations with H or NP-expressing DNAs. Protection with the H-expressing DNA was limited to viruses of the same subtype but was effective against highly virulent strains even following single deliveries of DNA. By contrast, protection with NP-expressing DNA required multiple deliveries of DNA and challenge with less virulent strains (Chen *et al.*, 1998; Robinson *et al.*, 1997a; Ulmer *et al.*, 1993). These strains, however, could be of a different subtype than the strain from which the NP was derived (Ulmer *et al.*, 1993). More recent studies suggest that the codelivery of H and NP-expressing DNAs will be more effective than either H or NP alone (Bot, Bot, and Bona, 1998; Donnelly *et al.*, 1995). In BALB/c mice, the protective effect contributed by the NP DNA is mediated by both CTL and Th (Ulmer *et al.*, 1998).

DNA vaccines for influenza virus have proved effective in a number of preclinical models. However, the first human trials of these vaccines, an H delivered by i.m. immunization, had disappointing results (Clements-Mann *et al.*, 1998).

DNA vaccines, given they can be as effective in humans as in preclinical models, have the potential to be an important factor in limiting future influenza pandemics. A major advantage of DNA vaccines over conventional vaccines is that they can be developed within weeks. Vaccine DNAs can be constructed using polymerase chain

reaction techniques on infected tissue. This eliminates the time and cost it takes to develop and produce a new virus in eggs or cell culture and eliminates the possibility of selection of mutant viruses in these systems.

## DEFICIENCIES IN NEONATAL IMMUNE SYSTEM

Neonates have immature immune systems with deficiencies in several components of inflammatory reactions, innate, and specific immunity. IgG responses often display slower kinetics and are lower in magnitude than corresponding adult responses. Additionally, human infants are incapable of generating antibody responses to carbohydrate determinants on bacterial capsules or to heavily glycosylated viral proteins, the basis of which is unknown (Siegrist and Lambert, 1997). Human neonates also have impaired monocyte/macrophage function (Johnston, 1998) that has been associated with reduced display of MHC II and costimulatory molecules, decreased ability to process antigen, and altered cytokine patterns (Siegrist and Lambert, 1997). Few studies have addressed the function of APC in neonates but it appears that in APC derived from both human cord blood and newborn mice, maturation is slow and functionally inadequate (Hunt *et al.*, 1994; Lu *et al.*, 1979), which may limit specific immunity. Similarly, human NK cell number and function are also impaired after birth, as assessed by IFNγ production and killing activity. The reason for NK impairment is unclear, but may be associated with deficient IL-12 production by monocytes/macrophages. Similarly, decreased IFNγ production by NK cells may contribute to the observed neonatal Th2-bias and inability to generate robust cellular immune responses (Siegrist and Lambert, 1997).

Several studies have addressed the ability to generate cellular immune responses. Classical early studies suggested the induction of tolerance following the injection of allogeneic cells. For example, one group showed that neonates exposed to allogeneic hematopoietic cells did not reject subsequent skin grafts as adults (Billingham, Brent and Medwar, 19560. These studies suggested a window of time in which lymphocyte exposure to foreign antigens resulted in deletion. However, very little was known about the biology of lymphocytes at the time. More recent studies showed that induction of cellular responses in neonatal mice was dependent on antigen dose, method of immunization, and presentation by professional APC. For instance, neonatal immunization with either low doses of antigen, or antigen emulsified in complete Freund's adjuvant, elicited Th1 and CTL induction; whereas high dose inoculation resulted in a Th2-biased response (Bot, Bot and Bona, 1996; Forsthuber, Yip and Lehman, 1996; Sarzotti, Robbins and Hoffman, 1996). H-Y-specific CTL were generated following transfer of adult male dendritic cells to newborn female mice, which indicates that presentation by professional APC leads to effective priming of cellular immune responses in neonates (Ridge, Fuchs and Matzinger, 1996). Taken together, these studies imply that low doses of antigen presented by professional APC generate Th1 and CTL induction upon immunization, whereas moderate or high doses of antigen are associated with the generation of Th2 cells. Thus, neonates exposed to antigens have the tendency to develop an immunodeviated response as opposed to true immunological tolerance (Bot, Antohi and Bona, 1997).

Neonatal mice develop preferential Th2-type responses to many vaccine antigens. Recent in vitro studies have shown that murine neonatal T cells develop preferentially into IL-4 and IL-5 secreting effector cells and respond to cytokines, such as IL-12, with production of both IFNγ and IL-4 (Siegrist and Lambert, 1997; Demeure *et al.*, 1995; Shu *et al.*, 1994, Yang *et al.*, 1996). In vitro studies are reflected in in vivo models as well. Neonatal mice typically develop Th2-biased responses following immunization with inactivated and live attenuated viral vaccines, live recombinant canarypox vaccines (Barrios *et al.*, 1996b; Siegrist *et al.*, 1998b) and peptides or proteins emulsified in Freund's complete adjuvant (Singh, Hahn, and Sercarz, 1996) or alum (Barrios *et al.*, 1996b). This Th2 bias places neonates at a greater risk for developing severe bacterial and viral infections, which are best controlled by Th1 cell-mediated immune responses.

## MATERNAL ANTIBODY INTERFERENCE WITH MANY VACCINES

The presence of high titer maternal antibody in newborns is the major form of protection from disease in early life. Maternal IgG antibody crosses the placenta from mother to fetus during human development (Leach *et al.*, 1996). The titers of transcytosed antibody in the newborn typically exceed the titer of the same antibody in the mother, but can range from 20% to 200% that of the mother (Siegrist *et al.*, 1998c). These levels of maternal IgG slowly decline over the first year of life, a time during which the infant's immune system matures, becomes more experienced, and develops its own repertoire of protective memory responses. Maternal antibody can interfere with

active immunization of the offspring (Albrecht *et al.*, 1977). Immunization protocols are often delayed several months and/or require multiple booster immunizations to achieve the desired protective immune response. This is exemplified by measles vaccination protocols, in which barely detectable levels of maternal antibody can prevent seroconversion following infant immunization (Albrecht *et al.*, 1977). This observation was the basis for recommending measles vaccination occur between 12-15 months of age (Siegrist *et al.*, 1998c). Thus, a window of time exists when maternal antibody levels are too low to reliably protect an infant from infectious disease but high enough to prevent vaccine responsiveness.

## POTENTIAL FOR DNA VACCINES IN NEONATES

DNA vaccination is an attractive method for neonatal immunization in the presence of maternal antibody. Maternal antibody is thought to interfere with traditional vaccine efficacy by reducing the amount of antigen available for processing and presentation by APC. The ability of DNA vaccines to directly transfect cells could bypass this problem. Additionally, the DNA vaccine itself will not be affected by the maternal antibody.

When this study was initiated, there were three conflicting studies published regarding DNA immunization of neonatal mice. Two of the studies, in which DNA expressing measles N (Fooks *et al.*, 1996) or influenza NP (Bot *et al.*, 1996) was inoculated by i.m., had success in eliciting antibody responses. The third study showed

long-term tolerance following i.m. inoculation at birth with DNA expressing the *P. yoelli* (mouse malaria) circumsporozoite protein (Mor *et al.*, 1996). Due to the importance of immunizing as soon as possible in early life, it was necessary to determine the effectiveness of these novel DNA-based vaccines in a neonatal animal model both in the presence and absence of maternal antibody. We wished to investigate whether tolerance was an exception or the rule in neonatal DNA vaccination. Further, if DNA vaccination was deemed possible, it was necessary to better characterize the associated DNA-raised immune responses.

## HYPOTHESIS

*DNA vaccines are effective in generating protective humoral and cellular immune responses in the presence or absence of maternal antibody and can circumvent the Th2-bias normally associated with neonatal immune responses to other vaccine preparations.*

## THESIS OBJECTIVES

The goal of this thesis was to characterize the long term humoral and cellular immune responses following neonatal DNA immunization. Although there was substantial data in the literature regarding the efficacy of DNA vaccines in adult animal models, relatively little was known about the longevity, and quantitative and qualitative differences in DNA-raised immune responses of neonatal animals. A paper by Mor et al. (1995) suggested that i.m. immunization of neonatal mice with a DNA expressing the

*Plasmodium yoelli* (mouse malaria) circumsporozoite protein led to long term

unresponsiveness of animals to a second exposure to antigen. To determine if these

results were antigen-specific or a general rule as applied to neonatal DNA

immunizations, I set out to address the following issues:

1.) The characterization of protective humoral immune responses following neonatal

immunization with influenza hemagglutinin DNA or protein.

2.) Effects of codelivery of Th1 cytokines on the generation of humoral and cellular

immune responses.

3.) The ability to generate protective humoral responses following immunization in the

presence of maternal or passively acquired anti-influenza antibody.

4.) An examination of the quantitative and qualitative differences in cellular immune

responses generated following DNA vaccination in the presence or absence of anti-

influenza antibody

# MATERIALS AND METHODS

## MATERIALS AND METHODS

*Cells.* The murine mastocytoma cell line, p815, was purchased from American Type Tissue Collection (# TIB 64, ATCC, Rockville, MD).

*Bacteria.* Competent HB101 or DH5α *E. coli* were used to amplify plasmid DNA.

*Virus.* A/PR/8/34 (H1N1) influenza virus was kindly provided by Dr. John Hermann (University of Massachusetts Medical Center, Worcester, MA) and was grown in the allantoic cavity of 10-day-old embryonic chicken eggs (SPAFAS, Preston, CT) at either the University of Massachusetts Medical Center, Worcester, MA or the Centers for Disease Control, Atlanta, GA. Virus used for coating ELISA plates was from allantoic fluid enriched for virus by sucrose gradient centrifugation.

*Mice.* BALB/c mice (Taconic Farms, Germantown, NY; Harlan Sprague Dawley, Indianapolis, IN) were housed in microisolator cages at either the University of Massachusetts Medical Center Animal Facility (Worcester MA) or the Emory University Rollins or Winship Animal Facilities (Atlanta, GA). Six to eight week old female mice were infected intranasally (i.n.) with a sublethal dose of influenza A/PR/8/34 and allowed to recover from infection. Approximately 3 months later, these influenza-immune mice, as well as naïve females, were bred. Pregnant females were separated into individual cages and monitored daily for births. Birth dates were recorded as the dates the litters

were discovered. Pups were weaned and sex separated at 3-4 weeks of age. Age at the time of immunization is as noted in the various chapters.

*Plasmid DNA.* pJW4303/HA (HA DNA) and pCMV/NP (NP DNA) plasmid vector construction and purification procedures have been previously described (Feltquate *et al.*, 1997; Pertmer *et al.*, 1995). The pIL-12 vector is pWRG 3169 mIL12 and is a bicistronic plasmid containing the sequences for p35 subunit, an internal ribosomal entry site, and the p40 subunit. The pIFN$\gamma$ is pWRG 3154 pCMV/mIFN-M gamma. Both cytokine vectors were kindly provided by Deb Fuller (Powderject Vaccines, Inc., Madison, WI). All vectors are under the transcriptional control of the cytomegalovirus immediate-early promoter. The empty pJW4303 vector was used as a negative control. Plasmids were grown in either DH5$\alpha$ or HB101 E. coli and purified using Qiagen (Chatsworth, CA) UltraPure-100 columns. All plasmids were verified by appropriate restriction enzyme digestion and agarose gel electrophoresis. Purity of DNA preparations was determined by optical density readings at 260 and 280 nm. All plasmids were resuspended in TE buffer and stored at $-20^{\circ}$C until use.

*Peptides.* The $K^d$-restricted $HA_{533-541}$ peptide (IYSTVASSL) (Winter, Fields, and Brownlee, 1981) or the $K^d$-restricted $NP_{147-155}$ peptide (TYQRTRALV) (Rotzschke *et al.*, 1990) were used for CTL lysis assays and CTL FACS analysis. Both peptides were synthesized at Emory University (Atlanta, GA).

*Subunit vaccine.* An influenza A/PR/8/34 (H1N1) subunit vaccine preparation was used

as described (Feltquate *et al.*, 1997). Briefly, the surface HA and neuraminidase (NA)

antigens from influenza virus particles were extracted using a nonionic detergent (7.5%

$N$-octyl-$\beta$-D-thioglucopyranoside). After centrifugation, the HA/NA-rich supernatant

(~55% HA) was used as the subunit vaccine. Doses of 10, 1, or 0.1$\mu$g were delivered in a

volume of 0.4 ml of sterile 0.9% saline.

*Preparation of DNA-coated gold beads for g.g immunizations.* Plasmid DNA was

precipitated onto the appropriate amount of 1 $\mu$m gold beads (BioRad, Hercules, CA).

One hundred microliters of 50 mM spermidine was added to the appropriate volume of

plasmid DNA and sonicated briefly. Two hundred microliters of 1 M $CaCl_2$ was then

added dropwise while vortexing. DNA was allowed to precipitate onto the gold beads for

~5 minutes. The beads were pelleted and washed 3 times with 100% EtOH and

resuspended in 100% EtOH to a final concentration of 7 mg DNA-coated gold beads per

1 ml of EtOH (Robinson and Pertmer, 1998). In situations where more than one vaccine

DNA was codelivered, separate DNA-coated gold bead preparations were made and then

mixed to a final concentration of 7 mg gold/ml EtOH; whereas when genetic adjuvants

were codelivered, DNAs were mixed and gold beads contained both DNAs (Robinson

and Pertmer, 1998).

*Preparation of DNA/gold-coated cartridges for g.g. immunizations.* To prepare the

cartridges for g.g. immunization, the DNA-coated gold beads must be spread evenly

around the inside of teflon tubing. The DNA/gold suspension was drawn into teflon tubing 1/8 inch in diameter.(McMaster-Carr, Chicago, IL) using a 10 cc syringe fitted with a tubing adapter. The tube was then quickly slid into a "tube turner" (Powderject Vaccines, Inc., Madison, WI) and allowed to settle for 5 minutes. The EtOH was drawn off at a rate of ~1 in./sec. using the syringe, and the tube was allowed to rotate at 20 rpm for 30 sec. to evenly coat the DNA-coated gold beads around the interior of the tubing. The tube was then dried by allowing nitrogen gas to flow through the tube at a rate of ~0.4 L/min. while continuously rotating. The tube was removed and cut into 1 inch. lengths using a cutting device (Powderject Vaccines, Inc., Madison, WI). Each cartridge contained 1 μg of total DNA per 0.5 mg of gold unless otherwise noted. The cartridges were stored in paraffin-sealed vials containing desiccant pellets at 4°C until use (Robinson and Pertmer, 1998).

***Gene gun DNA immunizations.*** Six- or 12-week-old young adult mice were anesthetized with 0.03-0.04 ml of a mixture of 5 ml ketamine HCl (100 mg/ml) and 1 ml xylazine (20 mg/ml). Gene gun immunizations were performed on abdominal skin using the hand-held Accell® gene delivery system (Powderject Vaccines, Inc., Madison, WI) as described previously (Pertmer *et al.*, 1995). Adult mice were anesthetized and abdominal skin was shaved with electric clippers. Neonatal mice were neither anesthetized nor shaved. Both groups of mice were immunized with a single gene gun dose containing a total of 1 μg of DNA per 0.5 mg 1 μm gold beads (BioRad, Hercules, CA) at a helium pressure setting of 400 psi (Robinson and Pertmer, 1998).

*Intramuscular immunizations.* Six- or 12-week-old young adult mice were anesthetized with 0.03-0.04 ml of a mixture of 5 ml ketamine HCl (100 mg/ml) and 1 ml xylazine (20 mg/ml). Intramuscular DNA immunizations involved injection of 0.04 ml of sterile 0.9% saline containing 50 μg total DNA into a surgically exposed quadriceps muscle (Pertmer, Roberts, and Haynes, 1996). One-day-old unanesthetized neonatal mice were injected with an equivalent DNA/saline injection into the gluteus maximus muscle. Surgical exposures were not performed in the neonatal animals (Robinson and Pertmer, 1998).

*Influenza A/PR/8/34 virus challenge.* Metofane-anesthetized mice were challenged by intranasal inoculation of 50 μl of influenza A/PR/8/34 (H1N1) virus containing allantoic fluid diluted $10^{-4}$ in PBS/2% BSA (50-100 $LD_{50}$; 0.25 HAU). Mice were weighed daily and sacrificed following >20% loss of pre-challenge weight. At this dose of challenge virus, 100% of naïve mice succumbed to influenza infection by 4-6 days. Sublethal infections were done similarly using a $10^{-7}$ dilution of virus.

*Detection of serum IgG by enzyme-linked immunosorbant assay (ELISA).* At various times pre- and/or post-immunization, mice from each group were bled, and individual mouse serum was assayed by standard quantitative ELISAs to assess anti-HA/NP-specific IgG, IgG1, IgG2a, IgG2b, IgG3, IgA or IgM levels in immune serum. Sera were collected individually and stored at –20°C until use. Sucrose gradient-purified A/PR/8/34 influenza virus was disrupted in flu lysis buffer (0.05 M Tris-HCL (pH 7.5-7.8), 0.5%

TritonX-100, 0.6 M KCl) for 5 minutes at room temperature. Ninety-six well ELISA

plates (Corning, Corning, NY) were coated with 200 HAU flu in carbonate buffer (0.8 g

$Na_2CO_3$, 1.47 g $NaHCO_3$, 500 ml $ddH_2O$, pH to 9.6) and incubated overnight 4°C. Plates

were blocked with 200 μl of 1% BSA in PBS for 1 hour at 37°C and washed 5 times with

PBS/0.025% Tween-20. Samples and standards were diluted in SDB (0.5% BSA in

PBS), added to microtiter plates at 50 μl per well, and incubated at 37°C for 90 min.

Following binding of antibody, plates were washed 5 times. Fifty microliters of HRP-

labeled goat anti-mouse Ig subtype antibody (Southern Biotechnology Associates) was

then added at optimized concentrations in SDB, and plates were incubated for 1 hour at

37°C. After washing plates 5 times, 100 μl of ABTS substrate (10 ml 0.05 M Citrate (pH

4.0), 5 ul 30% $H_2O_2$, 50 ul 40 mM ABTS) was added. Color was allowed to develop at

room temperature for 30 min., and the reaction was stopped by adding 10 μl of 10% SDS.

Plates were read at O.D.$_{405}$. Data were analyzed using Softmax Pro Version 2.21

computer software (Molecular Devices, Sunnyvale, CA). Data are shown as geometric

mean titers (GMT) unless otherwise indicated.


***Preparation and stimulation of splenocytes for cytokine production.*** Spleens were

harvested from groups of immunized mice (n=2-3) and pooled in p60 petri dishes

containing ~4ml RPMI-10 media (RPMI-1640, 10% fetal bovine serum, 50 μg/ml

gentamycin). All steps in splenocyte preparations and stimulations were done

aseptically. Spleens were minced with curved scissors into fine pieces and then drawn

through a 5 cc syringe attached to an 18G needle several times to thoroughly resuspend

cells. Then cells were expelled through a nylon mesh strainer into a 50 ml polypropylene tube. Cells were washed with RPMI-10, red blood cells were lysed with ACK lysis buffer (Sigma, St. Louis, MO), and washed 3 more times with RPMI-10. Cells were then counted by trypan blue exclusion, and resuspended in RPMI-10 containing 80 U/ml rat IL-2 (Sigma, St. Louis, MO) to a final cell concentration of $2 \times 10^7$ cells/ml. Cells to be used for intracellular cytokine staining were stimulated in 96-well flat-bottom plates (Becton Dickenson Labware, Lincoln Park, NJ), and cells to be used for cytokine analysis of bulk culture supernatants were stimulated in 96-well U-bottom plates (Becton Dickenson Labware, Lincoln Park, NJ). One hundred microliters of cells were dispensed into wells of a 96-well tissue culture plate for a final concentration of $2 \times 10^6$ cells/well. Stimulations were conducted by adding 100 μl of the appropriate peptide or inactivated influenza virus diluted in RPMI-10. CD8+ T cells were stimulated with either the $K^d$-restricted $HA_{533-541}$ peptide (IYSTVASSL) (Winter, Fields, and Brownlee, 1981) or the $K^d$-restricted $NP_{147-155}$ peptide (TYQRTRALV) (Rotzschke et al., 1990). CD4+ T cells were stimulated with inactivated influenza virus (13,000 HAU per well of boiled influenza virus plus 13,000 HAU per well of formalin-inactivated influenza virus) plus anti-CD28 (1 μg/ml) and anti-CD49d (1 μg/ml) (Waldrop et al., 1998). Negative control stimulations were done with media alone. Cells were then incubated as described below to detect extracellular cytokines by ELISA or intracellular cytokines by FACS staining.

***Chromium release assay for CTL.*** CTL responses to influenza HA and NP were measured as previously described (Fuller and Haynes, 1994), except the synthetic peptide

$HA_{533-541}$ IYSTVASSL (Winter, Fields, and Brownlee, 1981) or $NP_{147-155}$

TYQRTRALV (Rotzschke et al., 1990) was substituted for the human

immunodeficiency virus type 1 gp120 peptide in the target preparation step. Responder

splenocytes from each animal were washed with RPMI-10 and resuspended to a final

concentration of $6.3x10^6$ cells/ml in RPMI-10 containing 10 U/ml rat IL-2 (Sigma, St.

Louis, MO). Stimulator splenocytes were prepared from naïve, syngeneic mice and

suspended in RPMI-10 at a concentration of $1x10^7$ cells/ml. Mitomycin C was added to a

final concentration of 25 µg/ml. Cells were incubated at $37°C/5\%CO_2$ for 30 minutes and

then washed 3 times with RPMI-10. The stimulator cells were then resuspended to a

concentration of $2.4x10^6$ cells/ml and pulsed with HA peptide at a final concentration of

$9x10^{-6}M$ or with NP peptide at a final concentration of $2x10^{-6}M$ in RPMI-10 and 10 U/ml

IL-2 for 2 hours at $37°C/5\% CO_2$. The peptide-pulsed stimulator cells ($2.4x10^6$) and

responder cells ($6.3x10^6$) were then coincubated in 24-well plates in a volume of 2 ml SM

media (RPMI-10, 1 mM non-essential amino acids, 1 mM sodium pyruvate) for 5 days at

$37°C/5\%CO_2$. A chromium-release assay was used to measure the ability of the in vitro

stimulated responders (now called effectors) to lyse peptide-pulsed mouse mastocytoma

p815 cells. P815 cells were labeled with $^{51}Cr$ by taking 0.1 ml aliquots of p815 in RPMI-

10 and adding 25 µl FBS and 0.1 mCi radiolabeled sodium chromate (NEN, Boston, MA)

in 0.2 ml normal saline. Target cells were incubated for 2 hours at $37°C/5\%CO_2$, washed

3 times with RPMI-10 and resuspended in 15 ml polypropylene tubes containing RPMI-

10 plus HA ($9x10^{-6}M$) or NP ($1x10^{-6}$) peptide. Targets were incubated for 2 hours at

$37°C/5\%CO_2$ . The radiolabeled, peptide-pulsed targets were added to individual wells of

a 96-well plate at $5 \times 10^4$ cells per well in RPMI-10. Stimulated responder cells from individual immunization groups (now effector cells) were collected, washed 3 times with RPMI-10, and added to individual wells of the 96-well plate containing the target cells for a final volume of 0.2 ml/well. Effector to target ratios were 50:1, 25:1, 12.5:1 and 6.25:1. Cells were incubated for 5 hours at $37^\circ C/5\% CO_2$ and cell lysis was measured by liquid scintillation counting of 25 μl aliquots of supernatants. Percent specific lysis of labeled target cells for a given effector cell sample was 100x(Cr release in sample-spontaneous release sample) / (maximum Cr release-spontaneous release sample). Spontaneous chromium release is the amount of radioactive released from targets without the addition of effector cells. Maximum chromium release is the amount of radioactivity released following lysis of target cells after the addition of TritonX-100 to a final concentration of 1%. Spontaneous release was 13% of the maximum release.

***Detection of IFNγ or IL-5 in bulk culture supernatants by ELISA.*** Pooled splenocytes were incubated for 2 days at $37^\circ C$ in a humidified atmosphere containing 5% $CO_2$. Supernatants were harvested, pooled and stored at $-80^\circ C$ until assayed by ELISA. All ELISA antibodies and purified cytokines were purchased from Pharmingen (San Diego, CA). Fifty microliters of purified anti-cytokine monoclonal antibody diluted to 5 μg/ml (rat anti-mouse IFNγ) or 3 μg/ml (rat anti-mouse IL-5) in coating buffer (0.1 M $NaHCO_3$, pH 8.2) was distributed per well of a 96-well ELISA plate (Corning, Corning, NY) and incubated overnight at $4^\circ C$. Plates were washed 6 times with PBS/0.025% Tween-20 (PBS-T) and blocked with 250μl of 2% dry milk/PBS for 90 min. at $37^\circ C$. Plates were

washed 6 times with PBS-T. Standards (recombinant mouse cytokine) and samples were added to wells at various dilutions in RPMI-10 and incubated overnight at 4°C for maximum sensitivity. Plates were washed 6 times with PBS-T. Biotinylated rat anti-mouse cytokine detecting antibody was diluted in PBS-T to a final concentration of 2 µg/ml and 100 µl was distributed per well. Plates were incubated for 1 hr. at 37°C and then washed 6 times with PBS-T. Streptavidin-AP (Gibco BRL, Grand Island, NY) was diluted 1:2000 according to manufacturer's instructions, and 100 µl was distributed per well. Plates were incubated for 30 min. and washed an additional 6 times with PBS-T. Plates were developed by adding 100 µl/well of AP developing solution (BioRad, Hercules, CA) and incubating at room temperature for 50 minutes. Reactions were stopped by addition of 100 µl 0.4M NaOH and read at $OD_{405}$. Data were analyzed using Softmax Pro Version 2.21 computer software (Molecular Devices, Sunnyvale, CA).

*Intracellular cytokine staining and FACS analysis.* Pooled splenocytes were incubated for 5-6 hours at 37°C in a humidified atmosphere containing 5% $CO_2$. A Golgi transport inhibitor, Monensin (Pharmingen, San Diego, CA), was added at 0.14 µl/well according to the manufacturer's instructions, and the cells were incubated for an additional 5-6 hours (Waldrop *et al.*, 1998). Cells were thoroughly resuspended and transferred to a 96-well U-bottom plate. All reagents (GolgiStop kit and antibodies) were purchased from Pharmingen (San Diego, CA) unless otherwise noted, and all FACS staining steps were done on ice with ice-cold reagents. Plates were washed 2 times with FACS buffer (1x PBS, 2% BSA, 0.1% w/v sodium azide). Cells were surface stained with 50 µl of a

solution of 1:100 dilutions of rat anti-mouse CD8β-APC, -CD69-PE, and -CD16/CD32 (FcγIII/RII; 'Fc Block') in FACS buffer. For tetramer staining, cells were similarly stained with CD8β-TriColor, CD69-PE, CD16/CD32, and HA- or NP-tetramer-APC in FACS buffer. Cells were incubated in the dark for 30 min. and washed 3 times with FACS buffer. Cells were permeabilized by thoroughly resuspending in 100 μl of Cytofix/Cytoperm solution per well and incubating in the dark for 20 minutes. Cells were washed 3 times with Permwash solution. Intracellular staining was completed by incubating 50 μl per well of a 1:100 dilution of rat anti-mouse IFNγ-FITC in Permwash solution in the dark for 30 min. Cells were washed 2 times with Permwash solution and 1 time with FACS buffer. Cells were fixed in 200 μl of 1% paraformaldehyde solution and transferred to microtubes arranged in a 96-well format. Tubes were wrapped in foil and stored at 4°C until analysis (less than 2 days). Samples were analyzed on a FACScan® flow cytometer (Becton Dickenson, San Jose, CA). Compensations were done using single-stained control cells stained with rat anti-mouse CD8-FITC, -PE, -TriColor, or -APC. Results were analyzed using FlowJo Version 2.7 software (Tree Star, San Carlos, CA).

*Tetramers.* Tetramers were prepared essentially as described previously (Flynn *et al.*, 1998). The present experiments utilized the H-2K$^d$ MHC class I glycoprotein complexed the synthetic influenza A/PR/8/34 virus peptide HA$_{533-541}$ (IYSTVASSL) (Winter, Fields, and Brownlee, 1981) or NP$_{147-155}$ (TYQRTRALV) (Rotzschke *et al.*, 1990).

The K$^d$ vector was kindly provided by John D. Altman (Emory University Vaccine Research Center, Atlanta, GA).

***Statistical Analysis.*** Student's *t* test was calculated using Sigma Plot for Windows version 1.02 (Jandel Scientific, San Rafael, CA) or Microsoft Excel 97 SR-1 (Microsoft Corporation, Seattle, WA). Means and standard deviations were calculated using Microsoft Excel 97 SR-1 (Microsoft Corporation, Seattle, WA). Fisher Exact Test and ANOVA analyses of variance were kindly performed by Dr. James Herndon at Emory University (Atlanta, GA). Probabilities greater that $P = 0.05$ were considered insignificant.

# CHAPTER I:

# STUDIES ON ANTIBODY RESPONSES FOLLOWING

# IMMUNIZATION WITH HEMAGGLUTININ

# DNA OR PROTEIN

# CHAPTER I:

# STUDIES ON ANTIBODY RESPONSES FOLLOWING

# IMMUNIZATION WITH INFLUENZA DNA OR PROTEIN [1]

## HYPOTHESIS

*DNA vaccines are effective in eliciting protective IgG responses in neonatal mice and can circumvent the Th2-bias normally associated with neonatal immune responses to other vaccine preparations.*

## INTRODUCTION

Neonates have an immature immune system with deficiencies in several components of inflammatory reactions, and innate and specific immunity (Hunt *et al.*, 1994; Schelonka and Infante, 1998; Trivedi *et al.*, 1997). This immaturity is reflected in a Th2 bias in murine immune responses to several vaccine preparations. These vaccines include inactivated and live attenuated viral vaccines, live recombinant canarypox vaccines (Barrios *et al.*, 1996a; Siegrist *et al.*, 1998b), and peptides or proteins emulsified in Freund's complete adjuvant (Singh, Hahn, and Sercarz, 1996) or alum (Barrios *et al.*, 1996b). In mice, Th2 patterns of immune responses are characterized by a preferential production of complement-independent IgG1 antibodies with low production of complement-dependent IgG2a antibodies, whereas Th1 responses are characterized by production of IgG2a and not IgG1 antibody. Consistent with these

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[1] The data presented in this chapter have been published by Pertmer and Robinson, 1999.

isotype profiles, antigen-specific CD4+ Th2-type T helper cells produce IL-4, which

supports switching to IgG1 and IgE, while Th1-type T helper cells are characterized by

the generation of IFNγ which supports switching to IgG2a. Th2-type responses also have

different patterns of CD8+ CTL activity (Mosmann *et al.*, 1995; Mosmann *et al.*, 1997).

The Th2 bias of the infant immune system places neonates at a greater risk for

developing severe bacterial and viral infections, which are best controlled by Th1 cell-

mediated responses.

It has been well documented that immunization of adult animals with antigen-

expressing plasmid DNAs raise strong, long-lasting, protective antibody and CTL

responses (for reviews, see Donnelly *et al.*, 1997; Robinson and Torres, 1997; Robinson

and Pertmer, 2000). DNA vaccines have the advantage of allowing protein production to

occur within cells and thereby permits presentation of peptides in the context of both

MHC class I and class II molecules. For many antigens, the Th bias of DNA-raised

responses seems to be dependent on the method of DNA inoculation. For instance, i.m.

inoculation with influenza A/PR/8/34 nucleoprotein- or hemagglutinin (HA)- expressing

DNA in saline produces a strong Th1 bias; whereas g.g. DNA delivery produces a strong

Th2 bias (Feltquate *et al.*, 1997; Pertmer, Roberts, and Haynes, 1996). In addition, both

methods of DNA inoculation induce strong cytolytic T cell activity (Pertmer, Roberts,

and Haynes, 1996). It was therefore of interest to evaluate the efficacy of DNA

immunization of newborns and determine the capacity of DNA vaccines to overcome the

Th2 bias characteristic of neonatal immune responses.

All but one study utilizing i.m. inoculation of DNAs in young mice have raised

humoral and cell-mediated immune responses (Bot *et al.*, 1996; Butts *et al.*, 1998; Fooks

*et al.*, 1996; Hassett, Zhang, and Whitton, 1997; Sarzotti *et al.*, 1997; (Siegrist *et al.*,

1998b; Siegrist and Lambert, 1997; for exception see Mor *et al.*, 1996. Two groups

(Martinez *et al.*, 1997; Wang *et al.*, 1997) showed similar IgG subtypes elicited in adult

and newborn DNA-primed animals. In contrast, two other groups (Bot *et al.*, 1997;

Manickan, Yu, and Rouse, 1997) showed differences in the predominant IgG subtypes

between adults and neonates, with adults generating mainly a Th1 profile and pups

generating a mixed Th1/Th2 profile.


In this chapter, we compare i.m. and g.g. HA DNA vaccination of one-day-old

and young adult BALB/c mice. HA DNA-vaccinated mice were also compared with

groups vaccinated with various doses of an A/PR/8/34 virus subunit vaccine in saline.

Antibody isotyping was performed as an indirect measure of Th1- or Th2-type biased

responses (Feltquate *et al.*, 1997). Patterns of humoral responses also were evaluated

following a secondary immunization later in life to determine whether Th-types had been

fixed by the primary neonatal immunization and whether tolerance or anergy had

occurred in non-responders.

## RESULTS

*Immunization of 1-day-old neonates with HA DNA, but not HA protein, induces a strong IgG response.*

Mice immunized at one day of age with HA DNA, but not HA protein, generated anti-HA IgG responses similar in titer to mice immunized as adults (Figures 4 and 5). Mice were given a primary immunization at 1 day or 6 weeks of age. Intramuscular and gene gun HA DNA inoculations delivered 50 µg and 1 µg of HA DNA, respectively, whereas more conventional protein immunizations delivered 10, 1, or 0.1 µg of an influenza A/PR/8/34 subunit vaccine in saline. Serum samples were collected 4, 10 and 22 weeks later for assessment of titer and longevity of anti-HA-specific IgG responses.

HA DNA-raised antibody responses were similar in mice immunized as adults or neonates (Figure 4). Four weeks following the primary immunization, most of the mice had low to undetectable IgG responses (data not shown). These responses substantially increased by 10 weeks post-prime in both the neonatal and adult immunized groups revealing overall similar kinetics of the IgG responses. At 22 weeks post-prime, the IgG responses remained stable (Figure 4). The mean post-prime IgG titer of mice HA DNA-immunized as newborns by i.m. inoculation was $24.1 \pm 19.5$ µg/ml (n=11) and by g.g. delivery was $16.1 \pm 25.7$ µg/ml (n=11). Some of the mice HA DNA-primed at birth developed very low to undetectable levels of IgG (3/11 i.m. and 4/11 g.g.). The mean post-prime IgG titer in adult HA DNA-vaccinated mice was $17.8 \pm 10.5$ µg/ml (n=4) and $17.9 \pm 5.8$ µg/ml (n=6) for i.m. and g.g. inoculated mice, respectively. Differences in
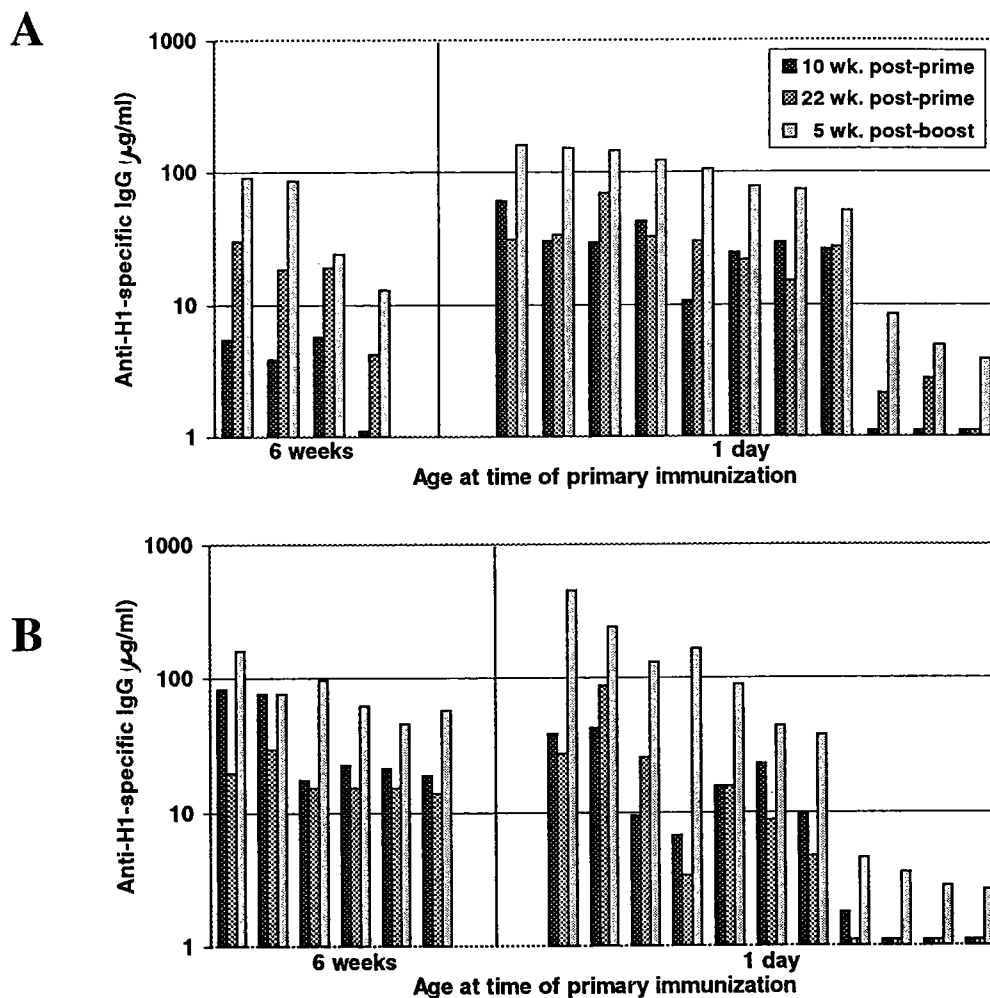
**Figure 4.** Anti-HA-specific IgG responses after i.m. or g.g. HA DNA immunization of 6-week-
or 1-day-old mice. Mice were immunized with (A) 50 μg of HA DNA dissolved in saline by i.m.
inoculation or (B) 1 μg HA DNA by g.g. delivery. Primary immunizations were given to mice at
either 6 weeks or 1 day of age. A second equivalent booster immunization was given 22 weeks
later. Serum samples were collected at 10 and 22 weeks post-prime and 5 weeks post-boost and
assayed for anti-HA-specific IgG by ELISA. Data are for individual mice.
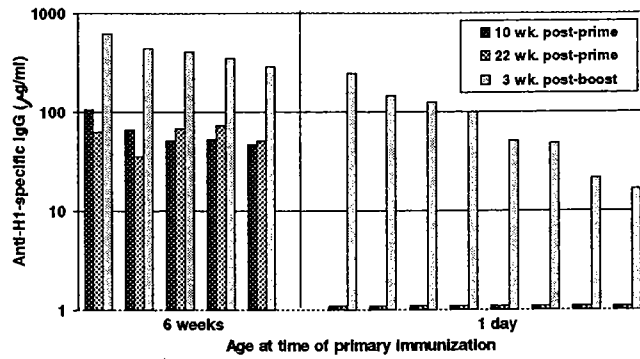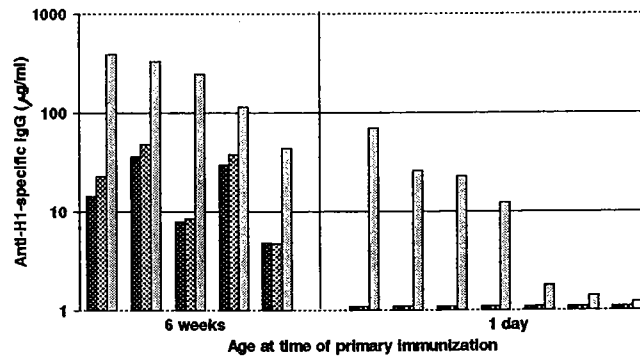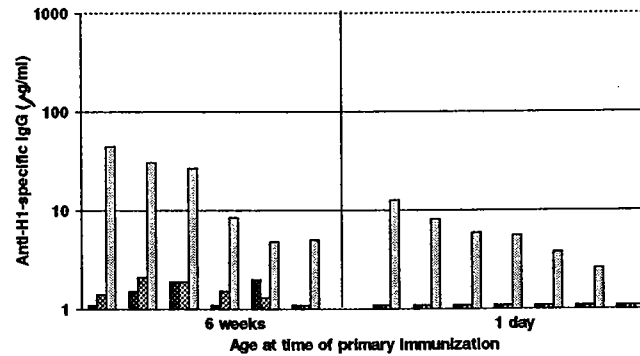
**Figure 5.** Anti-influenza-specific IgG responses after immunization of 6-week- or 1-day-old mice with various amounts of an A/PR/8/34 virus subunit vaccine. Mice were i.m. immunized with (A) 10 μg, (B) 1 μg, or (C) 0.1 μg of an A/PR/8/34 virus protein subunit vaccine preparation in 40 μl of saline. Primary immunizations were given at either 6 weeks or 1 day of age. A second equivalent booster immunization was given 22 weeks later. Serum samples were collected at 4 and 10 weeks post-prime and 3 weeks post-boost. Serum was assayed for anti-influenza-specific IgG Ab responses by ELISA. Data are for individual mice.

IgG titers between mice primed with HA DNA as newborns or adults were not statistically significant (i.m. p=0.57, g.g. p=0.86). Mice immunized with the pJW4303 vector DNA containing no insert had undetectable anti-HA-specific IgG levels (data not shown).

In contrast to the DNA immunizations, neonatal mice immunized with an influenza protein subunit vaccine had no detectable IgG responses (Figure 5). Adult mice immunized with 10 and 1 µg of the virus subunit vaccine generated high levels of anti-influenza-specific IgG, with 10 µg eliciting stronger IgG responses (64.6 ± 24.1 µg/ml, n=5) than 1 µg (18.7 ± 13.9 µg/ml, n=5). These responses persisted for greater than 22 weeks. The 0.1 µg dose did not generate IgG responses in mice primed either as adults or newborns.

Secondary HA DNA or protein immunizations given 22 weeks after priming caused an increase in anti-HA-specific IgG levels in all animals (Figures 4 and 5). All mice that had responded to the HA DNA as neonates had boosts in IgG titers (Figure 4). The magnitude of these boosts did not differ from that in adult-primed animals (i.m. p=0.4, g.g. p=0.7). The non-responding mice HA DNA primed as neonates generated low but detectable anti-HA-specific IgG responses 5 weeks following the second HA DNA immunization indicating tolerization had not occurred. These low responses were comparable to those attained in mice at 4 weeks post-prime (data not shown). All of the previously non-responding mice that were primed as neonates with 10 or 1 µg of viral

subunit developed post-boost mean titers of anti-influenza IgG that were comparable to responses generated in adults following the primary immunization (p=0.32 and p=0.82, respectively). Both non-responders primed as adults (6/6) or neonates (5/7) with 0.1 μg of subunit vaccine also generated low but detectable anti-influenza IgG responses after the booster immunization (Figure 5). The post-boost mean anti-influenza IgG titer for mice primed as adults was 20.2 ± 16.6 μg/ml (n=6) and primed as newborns was 5.7 ± 3.8 μg/ml (n=7). The differences in titers between the two groups was significant (p<0.03). This may reflect low-level priming in the adults following the first immunization.

*Neonatal HA DNA immunization elicits a mixed Th profile; whereas protein elicits a Th2-biased profile*

Mice HA DNA-immunized as neonates lacked the strong polarization of IgG profiles observed in mice immunized as adults with DNA or protein (Table 1). Sera from mice i.m. or g.g. HA DNA-immunized or protein immunized as pups or adults was further analyzed for anti-HA-IgG2a and -IgG1 profiles. These isotype profiles have been shown to correlate well with Th1 and Th2 cytokine profiles (Feltquate *et al.*, 1997). Mice HA DNA immunized as adults generated polarized IgG profiles that were dependent on the method of inoculation, whereas mice immunized as pups did not (p<0.001; Table 1). As expected, at 22 weeks post-prime adult mice i.m. DNA-immunized generated a strong IgG2a (3/4) response and g.g. DNA-immunized generated a strong IgG1 (6/6) response. The strong isotype polarization seen in mice HA DNA-

**Table 1.** Isotype profiles of mice immunized at 6 weeks or 1 day of age by i.m. or g.g. inoculation of HA-DNA or i.m. with various amounts of an A/PR/8/34 virus subunit vaccine in saline.

| | Age at time of primary | Assay time | Isotype profile[a] | | |
|---|---|---|---|---|---|
| Intramuscular DNA[d] | 6 weeks | 22 wk. PP | --- | 1 / 4 | 3 / 4 |
| | | 5 wk. PB | --- | --- | 4 / 4 |
| | 1 day | 22 wk. PP | 5 / 9 | 2 / 9 | 2 / 9 |
| | | 5 wk. PB | 1 / 9 | 6 / 9 | 2 / 9 |
| Gene gun DNA[d] | 6 weeks | 22 wk. PP | 5 / 6 | 1 / 6 | --- |
| | | 5 wk. PB | 5 / 6 | 1 / 6 | --- |
| | 1 day | 22 wk. PP | 1 / 7 | 3 / 7 | 3 / 7 |
| | | 5 wk. PB | 3 / 9 | 1 / 9 | 5 / 9 |
| Protein - 10 µg | 6 weeks | 22 wk. PP | 5 / 5 | --- | --- |
| | | 3 wk. PB | 5 / 5 | --- | --- |
| | 1 day | 22 wk. PP | <[c] | < | < |
| | | 3 wk. PB | 5 / 8 | 3 / 8 | --- |
| Protein - 1 µg | 6 weeks | 22 wk. PP | 5 / 5 | --- | --- |
| | | 3 wk. PB | 5 / 5 | --- | --- |
| | 1 day | 22 wk. PP | < | < | < |
| | | 3 wk. PB | 7 / 7 | --- | --- |
| Protein - 0.1 µg | 6 weeks | 22 wk. PP | < | < | < |
| | | 3 wk. PB | 5 / 6 | --- | 1 / 6 |
| | 1 day | 22 wk. PP | < | < | < |
| | | 3 wk. PB | 1/1 | < | < |

[a]Mice were immunized and serum was collected as given in Figures 4 and 5. Serum was assayed for anti-H1-specific IgG1 and IgG2a Ab by ELISA. IgA, IgM, IgG2b and IgG3 levels were below the level of detection (data not shown). Data are shown as number of animals with a specific predominant isotype over total number of animals observed. IgG1, an IgG1 to IgG2a ratio of >2; Mix, an IgG1 to IgG2a ratio of 0.5-2; IgG2a, an IgG1 to IgG2a ratio of <0.5.

[b]PP, post-prime; PB, post-boost.

[c]<, below detection.

[d]These data, pooled with independent data from similar groups in Tables 2 and 3, showed that differences in IgG isotype patterns between one-day-old and young adult immunizations following both i.m. and g.g. were significant (Fisher Exact Test, alternative hypothesis, two-sided, p<0.001).

primed as adults was in contrast to mice that were DNA-primed within 1 day of birth.

Mice receiving a primary HA DNA immunization at birth had no apparent pattern of Th-

bias regardless of the method used. At 22 weeks post-prime, 5/9 mice that responded to

the neonatal i.m. HA DNA immunization had an IgG1 predominance, 2/9 had a mixed

(1:1) IgG1:IgG2a response, and 2/9 had an IgG2a bias. It could be argued that the lack of

strong IgG2a predominant responses in these mice is due to the Th2-bias of neonatal

mice. However, at 22 weeks post-prime, mice HA DNA immunized as newborns using

the gene gun also failed to show an isotype bias. In the groups of responding mice, 1/7

had an IgG1 predominance, 3/7 had a mixed response, and 3/7 had an IgG2a

predominance. These data were surprising due to the strong Th2 bias in adult immune

responses raised by gene gun DNA inoculations (Feltquate et al., 1997; Pertmer,

Roberts, and Haynes, 1996; Robinson and Torres, 1997). Isotype profiles established in

both neonates and adults were maintained at boost in agreement with previous studies

showing Th cell bias is maintained once Th1 or Th2 fate has been determined (Barrios

et al., 1996b; Barrios et al., 1996a; Feltquate et al., 1997; Pertmer, Roberts, and Haynes,

1996).

All adult mice that received either 10 or 1 µg of an A/PR/8/34 protein subunit

vaccination generated the expected IgG1 antibody isotype predominance, which

remained stable following a boost (Table 1). Although mice primed as neonates did not

generate a detectable primary antibody response, a boost given at 22 weeks of age

generated an IgG1 predominance. Most (5/8) of the neonates that received 10 µg of the

viral protein subunit vaccine had an IgG1-predominance, whereas a mixed IgG1/IgG2a

response was seen in the remaining mice (3/8). In the group that received a 1 μg protein

booster immunization, an expected IgG1 predominance was observed in all mice (n=5).

The difference in the appearance of IgG2a in the 10 μg versus the 1 μg group may reflect

previous findings in which high doses of soluble protein elicit Th1-type responses,

whereas low doses generate Th2-type responses in neonates (Constant and Bottomly,

1997).

### *IgG profile polarization following HA DNA immunization and efficacy of protein*
### *subunit vaccination is determined by age.*

Age at the time of vaccination determined both the pattern of IgG profiles

following HA DNA immunization and the effectiveness of protein immunization. To

examine the effect of age on the generation of polarized IgG2a and IgG1 responses

following i.m. and g.g. DNA inoculation or on the responsiveness of young mice to the

protein subunit vaccine, groups of mice were immunized by i.m. or g.g. HA DNA

inoculation or with 10, 1, or 0.1 μg of protein subunit in saline at 1, 7, or 14 days of age.

Serum samples were collected 4-6 weeks post-prime and assayed for specific IgG, IgG1,

and IgG2a by ELISA.

Polarized patterns of IgG responses appeared by 2 weeks of age (Table 2). As

seen previously (Table 1), mice i.m. or g.g. DNA-immunized at birth generated a variety

of responses, with some having predominantly IgG1, some having predominantly IgG2a,

**Table 2.** Isotype profiles of mice immunized at various ages by i.m. or g.g. inoculation of HA-DNA.

| Method of DNA inoculation | Age at time of primary immunization | Isotype profile[a,b] | | |
|---|---|---|---|---|
| | | IgG1 | Mix | IgG2a |
| Intramuscular | 6 weeks | --- | 1 / 9 | 8 / 9 |
| | 14 days | --- | --- | 5 / 5 |
| | 7 days | --- | 1 / 5 | 4 / 5 |
| | 1 day | 6 / 10 | 4 / 10 | --- |
| Gene gun | 6 weeks | 12 / 12 | --- | --- |
| | 14 days | 6 / 6 | --- | --- |
| | 7 days | 5 / 9 | 3 / 9 | 1 / 9 |
| | 1 day | 3 / 9 | 2 / 9 | 4 / 9 |

[a]Mice of various ages were immunized as given in Figure 4, and serum was collected at 4-6 weeks post-prime. Serum was assayed for anti-H1-specific IgG1 and IgG2a Ab by ELISA. Isotype profiles were determined by comparing IgG1:IgG2a ratios of mice with detectable IgG. The number of individual mice which scored IgG1 (ratio >2), IgG2a (ratio <0.5), or Mix (ratio between 0.5 and 2) are shown as a fraction of the total tested. Results are combined data from two independent experiments.

[b]These data, pooled with independent data from similar groups in Tables 1 and 3 showed that differences in IgG isotype patterns between one-day-old and young adult immunizations following both i.m. and g.g. were significant (Fisher Exact Test, alternative hypothesis, two-sided, p<0.001).

and some having approximately equal mixtures of IgG1 and IgG2a isotypes. However, mice immunized at 2 weeks of age had the expected polarization of responses with 6/6 of g.g. immunized mice developing IgG1-biased responses and 5/5 i.m. immunized mice developing IgG2a-biased responses.

A similar age dependence was observed for the ability to immunize with 10 or 1 μg of protein subunit (Figure 6). As expected, mice immunized at birth did not develop an IgG response following immunization with 10 or 1 μg of protein confirming previous data (Figure 5). Immunizations performed at 14 days of age with either of the two doses produced low anti-influenza IgG in 7/10 (10 μg) and 5/10 (1 μg) mice. All six-week-old adult mice immunized with protein subunit generated strong anti-influenza IgG responses 4 weeks post-prime, with 10 μg eliciting better responses ($28.0 \pm 11.9$ μg/ml; n=6) than 1 μg ($9.1 \pm 6.0$ μg/ml; n=5).

### Mice HA DNA immunized at birth are protected from A/PR/8/34 challenge

Anti-HA-specific IgG responses generated by a single neonatal i.m. or g.g. HA DNA inoculation afforded long-term protection from influenza A/PR/8/34 virus challenge (Figure 7). One-day- and 8-week-old mice were i.m. (25 μg) or g.g. (1 μg) HA DNA-immunized to compare the long-term protective efficacy of a single immunization. Eight to nine months later, mice were assayed for anti-HA-IgG responses and challenged intranasally (i.n.) with a lethal dose of influenza A/PR/8/34. All mice i.m. or g.g.
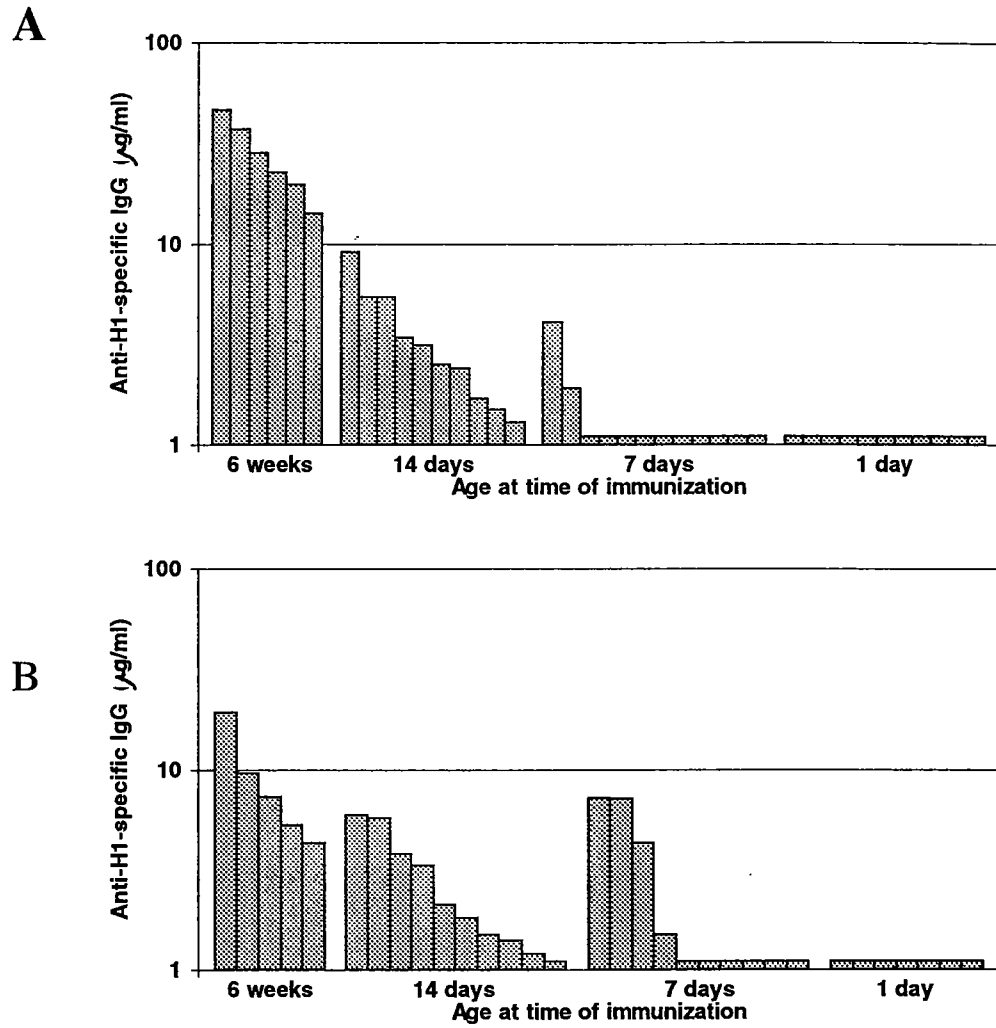
**Figure 6.** Anti-influenza-specific IgG responses after immunization of 1-, 7-, 14-day- or 6-week-old mice with 10 or 1 µg of an A/PR/8/34 virus subunit vaccine. Mice were i.m. immunized with either (A) 10 µg or (B) 1 µg of an A/PR/8/34 virus protein subunit vaccine preparation in 40 µl of saline. Immunizations were given at 6 weeks, 14, 7 or 1 day of age. Serum was collected at 4 weeks post-prime and assayed for anti-influenza-specific IgG Ab responses by ELISA. Data are for individual mice.

immunized with the pJW4303 empty vector DNA succumbed to flu infection by 4-6

days post-challenge. Survival of both adult and neonatal HA DNA-immunized mice

was dependent on anti-HA-specific IgG pre-challenge titers. Similar titers of

antibody conferred protection in both groups (Figure 7).


## DISCUSSION

In this chapter we show that both intramuscular and gene gun immunization of

newborn mice with HA DNA elicits vigorous, long-lasting, protective anti-HA-

specific IgG responses comparable in titer, but different in isotype, to those generated

in adult HA DNA-immunized mice. In contrast to the strongly polarized IgG2a and

IgG1 responses following adult i.m. and g.g. DNA inoculations, newborns generated

a variety of IgG1, IgG2a and mixed IgG1/IgG2a responses regardless of the method

of DNA inoculation. Also in contrast to adult immunizations, neonatal mice failed to

respond to various doses of an A/PR/8/34 subunit vaccine. Responsiveness to protein

vaccination and development of polarized patterns of T help following DNA

immunization appeared by 2 weeks of age.


Mice DNA-immunized as adults and neonates had similar antibody titers but

different isotype profiles. Adult HA DNA immunized mice generated the expected

strongly polarized IgG2a and IgG1 responses following i.m. and g.g. inoculation,

respectively. Interestingly, both i.m. and g.g. inoculation of neonatal mice generated
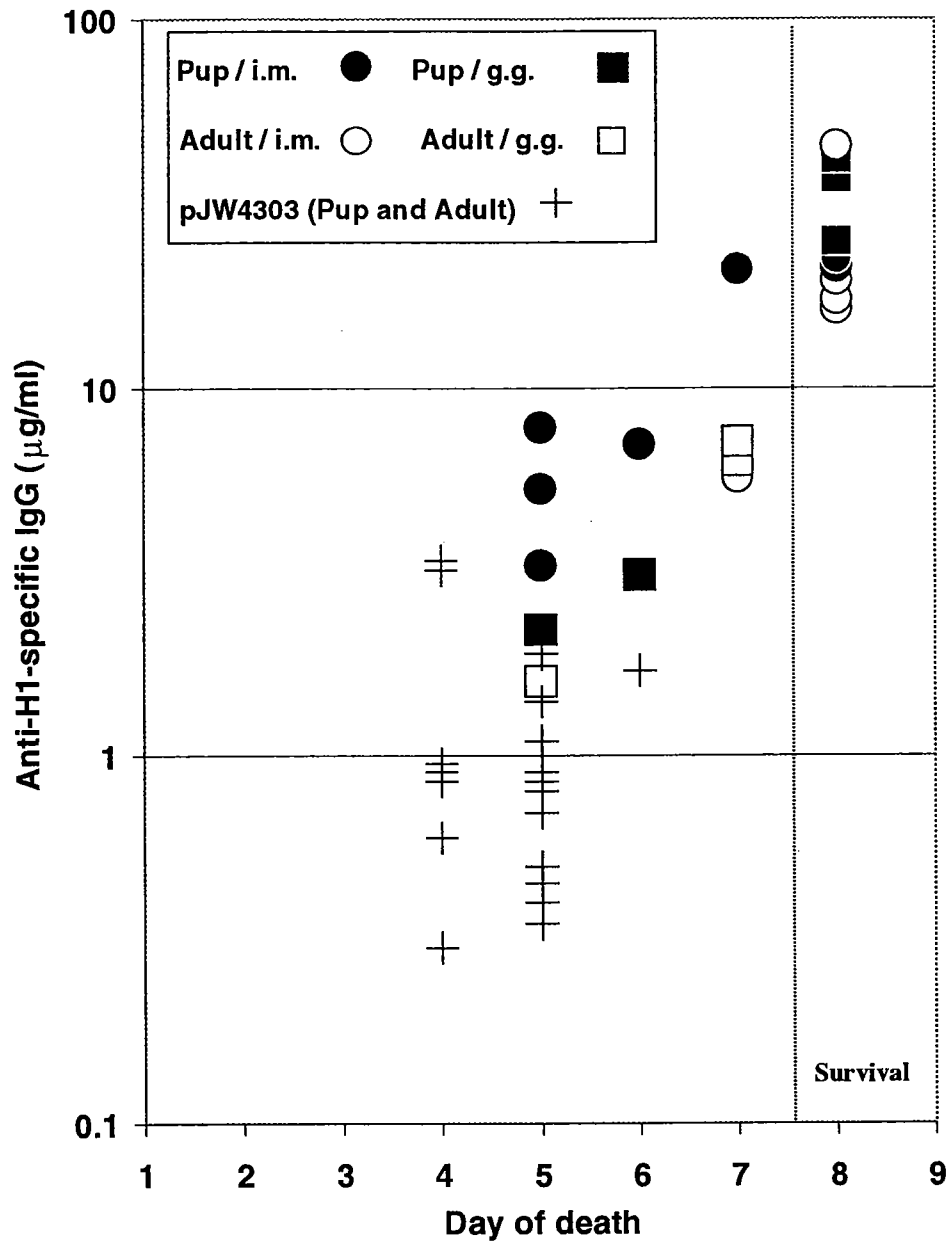
**Figure 7.** Correlation of anti-HA-specific IgG levels and protection from influenza challenge. One-day- and 8 week-old mice were immunized with HA-DNA by i.m. (25 μg) or g.g. (1 μg). Eight to 9 months later, mice were challenged i.m. with a lethal dose of A/PR/8/34 and weighed daily. Mice were sacrificed following >20% loss of pre-challenge body weight. Serum was collected and assayed for anti-HA-specific IgG titers by ELISA prior to virus challenge.

a variety of IgG1, IgG2a, and mixed IgG1/IgG2a responses. The profiles raised by the prime persisted following the boost. The combination of both IgG1 and IgG2a responses following g.g. DNA immunization was particularly unexpected. Neonates typically have IgG1 biased responses following immunization with many types of vaccines (Barrios *et al.*, 1996b; Siegrist *et al.*, 1998b; Singh, Hahn, and Sercarz, 1996), and g.g. DNA inoculations typically raise Th2 biased responses in adult animals (Feltquate *et al.*, 1997; Pertmer, Roberts, and Haynes, 1996).

We do not understand the differences in the biases of the IgG responses that occurred within the same litter using identical delivery conditions. Very few APC may be required to initiate a response following DNA immunization in the neonate. APC as well as the lymphoid organs are still undergoing development at birth. Macrophages and B cells, for instance, are known to be deficient in antigen processing and presentation functions including co-stimulation (Lu, Calamai, and Unanue, 1979; Schelonka and Infante, 1998; Trivedi *et al.*, 1997). The various Th types of the DNA-raised responses may reflect this ongoing development. The specific APC transfected within a particular neonate may be in a different stage of development than the APC in a littermate, which may subsequently affect IgG isotype development. In contrast, adults have fully differentiated APC and lymphoid organs, which may account for the highly polarized responses raised in these animals.

Previous studies have shown different dominant Th types elicited by i.m. DNA-immunization of neonatal mice. Two reports show differences in IgG isotype responses from mice DNA-immunized as pups or in adulthood. DNA encoding the herpes simplex virus gB elicited mainly an IgG2a response in adult-immunized animals and a mixed IgG1/IgG2a response in pooled serum from mice immunized as newborns (Manickan, Yu, and Rouse, 1997). Likewise, adult mice immunized with DNA encoding hemagglutinin from the WSN strain of influenza gave a predominant IgG2a response, and mice immunized as neonates gave a mixed IgG1/IgG2a response (Bot *et al.*, 1997). These two reports parallel the data shown in Tables 2 and 3. However, Wang *et al.* (1997) reported a strong IgG2a profile in pooled serum from mice immunized with a rabies virus gp-DNA at 1 day or 6 weeks of age. Martinez *et al.* (1997) also showed equivalent IgG subtypes in pooled serum from mice immunized as adults or pups, but subtypes were dependent on the type of antigen being encoded by the plasmid DNA. Both measles virus hemagglutinin and Sendai virus nucleoprotein DNAs generated IgG2a responses, whereas DNA encoding the C fragment of tetanus toxin elicited a mixed IgG1/IgG2a profile. Differences observed in dominant Th profiles in different experimental models may be antigen-specific and/or may reflect the use of pooled vs. individual mouse serum. Importantly, none of the groups showed a consistent, predominant IgG1 profile which indicate DNA vaccines are capable of circumventing the Th2 bias elicited in newborns with other vaccine antigens (Barrios *et al.*, 1996b).

Inherent defects in neonatal APC may account for the differences in efficacy of neonatal protein and DNA immunizations (Schelonka and Infante, 1998; Trivedi *et al.*, 1997). Studies using soluble pigeon cytochrome show that splenic B cells are unable to internalize, process and present the antigen until 10-14 days of age and adult levels do not occur until 21-28 days of age. Furthermore, presentation of a pigeon cytochrome peptide, which does not require processing, occurs at 50% of adult levels by 7 days and is at 100% by 14-18 days of age. These data also correlate with temporal MHC class II, ICAM-1 and LFA-1 cell surface expression (Hoyer, Morris, and Pierce, 1992; Morris, Hoyer, and Pierce, 1992). This time course supports data presented in Figure 6 which shows that HA protein-immunized mice generate low-level IgG responses when immunized between 7 and 14 days of age. In contrast to protein that must be taken up by cells for presentation, plasmid DNA can directly transfect APC that are either resident in or circulating through the target tissue. DNA vaccines are also different from protein vaccines in that the DNA-encoded protein is expressed for relatively long periods of time (Williams *et al.*, 1991; Wolff *et al.*, 1990); T. Pertmer, unpublished observations). Therefore, the presence of newly generated antigen at later times in ontogeny may circumvent the defects associated with APC at earlier time points and allow for antigen presentation. The long-term presence of antigen may also account for the longevity and stability of the humoral responses generated following DNA immunizations.

## CONCLUSION

These findings suggest that newborn mice developed stable, long-lived, protective anti-HA-specific IgG responses similar in titer to those of adult DNA-immunized mice. However, unlike the adult i.m. and g.g. DNA immunizations, which develop polarized IgG2a and IgG1 responses respectively, mice immunized as neonates developed a variety of IgG1-, IgG2a-, and mixed IgG1/IgG2a responses, regardless of the inoculation method. Boosting increased but did not change these antibody profiles. In contrast to the DNA immunizations, inoculations of newborn mice with an A/PR/8/34 viral protein subunit preparation failed to elicit an antibody response. Temporal studies revealed that both responsiveness to protein vaccination and development of polarized patterns of T help following DNA immunization appeared by 2 weeks of age.

# CHAPTER II:

# Th1 GENETIC ADJUVANTS MODULATE IMMUNE

# RESPONSES IN NEONATAL MICE

# CHAPTER II:

# TH1 GENETIC ADJUVANTS MODULATE IMMUNE RESPONSES

# IN NEONATAL MICE[2]

## HYPOTHESIS

*The disparity between adult and neonatal DNA-raised IgG responses is due to a deficiency in Th1-type cytokines at birth, and thus, it is possible to overcome this deficiency by codelivering Th1 genetic adjuvants at the time of DNA immunization.*

## INTRODUCTION

In the previous chapter, we examined the ability of neonatal mice to generate protective IgG responses following DNA immunization. We showed that newborn mice developed stable, long-lived, protective anti-HA-specific IgG responses similar in titer to those of adult DNA-immunized mice. However, unlike the adult i.m. and g.g. DNA immunizations which develop polarized IgG2a and IgG1 responses, respectively, mice immunized as neonates developed a variety of IgG1-, IgG2a-, and mixed IgG1/IgG2a responses, regardless of the inoculation method. It is unclear why this disparity in IgG subtype profiles exists.

One theory that accounts for the disparity between neonatal and adult IgG subtype responses is related to their deficiencies in many components of innate, specific, and

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[2] The data presented in this chapter have been submitted for publication.

inflammatory immune responses. For instance, one of the areas of deficiency is in Th1-type cytokines. This is exemplified in one study, which addressed the expression and production of IL-12 in human cord blood cells following LPS stimulation. When cord blood cells were compared to adult PBMC, it was found that the levels of p40 mRNA were similar in both groups, but cord blood cells produced p40 mRNA transcripts with a shorter half life. Thus, the deficiency in neonatal IL-12 production was due to post-transcriptional regulation (Suen *et al.*, 1998). Similarly, another group (Pioli *et al.*, 1998) showed that older mice produce more IFNγ than young mice, and the difference was attributed to different IFNγ mRNA stability patterns.

The studies in this chapter were undertaken to determine if it possible to overcome the Th1 cytokine deficiency in neonates. IL-12 is the predominant force behind Th1-type immune responses. IL-12 induces IFNγ production by both NK and T cells and has multiple biological effects on the immune system. Thus, both of these cytokines have obvious potential benefits for overcoming the Th1 deficiency in neonates; however there are very little data to support the use of these cytokines in neonates. The use of DNA as vaccine and adjuvant may be better suited for use in neonates, as the Th1 cytokines will co-localize with the vaccine antigen at the site of antigen presentation. Although effective (Arulanandam, Cleave, and Metzger, 1999; Kovarik *et al.*, 1999), recombinant cytokines are typically administered systemically and have the potential for serious side effects in infants (Kovarik *et al.*, 2000). We show that both codelivered IFNγ and IL-12 DNA polarized the isotype patterns of anti-HA antibody in neonates to

IgG2a, but did not affect isotype patterns in adults. Additionally, we show that codelivery of the Th1 genetic adjuvants biased the pattern of cytokine production by restimulated splenocytes more strongly toward IFNγ in neonates than adults.

## RESULTS

### *Neonatal mice immunized with HA DNA lack polarized IgG responses.*

One-day-old and 12-week-old mice immunized by either i.m. (10 µg HA DNA) or g.g. (1 µg HA DNA) inoculation generated HA-specific IgG responses that were comparable in magnitude but had different isotype profiles (Table 3). Mice immunized at 12 weeks of age by i.m. inoculation had a dominant IgG2a response; whereas g.g. immunized mice had a dominant IgG1 response. In contrast, mice immunized at 1 day of age differed in the isotype profile of their responses, with some mice generating predominantly IgG1, some, predominantly IgG2a, and some overall similar levels of IgG1 and IgG2a irrespective of the method of immunization. The observed isotype profiles of each group were stable for at least 1 year and confirmed our previous results from Chapter 1 (Pertmer and Robinson, 1999).

### *Neonatal mice coimmunized with DNAs expressing HA and Th1-promoting cytokines develop polarized IgG2a responses.*

To determine if the deficiency of Th1-promoting cytokines at birth played a role in the lack of polarized IgG responses in neonatal mice, newborn mice were coimmunized with DNAs expressing HA and the Th1-type cytokines, IL-12 or IFNγ.

**Table 3.** Antibody isotype profiles following neonatal and adult i.m. or g.g. codelivery of HA- and IL-12- or IFNγ-DNAs[a].

| Age at Immunization | Codelivered DNA | Method | IgG (µg/ml)[d] | IgG isotype profiles[b,c] | | | IgG (µg/ml)[d] | IgG isotype profiles[b,c] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | IgG1 | Mix | IgG2a | | IgG1 | Mix | IgG2a |
| 1 day | --- | i.m. | 4.9 (3.0, 8.1) | 5/12 | 4/12 | 3/12 | 7.9 (5.7, 11.0) | 5/12 | 4/12 | 3/12 |
| | | g.g. | 3.5 (1.6, 7.6) | 1/7 | 3/7 | 3/7 | 7.3 (4.7, 11.3) | 2/7 | 2/7 | 3/7 |
| | Control | i.m. | 1.6 (1.1, 2.2) | 1/5 | 2/5 | 2/5 | 3.7 (2.2, 6.2) | 1/5 | 1/5 | 3/5 |
| | | g.g. | 1.6 (1.5, 1.9) | 2/6 | 2/6 | 2/6 | 1.2 (0.8, 1.9) | 3/6 | 1/6 | 2/6 |
| | IL-12 | i.m. | 6.0 (5.4, 6.8) | 1/9 | --- | 8/9 | 5.1 (4.2, 6.1) | 2/9 | 3/9 | 4/9 |
| | | g.g. | 3.1 (2.4, 4.1) | --- | --- | 10/10 | 12.6 (9.6, 16.5) | 1/10 | --- | 9/10 |
| | IFNγ | i.m. | 3.8 (3.4, 4.3) | 1/7 | --- | 6/7 | 2.5 (1.4, 4.4) | --- | 1/7 | 6/7 |
| | | g.g. | 1.5 (1.3, 2.0) | --- | --- | 3/3 | 2.0 (1.5, 3.0) | --- | --- | 3/3 |
| 12 weeks | --- | i.m. | 7.0 (4.6, 10.8) | --- | --- | 5/5 | 12.6 (7.6, 20.2) | --- | --- | 5/5 |
| | | g.g. | 9.1 (6.5, 12.7) | 5/5 | --- | --- | 13.3 (10.2, | 5/5 | --- | --- |
| | Control | i.m. | 3.2 (1.8, 5.6) | --- | --- | 3/3 | 4.7 (1.9, 11.6) | --- | --- | 3/3 |
| | | g.g. | 1.5 (1.3, 1.7) | 2/2 | --- | --- | 3.5 (2.7, 4.6) | 3/3 | --- | --- |
| | IL-12 | i.m. | 3.0 (2.3, 4.1) | --- | --- | 4/4 | 5.4 (3.5, 8.4) | --- | --- | 5/5 |
| | | g.g. | 2.3 (1.7, 3.2) | 5/5 | --- | --- | 6.4 (4.8, 8.6) | 5/5 | --- | --- |
| | IFNγ | i.m. | 5.3 (3.6, 7.6) | --- | 1/5 | 4/5 | 6.0 (3.3, 11.0) | --- | 1/4 | 3/4 |
| | | g.g. | 1.6 (1.3, 2.1) | 4/4 | --- | --- | 3.0 (2.3, 3.9) | 5/5 | --- | --- |

[a]Mice of various ages were immunized as given in the text, and serum was collected at 16 and 52 weeks post-prime. Serum was assayed for anti-HA-specific IgG, IgG1 and IgG2a Ab by ELISA. Isotype profiles were determined by comparing IgG1:IgG2a ratios of mice with detectable IgG. The number of individual mice which scored IgG1 (ratio >2), IgG2a (ratio <0.5), or Mix (ratio between 0.5 and 2) are shown as a fraction of the total tested

[b]These data, pooled with independent data from similar groups in Tables 1 and 2 showed that differences in IgG isotype patterns between one-day-old and young adult immunizations following both i.m. and g.g. were significant (Fisher Exact Test, alternative hypothesis, two-sided, p<0.001).

[c]Data for IL-12 and IFNγ codeliveries were compared to pooled data for one-day-old control animals from Tables 1-3 and showed significance of: i.m. plus IL-12, p<0.001; g.g. plus IL-12, p<0.02; i.m. plus IFNγ, p=0.001; g.g. plus IFNγ, p=0.2 (Fisher Exact Test, alternative hypothesis, two-sided).

[d]Data are shown as GMT IgG (95% confidence limits).

The IL-12 plasmid was bicistronic and encoded both the p35 and p40 subunits of IL-12.

Mice immunized by i.m. inoculation received 10 μg of HA- and 40 μg of cytokine-

encoding DNA, while g.g.-immunized mice received 1 μg of HA- and 4 μg of cytokine-

encoding DNA. Control groups received empty vector DNA (control DNA) in the place

of the cytokine DNA. Results are from a single experiment.


In contrast to the lack of polarized IgG responses in HA DNA immunized

neonates, mice coimmunized on the day of birth with HA DNA and IL-12- (i.m.,

p<0.001; g.g., p=0.02) or IFNγ- (i.m., p=0.001; g.g., p=0.2) DNA developed strongly

polarized IgG2a responses (Figure 8, Table 3). This occurred in groups receiving either

i.m. or g.g. inoculations. For instance, in mice coimmunized on the day of birth with IL-

12 DNA, 8/9 i.m. immunized and 10/10 g.g.-immunized mice developed predominantly

anti-HA IgG2a responses. The IgG2a polarization seen following neonatal i.m.

codelivery of IL-12 DNA was transient. By 1 year post-prime, IgG profiles had reverted

to the mixture of responses seen in mice immunized with HA DNA alone (Table 3). This

reversion was not seen in the neonatal g.g.-immunized mice, where the IgG2a-

predominance remained stable in 9/10 mice for up to 1 year. The IgG2a polarization of

responses also was stable over the course of a year in mice receiving codelivered IFNγ

DNA (Figure 8, Table 3).


Unlike neonatal mice, codelivery of HA DNA and IL-12- or IFNγ-DNA in adult

mice had no effect on the polarized patterns of IgG responses in i.m. or g.g. DNA-

inoculated mice (Figure 8, Table 3). As expected, virtually all i.m.-inoculated mice had

polarized IgG2a patterns. However, 100% of the adult mice coimmunized by g.g. with

IL-12- or IFNγ-DNA retained an IgG1-biased anti-HA antibody response. These

polarized patterns did not change over the course of a year. Thus, Th1-promoting DNA

adjuvants had a greater influence on IgG subtype polarization in neonates than adults.

***Both neonatal and adult mice coimmunized with Th1 cytokines have increased Th1-type cellular immune responses.***

We further addressed the effect of Th1 cytokine DNA codelivery on immune

responses by testing for cytokine responses following stimulation of PBMC with

inactivated influenza virus. Vaccinated mice were challenged with a sublethal dose of

influenza virus. One week later, splenocytes were pooled from 2-3 mice and cultured for

2 days with inactivated influenza virus. Culture supernatants were harvested, pooled and

assayed by ELISA for IFNγ (indicative of a Th1 response) and IL-5 (indicative of a Th2

response) (Figure 9, Table 4). This assay tested for lymphokine patterns of the secondary

response recalled by a viral infection.

Codelivered IL-12 DNA had a stronger effect on the lymphokine responses of

restimulated splenocytes than codelivered IFNγ DNA (Table 4). In both neonates and

adults, codelivery of IL-12 DNA completely inhibited the ability of recalled splenocytes

to produce IL-5. This was true for i.m. (adults, p<0.01; pups, p<0.02) and g.g (adults, p
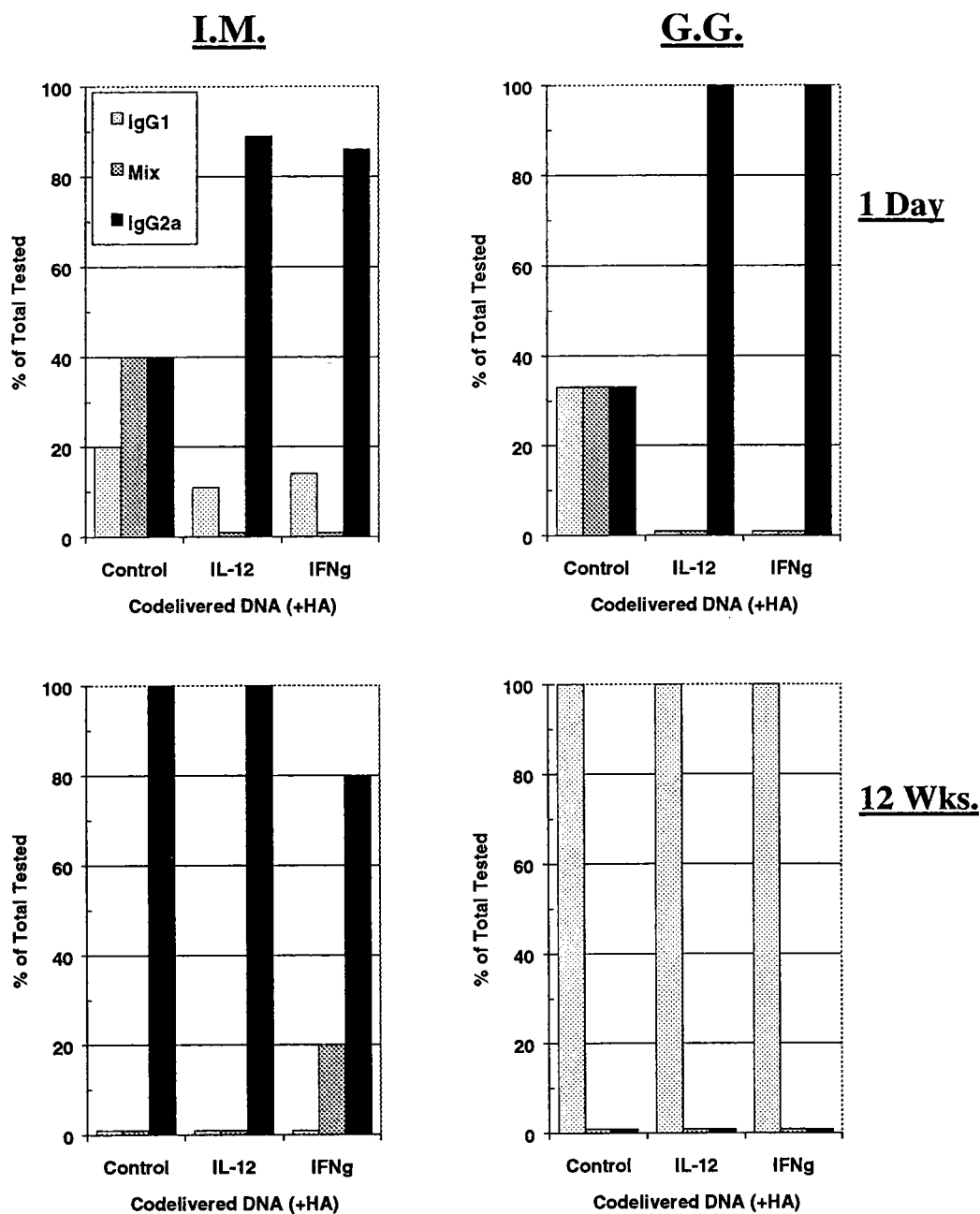
**Figure 8.** Isotype profiles of mice immunized with HA DNA with or without Th1 genetic adjuvants. One-day-old and 12-week-old mice were i.m. or g.g. immunized with DNAs expressing HA and either pJW, IL-12 or IFNγ. Serum was collected at 16 weeks post-prime and assayed by ELISA for IgG1 and IgG2a. Mice were scored IgG1-predominant if the ratio of IgG1 to IgG2a was ≥ 2.0, scored IgG2a-predominant if the ratio of IgG1 to IgG2a was ≤ 0.5, and scored 'Mix' if the ratio of IgG1 to IgG2a was between 0.5 and 2.0. See also Table 3.

<0.01; pups, p=0.05) deliveries of DNA. In contrast, codelivery of IFNγ DNA completely inhibited IL-5 production in recall responses in neonates (p=0.002) but not adults (p=0.24). In i.m-immunized adults, the codelivered IFNγ DNA had no effect on the relative production of IFNγ (p=0.34) and IL-5 (p=0.13) in the recall responses. However, in g.g.-immunized adults codelivery of IFNγ DNA did increase the ratio of IFNγ to IL-5.

## DISCUSSION

We have previously shown in Chapter 1 that both i.m. and g.g. immunization of neonatal mice generate a variety of IgG1, IgG2a and mixed IgG1/IgG2a responses; whereas adult mice have polarized IgG2a- and IgG1-predominant responses following i.m. and g.g. immunization, respectively (Pertmer and Robinson, 1999). In this chapter, we evaluate the ability of codelivered Th1-promoting cytokine DNAs to bias immune responses toward Th1. Our results show that codelivery of HA and IL-12 or IFNγ DNA in neonates elicits predominantly IgG2a responses, while codelivery in adults has no effect on IgG subtype patterns (Figure 8, Table 3). Our results also show that codelivery of IL-12- or IFNγ -DNA in neonates causes a marked shift towards production of IFNγ; whereas codelivery of IL-12 DNA has a much stronger effect than codelivery of IFNγ DNA in adults (Figure 9, Table 4).
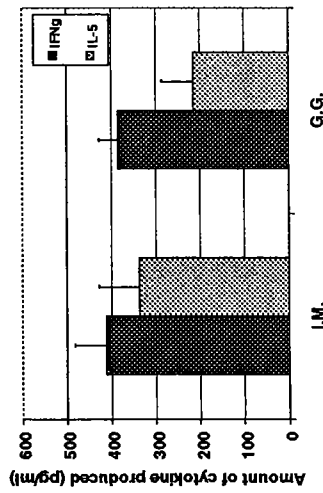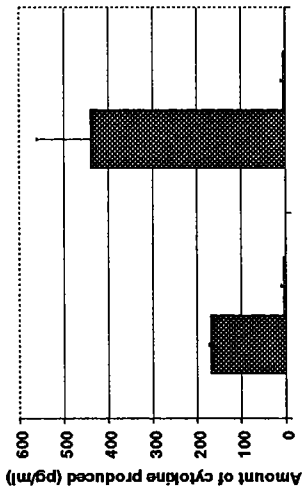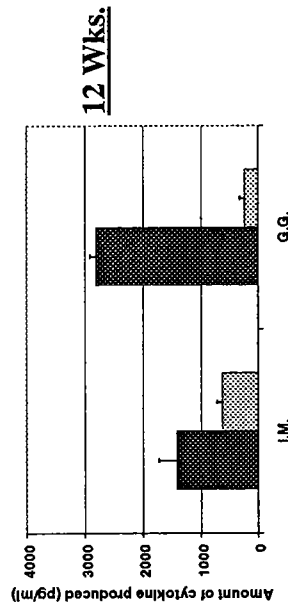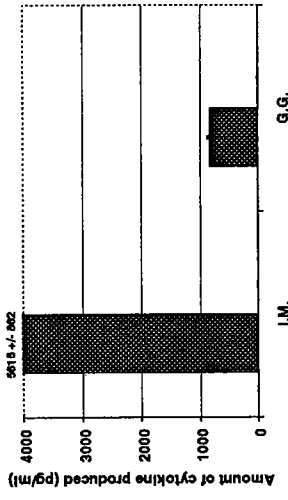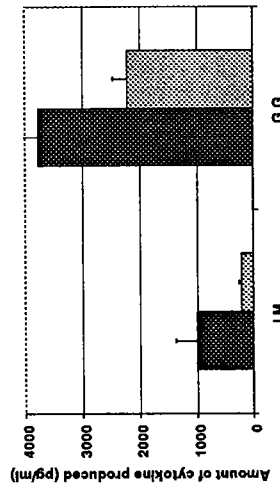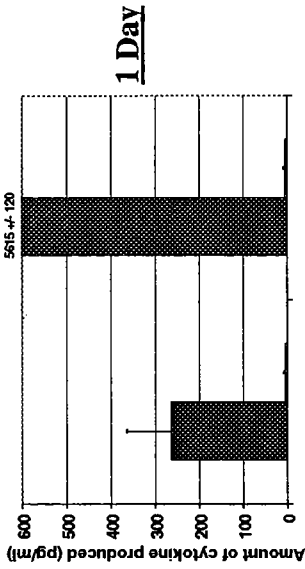
**Figure 9.** Effects of Th1-cytokine DNA codelivery on CD4+ T cell responses. Splenocytes from the same mice used in Figure 8 were cultured for 2 days with inactivated influenza virus. Bulk culture supernatants were harvested and pooled and assayed for IFNγ and IL-5 production by ELISA. Results are shown as mean +/- SD IFNγ and IL-5 levels in culture supernatants.

**Table 4.** CD4+ T cell responses following immunization with DNAs expressing influenza HA and Th1 cytokines[a].

| Codelivered DNA | Age at immunization | Method | IFNγ (pg/ml) | IL-5 (pg/ml) | Ratio (IFNγ/IL-5) |
|---|---|---|---|---|---|
| Control | 1 day | i.m. | 445 / 380* | 395 / 280* | 1.1/1.3 |
|  |  | g.g. | 385 | 215 | 1.8 |
|  | 12 weeks | i.m. | 1005 | 220 | 4.6 |
|  |  | g.g. | 3775 | 2220 | 1.7 |
| IL-12 | 1 day | i.m. | 170 | < | >170 |
|  |  | g.g. | 440 | < | >440 |
|  | 12 weeks | i.m. | 5615 | < | >5615 |
|  |  | g.g. | 830 | < | >630 |
| IFNγ | 1 day | i.m. | 265* | <* | >265 |
|  |  | g.g. | 1665 | < | >1665 |
|  | 12 weeks | i.m. | 1420 | 625 | 2.3 |
|  |  | g.g. | 2815 | 240 | 11.7 |
| Control only |  | i.m./g.g | < / <* | < / <* | --- |

*Results from an independent experiment

[a]Splenocytes from the same mice used in Figure 8 were cultured for 2 days with inactivated influenza virus. Bulk culture supernatants were harvested and pooled and assayed for IFNγ and IL-5 production by ELISA. Results are shown as mean IFNγ and IL-5 levels in culture supernatants. See also Figure 9.

Codelivery of Th1 cytokine DNA affected IgG isotype profiles in neonates, but not adults. Neonatal mice immunized with HA and control DNA had a variety of IgG1, IgG2a and mixed IgG1/IgG2a response, as opposed to the strongly polarized responses of adult immunized mice (Table 3, Figure 8). Codelivery of either IL-12- or IFNγ-DNA in neonates directed the isotype responses to an IgG2a-predominance. These results are consistent with a recent study showing that administration of recombinant IL-12 with protein antigens in neonatal mice redirect an IgG1 to an IgG2a-predominant response (Arulanandam, Cleave, and Metzger, 1999). In contrast to the studies in neonates, codelivery of the Th1 cytokine DNAs failed to redirect IgG1 responses to IgG2a in adults. These results imply that antibody responses generated by immature immune systems are more susceptible to modulation by exogenous IL-12 and IFNγ than antibody responses raised by mature immune systems. This could reflect exogenously introduced cytokines having larger impacts on lymphokine levels in neonates because of the low levels of expression of endogenous IL-12 and IFNγ in immature murine lymphoid cells (Pioli *et al.*, 1998; Suen *et al.*, 1998).

Lymphokine responses of recall splenocytes were more sensitive to immunizations in the presence of codelivered cytokines than isotype responses. Whereas effects on isotype profiles were limited to neonates, effects on cytokine production were observed in both neonates and adults. Codelivered IL-12 DNA resulted in the absence of IL-5 production by recall splenocytes in both age groups (Table 4, Figure 9). A recent study by Kovarik, et al. also showed a decrease in IL-5 responses following codelivery of

IL-12 DNA (Kovarik *et al.*, 1999). Codelivered IFNγ DNA had a stronger effect in neonates than adults, blocking IL-5 production by recall splenocytes in mice immunized as neonates but only modulating the recall response in mice immunized as adults (Table 4, Figure 9).

The effects of codelivered cytokines on antibody and T cell responses revealed isotype patterns that did not necessarily correlate with cytokine responses. In adult mice coimmunized with IL-12 DNA, a predominantly IgG1 antibody response occurred in mice whose recall splenocytes produced high levels of IFNγ but undetectable levels of IL-5. Thus, a Th2 isotype (IgG1) was associated with a Th1 (IFNγ) pattern of lymphokines. This "dichotomy" in isotype and lymphokine responses was also observed in neonates immunized in the presence of codelivered IL-12. By one year of age, IgG isotypes had reverted to a mixed pattern, whereas the cytokines remained exclusively IFNγ. These results imply that recall T cells do not necessarily reflect the population of T cells that provided help for the memory B cell response. Similar results have been observed previously (Laylor *et al.*, 1999; Pertmer, Roberts, and Haynes, 1996).

Coadministration of genetic adjuvants may have a stronger effect on the establishment of recall responses than coadministration of lymphokine proteins. IL-12 coadministration with a protein antigen induces a primary Th1 response in adult mice; however, the recall response was a Th0 response despite subsequent immunizations

with IL-12 (Bliss *et al.*, 1996; Wolf *et al.*, 1995). In our study, mice coimmunized with HA and IL-12 or IFNγ DNAs had a Th1-predominant recall response (Figure 9, Table 4).

Despite the dichotomies in the phenotypes of some of the memory B and T cell responses in our study (see above), our results clearly demonstrate that codelivery of Th1 genetic adjuvants can direct neonatal immune responses in mice towards type 1 T cell help. Interestingly, within our sample sizes, the most definitive and consistent long term establishment of Th1 responses in neonates was achieved by coinoculation of IFNγ DNA, not IL-12 DNA, as evidenced by the production of IgG2a antibody and the presence of recall IFNγ-producing splenocytes. In contrast, the most pronounced long term effects on the Th-type of the raised response in adults was seen for coinoculated IL-12 DNA.

## CONCLUSION

In this chapter, we addressed the ability of DNA encoding Th1 cytokines to bias the immune responses raised by neonatal DNA immunization. These studies were undertaken to determine if it possible to overcome the Th1 cytokine deficiency in neonates. Neonatal mice coimmunized with HA DNA and either IL-12 or IFNγ-expressing DNA developed IgG2a-biased immune responses, regardless of inoculation method. In contrast, the Th1 genetic adjuvants had no effect on IgG subtype patterns in adults. In neonatal mice, the Th1 genetic adjuvants also shifted the pattern of lymphokine production by recall splenocytes from a mixed response of IFNγ and IL-5 to exclusively IFNγ. In adults, despite the failure to change the isotype pattern of the

antibody response, a shift towards IFNγ production also occurred for recall splenocytes following coimmunzation with IL-12. Thus, coinoculation of Th1 genetic adjuvants had greater effects on the nature of the immune response in the neonate and were more marked than the effects in adults.

# CHAPTER III:

# CHARACTERIZATION OF IMMUNE RESPONSES TO

# INFLUENZA DNA VACCINES

# IN THE PRESENCE OF MATERNAL ANTIBODY

# CHAPTER III:

# CHARACTERIZATION OF IMMUNE RESPONSES TO

# INFLUENZA DNA VACCINES

# IN THE PRESENCE OF MATERNAL ANTIBODY[3]

## HYPOTHESIS

*DNA vaccines are effective in generating both humoral and cellular immune responses in the presence of maternal antibody.*

## INTRODUCTION

Neonates are deficient in several components of inflammatory and innate and specific immune responses. The presence of high titer maternal antibody in newborns is the major form of protection from disease in early life. Maternal IgG crosses the placenta from mother to fetus during development (Leach *et al.*, 1996) and typically exceeds titers of the same antibody in the mother. This passive antibody slowly declines over the first year of life, a period during which the infant's immune system matures, becomes more experienced, and develops it's own repertoire of protective memory immune responses. However, maternal antibody can also interfere with active immunization of the offspring (Albrecht *et al.*, 1977). Immunization protocols are often delayed several months and/or require multiple booster immunizations to achieve the desired protective immune

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[3] The data in this chapter have been submitted for publication.

response.  Thus, a window of time exists when maternal antibody levels are too low to reliably protect an infant from infectious disease but are high enough to prevent responses to vaccines.

DNA vaccination is an attractive method for immunization in the presence of maternal antibody.  Maternal antibody is thought to interfere with traditional vaccine efficacy by reducing the amount of antigen available for processing and presentation by antigen presenting cells.  The ability of DNA vaccines to directly transfect cells bypasses this problem.  The maternal antibody will not inhibit the DNA vaccine itself because antigen is not available until *de novo* synthesis occurs.  Both DNA and subsequent antigen expression persists for several weeks (Doe *et al.*, 1996).  Thus, DNA-raised immune responses could occur as maternal antibody titers wane.  Some groups have reported success following neonatal DNA immunization in the presence of maternal antibody (Manickan, Yu, and Rouse, 1997), while others have failed   (Le Potier *et al.*, 1997; Monteil *et al.*, 1997; Siegrist *et al.*, 1998a; Wang *et al.*, 1998).

We have previously shown in Chapter 1 that i.m. and g.g. immunization of mice as neonates or adults with an influenza hemagglutinin (HA)-expressing DNA generates long-lasting protective IgG responses (Pertmer and Robinson, 1999).  In this study, we address the ability of DNAs expressing HA and nucleoprotein (NP) to generate humoral and cellular responses in the presence of maternal antibody.  When expressed in a DNA vaccine, the influenza HA localizes to the plasma membrane while the influenza NP is

intracellular. Our results show an inhibition of DNA-raised antibody responses to HA, which correlates with the amount of maternal antibody at the time of immunization. However, the presence of maternal antibody did not affect the generation of antibody to NP or the generation of long-lived cellular immune responses to HA or NP.


## RESULTS

### *Maternal antibody inhibits IgG responses to HA but not NP.*

Mice born to influenza-immune mothers vaccinated on the day of birth were deficient in the generation of DNA-raised IgG to HA but not NP (Figure 10, Table 5). One-day old mice born to naïve mothers or influenza-immune mothers were immunized on the day of birth by i.m. or g.g inoculation with HA, NP or HA plus NP DNA. At the time of DNA immunization, the geometric mean titer (GMT) of anti-influenza maternal IgG in the neonates was 128.5 μg/ml (107.2, 150.2, 95% confidence limits). By 12 weeks of age, the titers had fallen to background levels. At 30 weeks post-prime, the neonates immunized in the presence of maternal antibody showed different patterns of IgG responses to HA and NP than those immunized in the absence of maternal antibody. In the maternal antibody positive groups, the highest antibody responses were seen for NP, the second highest for HA plus NP, whereas no responses were seen for HA (Figures 11, AB). By contrast, in the maternal antibody negative groups, the highest antibody responses were seen for HA plus NP, the second highest for HA, and the lowest for NP (Figure 10, AB).
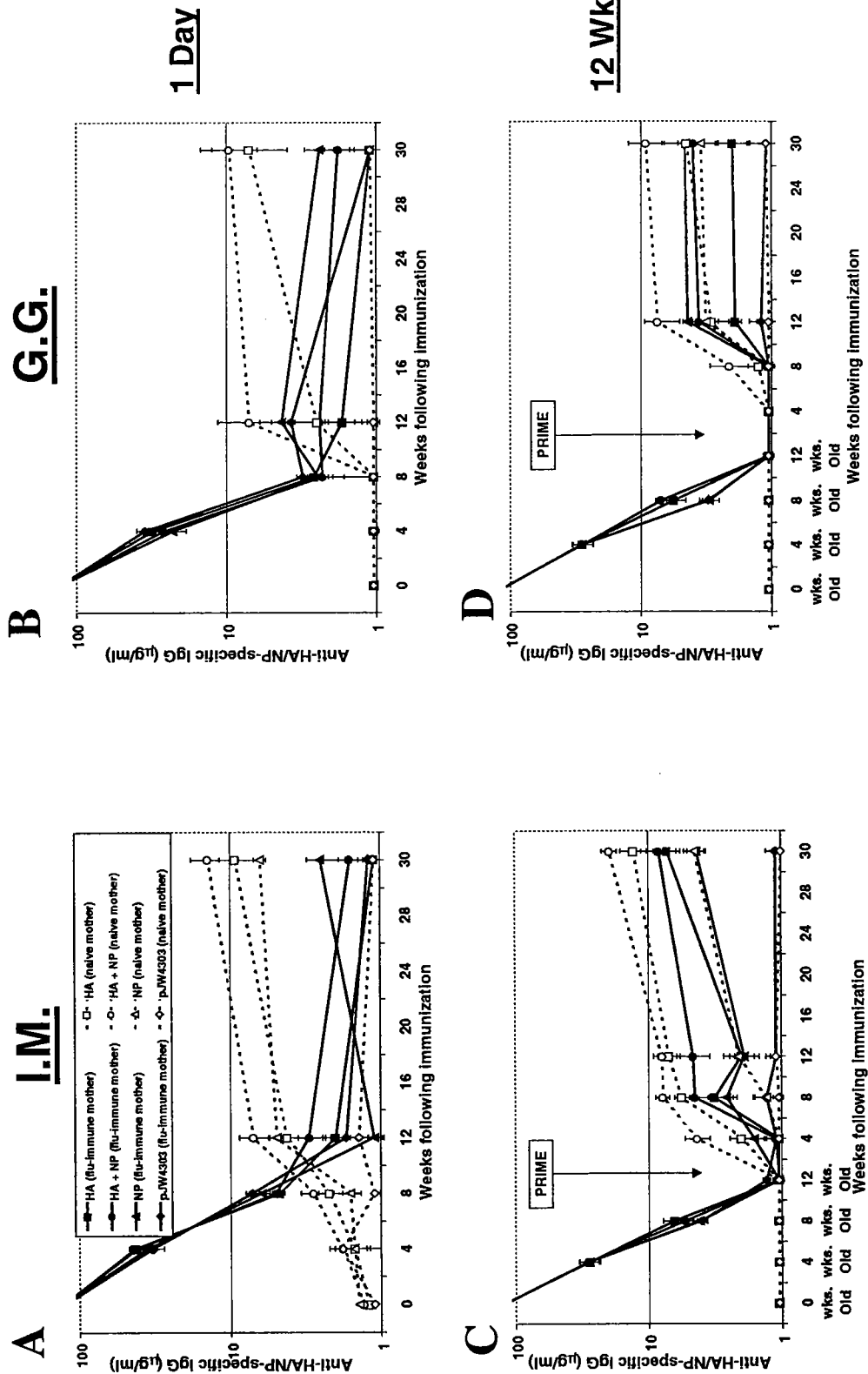
**Figure 10.** Effects of maternal antibody on DNA-raised IgG responses. Mice born to influenza-immune or naïve mothers were immunized on the day of birth by (A.) i.m. or (B.) g.g. at 12 weeks of age by (C.) i.m. or (D.) g.g. with HA-, NP- or HA plus NP-DNAs. Serum samples were tested over a course of 30 weeks for anti-HA/NP IgG levels by ELISA. Results are shown as GMT (95% confidence limits) IgG for groups of 7-14 mice.

**Table 5.** IgG responses following DNA immunization in the presence or absence of maternal antibody[a].

| Age at immunization | DNA | Method | IgG (μg/ml) Maternal Ab present[b] | Maternal Ab absent[c] | Ratio[d,e] |
|---|---|---|---|---|---|
| 1 day | HA | i.m. | 0.6 (0.2, 0.9) | 9.2 (6.8, 12.3) | **0.06** |
| | | g.g. | 0.4 (0.1, 0.7) | 7.1 (3.9, 12.4) | **0.06** |
| | NP | i.m. | 2.5 (1.9, 3.1) | 6.3 (5.7, 6.9) | **0.4** |
| | | g.g. | 2.4 (1.9, 3.0) | 1.1 (0.9, 1.4) | **2.2** |
| 12 weeks | HA | i.m. | 7.2 (5.7, 10.0) | 12.9 (10.2, 16.3) | **0.6** |
| | | g.g. | 2.0 (1.5, 2.6) | 4.5 (2.7, 6.9) | **0.4** |
| | NP | i.m. | 4.3 (3.6, 5.0) | 4.5 (3.8, 5.4) | **1.0** |
| | | g.g. | 4.6 (4.0, 5.2) | 3.5 (2.6, 4.5) | **1.3** |

[a] Mice were immunized and IgG levels were assayed as given in Figure 1. Results are shown as GMT of IgG for groups of 7-14 mice at 30 weeks post-prime.
[b] GMT of IgG of mice born to influenza-immune mothers (95% confidence limits).
[c] GMT of IgG of mice born to naïve mothers (95% confidence limits).
[d] Ratio = (IgG levels in DNA-immunized mice born to influenza-immune mothers) : (IgG levels in DNA-immunized mice born to naïve mothers).
[e] Differences between the effects of maternal antibody on HA- and NP-raised IgG responses was significant: i.m., $p<0.02$; g.g., $p<0.001$ (ANOVA replicated, two-way analysis of variance).

Direct comparison of the titers of the antibody responses for HA and NP in the groups immunized as neonates revealed that maternal antibody had limited antibody responses to HA but not NP (Table 5). In both g.g. and i.m. immunized neonates, the levels of anti-HA IgG had been reduced by 16-fold. By contrast, the maternal antibody had not significantly reduced antibody responses in the groups immunized with NP. Differences between the effects of maternal antibody on HA- and NP-raised IgG responses was significant (i.m., $p < 0.02$; g.g., $p < 0.001$; ANOVA replicated two-way analysis of variance).

Mice born to influenza-immune mothers and DNA-immunized as young adults, when maternal antibody had decreased to background levels, generated antibody to both HA and NP (Figure 10,CD). The ratios of antibody responses to HA and NP revealed that the blocking effect of maternal antibody on the raising of anti-HA IgG had been largely lost (Table 5).

Analysis of the kinetics of antibody responses in the various groups revealed that the method of DNA delivery, the age of the mouse at the time of DNA immunization, and the presence or absence of maternal antibody all affected the time course of the appearance of antibody (Figure 10). Naïve mice i.m. immunized on the day of birth developed IgG responses within 8 weeks, whereas g.g.-immunized mice did not generate detectable responses until 12 weeks of age (Figure 10, AB). Mice immunized as neonates required approximately 4 weeks longer to generate IgG than mice immunized as

young adults. Neonates developed antibody by 8 weeks following i.m. delivery of DNA; whereas young adults developed antibody by 4 weeks. Following gene gun immunizations, neonates developed antibody by 12 weeks; whereas young adults generated antibody within 8 weeks. The presence of residual maternal antibody in the young adults also slowed the appearance of antibody by about 4 weeks (Figure 10, CD).

### *Optimization of methods to characterize CD8+ T cell responses.*

We next wished to determine if anti-influenza maternal antibody was capable of preventing DNA-raised T cell responses; however, it was first necessary to optimize the method for detecting these responses. The popular method of determining the magnitude of an antigen-specific CTL response by $^{51}$Cr release is qualitative. Until recently, the only way to determine the frequency of CTL was by limiting dilution assay (LDA). However, this method has the potential to underestimate the number of antigen-specific T cells because it does not detect non-proliferating cells (Altman *et al.*, 1996; Murali-Krishna *et al.*, 1998). Although LDA has been sufficient for quantitating CTL in acute viral infections, it is likely not the best method for determining the expected lower frequencies of CTL following DNA-based immunizations. Recently, a novel method of detecting antigen-specific T cell response has been developed cells (Altman *et al.*, 1996; Murali-Krishna *et al.*, 1998). This method uses soluble tetrameric MHC class I-peptide complexes to bind specifically and stably to the appropriate MHC class I-peptide-specific CD8+ T cells. This technique allows for direct visualization of antigen-specific T cells and requires no in vitro restimulation.

In a preliminary experiment, mice that had been either i.m. or g.g. coimmunized with HA plus NP DNAs were sublethally infected ~35 weeks post-immunization to activate memory T cells. Seven days later, splenocytes were prepared and cultured for 10-12 hours with $K^d$-restricted HA or NP peptides, or in media without peptides. The final 5-6 hours of culture included Monensin (Pharmingen, San Diego, CA), which inhibits protein transport and allows for the accumulation of intracellular cytokines. The cells were surface stained for CD8 and 1 of 2 types of tetrameric complexes, $H\text{-}2K^d\text{-}HA_{533\text{-}541}$ (HA-tet) or $H\text{-}2K^d\text{-}NP_{147\text{-}155}$ (NP-tet). The cells were then permeabilized, stained for intracellular IFNγ and analyzed by flow cytometry.

Mice DNA-immunized with HA plus NP develop antigen-specific CD8+ T cell responses (Figures 11 and 12). As shown in Figure 11, mice immunized by i.m. or g.g. inoculation had 2.09% and 0.83% of CD8+ cells that were HA-tet$^+$, respectively. The HA-tet$^+$ cells were IFNγ$^-$ following stimulation with media only. However, following HA peptide stimulation, there was an expected TCR down-regulation and a shift to HA-tet$^-$, IFNγ$^+$ phenotype in both i.m. and g.g.-immunized mice. Similar, yet more pronounced, results were seen using NP tetramers (Figure 12). Mice immunized by i.m. or g.g. inoculation had NP-tet$^+$ frequencies of 4.26% and 1.67%, respectively. As seen with HA, NP peptide stimulation caused a massive TCR down-regulation and a shift to NP-tet$^-$, IFNγ$^+$ phenotype. In both cases, the frequencies of IFNγ$^+$ CD8+ cells following peptide stimulation corresponded with the frequencies of tet$^+$ cells prior to peptide

I.M. HA+NP /
CHALLENGE

G.G. HA+NP /
CHALLENGE

NAIVE

K<sup>d</sup>/HA533-541 tetramer

Media

IFNγ

HA peptide

IFNγ

Media

IL-4

HA peptide

IL-4
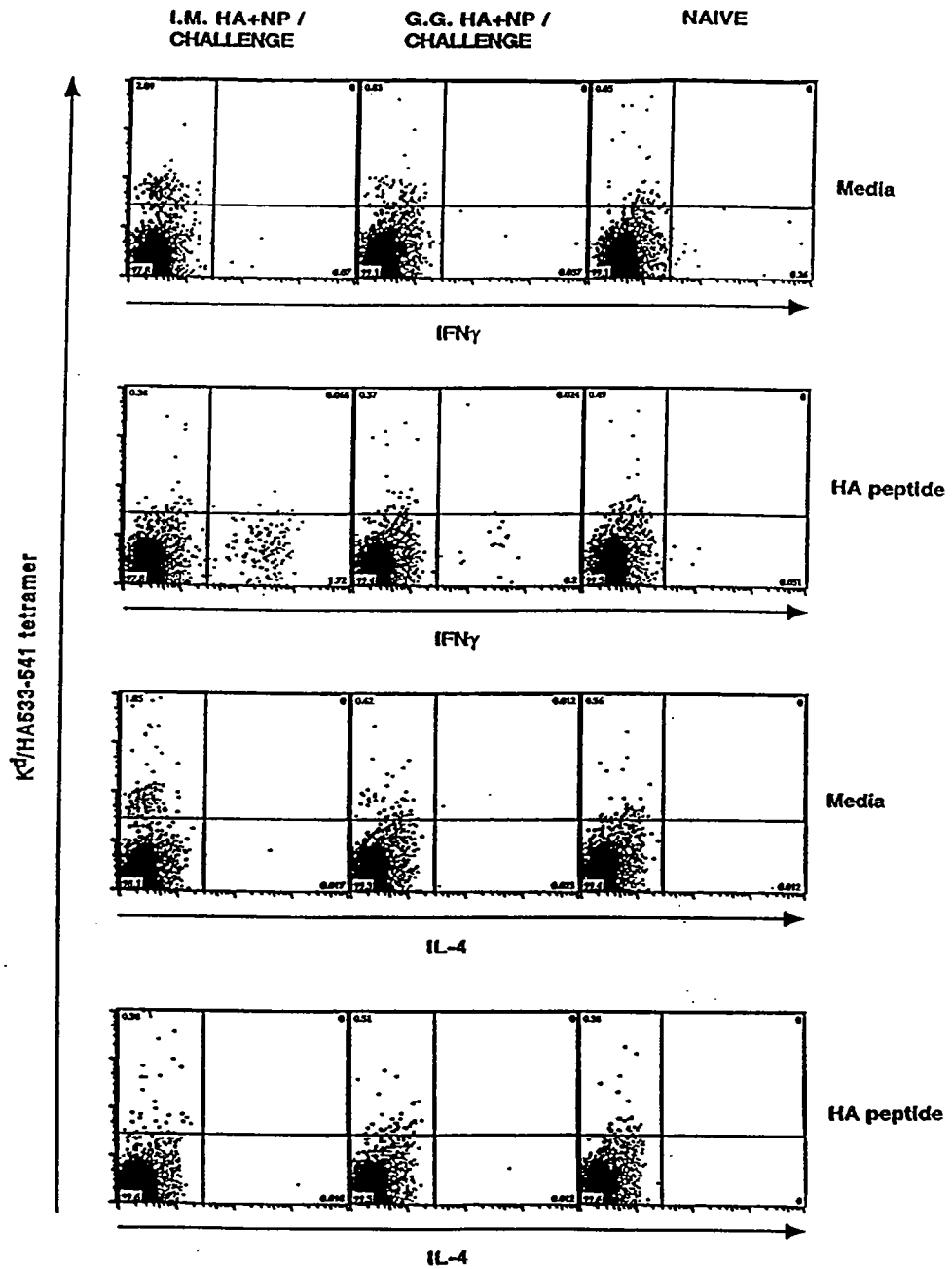
Figure 11. HA tetramers to quantitate HA-specific CD8+ T cell responses following HA plus NP DNA codelivery. Mice were i.m. or g.g. coimmunized with HA plus NP and sublethally infected with influenza virus ~35 weeks later to activate memory cells. Seven days later, splenocytes were cultured for 10-12 hours with media or a $K^d$-restricted HA peptide. The cells were surface stained with CD8-PE and APC-labeled HA tetramers. The cells were permeabilized, stained for intracellular IFNγ or IL-4 and analyzed by flow cytometry. One hundred thousand total cells were counted and gates were placed on CD8+ cell population. Results are shown as dot plots with numbers in quadrants representing the respective percent of the gated CD8+ population.
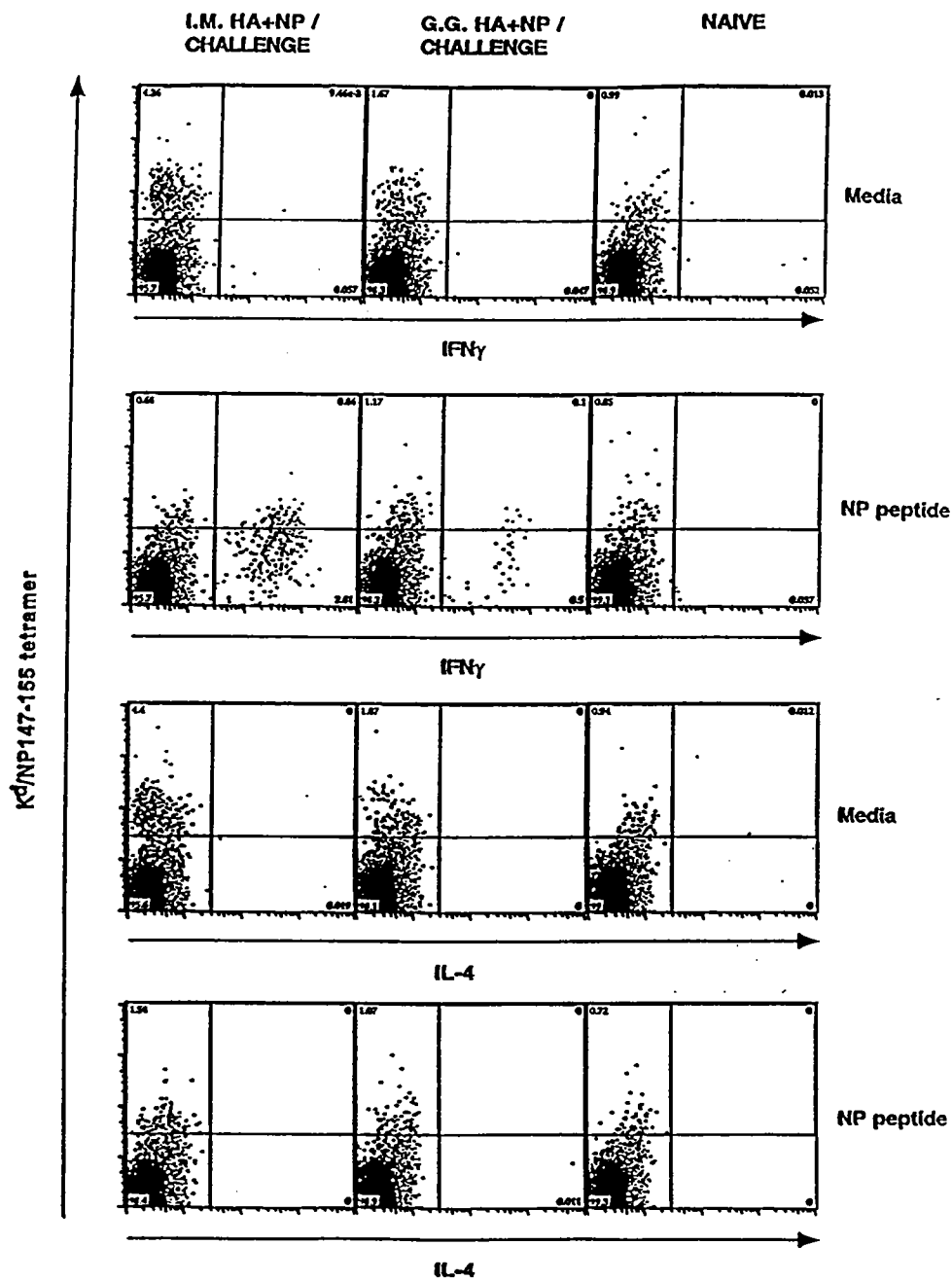
**Figure 12.** NP tetramers to quantitate NP-specific CD8+ T cell responses following HA plus NP DNA codelivery. Aliquots of splenocytes from mice described in Figure 11 were cultured for 10-12 hours with media or a $K^d$-restricted NP peptide. The cells were surface stained with CD8-PE and APC-labeled NP tetramers. The cells were permeabilized, stained for intracellular IFNγ or IL-4 and analyzed by flow cytometry. One hundred thousand total cells were counted and gates were placed on CD8+ cell population. Results are shown as dot plots with numbers in quadrants representing the respective percent of the gated CD8+ population.

**Table 6.** Tetramer positive cells correlates with IFNγ positive cells following peptide stimulation[a].

| | | HA | | | NP | | |
|---|---|---|---|---|---|---|---|
| | | FACS (%)[d,e] | | % | FACS (%) | | % |
| Method | Stimulation | HA-tet+[b] | IFNγ+ | Specific lysis[f] | NP-tet+[c] | IFNγ+ | Specific lysis |
| i.m. | Media | 1.5 | 0 | | 3.3 | 0 | |
| | | | | 52 % | | | 34 % |
| | Peptide | 0 | 1.7 | | 0 | 3.7 | |
| g.g. | Media | 0.2 | 0 | | 0.7 | 0 | |
| | | | | 11 % | | | 44 % |
| | Peptide | 0 | 0.2 | | 0.2 | 0.7 | |

[a]Mice were coimmunized with HA plus NP and sublethally challenged ~35 weeks later. Splenocytes were prepared and stimulated as given in Figures 11 (HA) and 12 (NP).
[b]HA-tet+, HA-tetramer positive.
[c]NP-tet+, NP-tetramer positive.
[d]Data are shown as (% Tet+ in immunized mice) minus (average % Tet+ of naïve mice) both before and after stimulation with appropriate peptide.
[e]Data are shown as (% IFNγ+ in immunized mice) minus (average % IFNγ+ in naïve mice) both before and after stimulation with the appropriate peptide.
[f] Data are for the 12.5:1 effector:target ratios of $^{51}$Cr-release assay shown in Figure 13. Data are (% specific lysis of immunized mice) minus (% specific lysis of naïve control).

stimulation (Table 6). There was only minimal background staining in naïve animals and none of the groups had a Tc2 phenotype (Mosmann *et al.*, 1995; Mosmann *et al.*, 1997) as determined by IL-4 intracellular staining (Figures 11 and 12).

Frequencies of tetramer positive cells correlated with killing activity (Figure 13). Aliquots of cells used in Figures 11 and 12, were further analyzed by chromium-51 ($^{51}$Cr) release for antigen-specific killing activity. Target p815 cells were pulsed with the $K^d$-restricted peptides $HA_{533-541}$ or $NP_{147-155}$. Gene gun immunized mice had HA CTL responses slightly above background, yet very high NP CTL responses, which correlates with the lower frequency of HA-tet$^+$ cells and high frequency of NP-tet$^+$ cells shown in Figures 11 and 12. Similarly, i.m. inoculated mice had good killing of both HA and NP targets. Taken together, these data suggest that tetramer staining is a good measure of antigen-specific T cells, and that the frequencies of tet$^+$ T cells (or IFN$\gamma^+$) T cells correlate with antigen-specific killing activity.

***Maternal antibody does not block the generation of long-lived CD8+ T cell responses.***

We next determined if anti-influenza antibody was capable of preventing DNA-raised T cell responses. Mice born to naïve or influenza-immune mothers were DNA-immunized as described in Figure 10 by i.m. or g.g. with HA- and/or NP-expressing DNA. One year later, mice were inoculated with a sublethal dose of influenza A/PR/8/34 virus to activate memory T cells. Mice were sacrificed 7 days later, and spleens from each group (n=2-3) were pooled. Splenocytes were cultured for 10-12 hours with $K^d$-
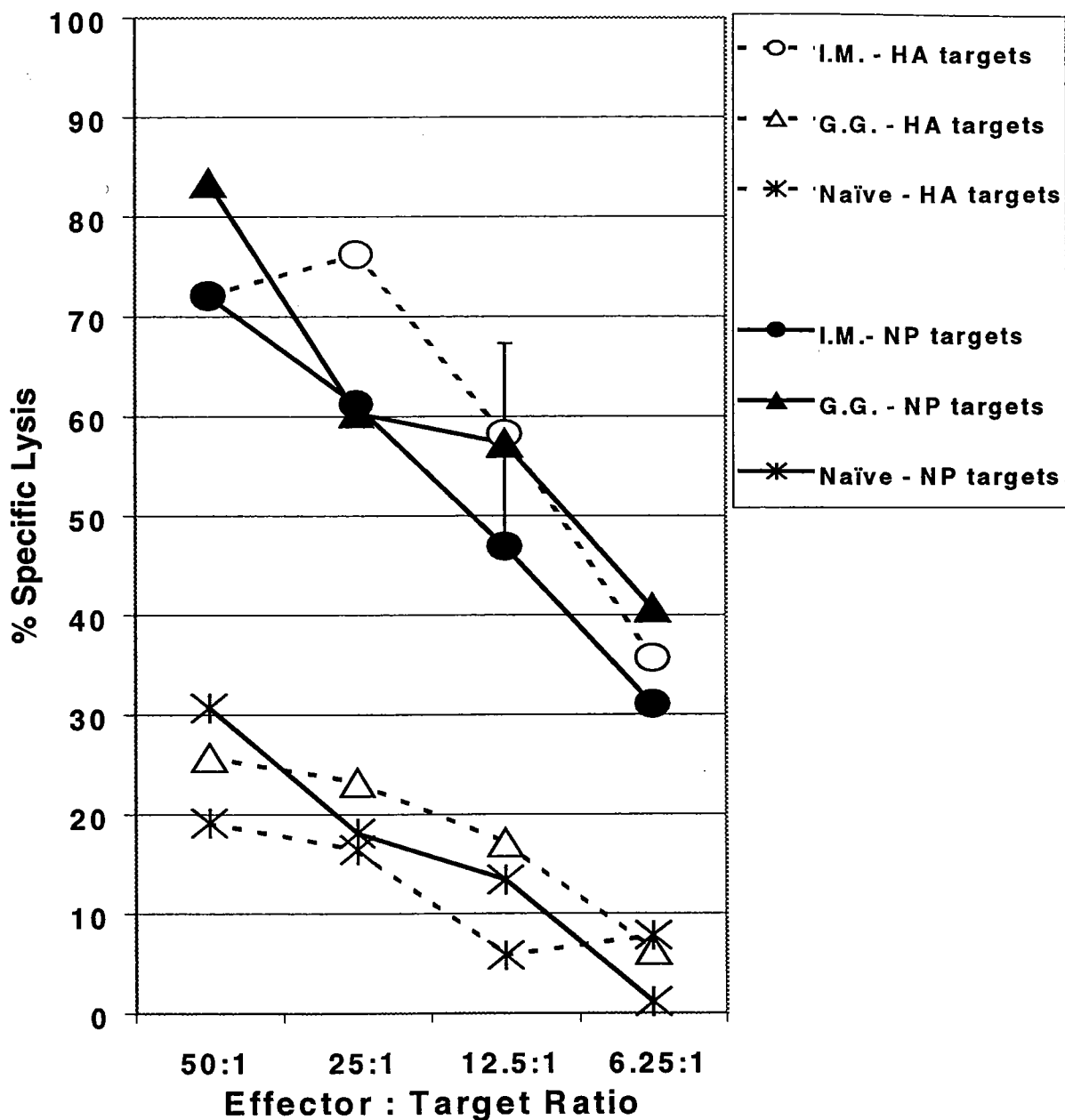
**Figure 13.** HA- and NP-specific killing following HA plus NP DNA coimmunization. Aliquots of splenocytes from mice described in Figures 11 and 12 were further analyzed in a chromium release assay. The splenocytes from these mice were cultured with the $K^d$-restricted HA or NP peptide-pulsed stimulator splenocytes from syngeneic mice. Five days later, the effectors were cultured for 4 hours with $^{51}$Cr-labeled, HA- or NP-peptide pulsed p815 target cells. Culture supernatants were measured for radioactivity in a scintillation counter. Results are shown as percent specific lysis +/- SD at various effector to target rations (see Materials and Methods).

restricted HA or NP peptides. The final 5-6 hours of culture included Monensin

(Pharmingen, San Diego, CA), which inhibits protein transport and allows for the

accumulation of intracellular cytokines. Because it was shown previously (Figures 11-

12) that tetramer positive CD8+ T cells prior to peptide stimulation corresponded with

IFNγ+ following peptide stimulation, we decided to rely on IFNγ staining only, as both

HA and NP tetramers were unstable. Thus, the peptide stimulated cells were instead

surface stained for CD8β and CD69 (an early activation antigen), permeabilized, stained

for intracellular IFNγ and analyzed by flow cytometry. Results are from a single

experiment.

Maternal antibody did not block the generation of long-term CD8+ T cell

responses to DNA expressed HA or NP (Figures 14 and 15, Table 7). Immunizations

with HA, NP, or HA plus NP DNA in the presence or absence of maternal antibody

generated overall comparable frequencies of antigen-specific CD8+ T cells. Variation

was seen between groups in the frequencies of responding T cells. However, this

variation had no consistent pattern between groups born to naïve or influenza-immune

mothers. Data were also similar between mice immunized as neonates or as young

adults. Analysis of the geometric means of the frequencies of responding T cells (Table

7) revealed higher frequencies in the g.g.-immunized groups ($p<0.01$). Overall, the

frequencies of responding cells for HA and NP were not affected by HA- plus NP DNA

codelivery (HA, $p=0.6$; NP, $p=0.5$).

# Intramuscular DNA Immunization



**Figure 14.** Effects of maternal antibody on i.m. DNA-raised CD8+ T cell responses, as determined by intracellular cytokine staining. Mice born to naïve or influenza-immune mothers were immunized on the day of birth or in adulthood by (top) i.m. inoculation with HA and/or NP DNA or (bottom) pJW4303 control DNA. One year later, mice were inoculated with a sublethal dose of influenza virus. Mice were sacrificed 7 days later, and spleens from each group (n=2-3) wer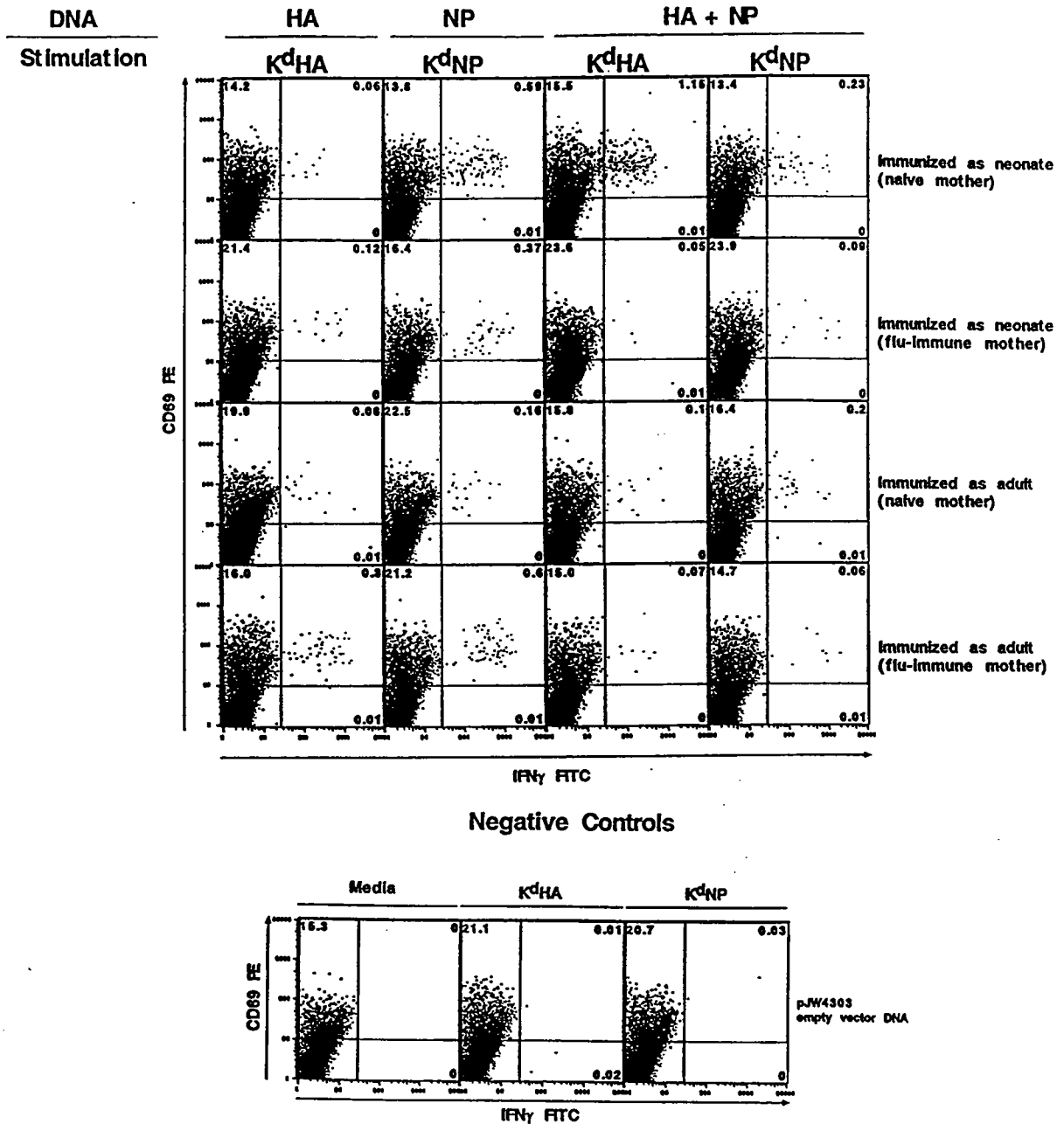e pooled. Splenocytes were cultured for 10-12 hours with a $K^d$-restricted HA or NP peptide as appropriate. The final 5-6 hours of culture included Monensin, which inhibits protein transport by golgi and allows for the accumulation of intracellular cytokines. The cells were surface stained for CD8 and CD69 (early activation antigen) then permeabilized and stained for intracellular IFNγ and analyzed by flow cytometry. Data are shown as dot plots with numbers in quadrants representing the respective percent of the CD8+ cell population.

# Gene Gun DNA Immunization
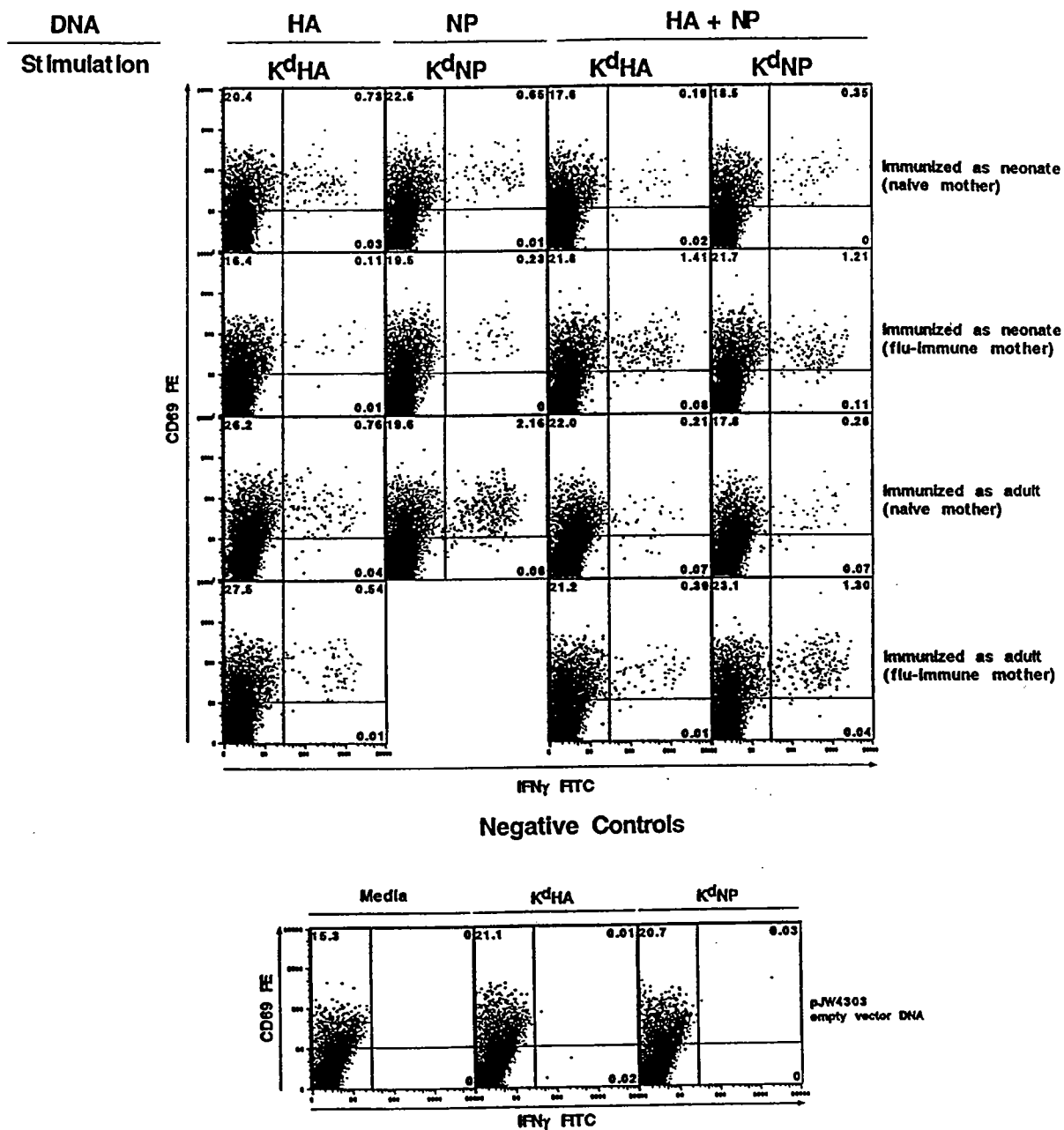


Figure 15. Effects of maternal antibody on g.g. DNA-raised CD8+ T cell responses, as determined by intracellular cytokine staining. Mice born to naïve or influenza-immune mothers were immunized on the day of birth or in adulthood by (top) g.g. inoculation with HA and/or NP DNA or (bottom) pJW4303 control DNA. One year later, mice were inoculated with a sublethal dose of influenza virus. Mice were sacrificed 7 days later, and spleens from each group (n=2-3) were pooled. Splenocytes were cultured for 10-12 hours with a $K^d$-restricted HA or NP peptide as appropriate. The final 5-6 hours of culture included Monensin, which inhibits protein transport by golgi and allows for the accumulation of intracellular cytokines. The cells were surface stained for CD8 and CD69 (early activation antigen) then permeabilized and stained for intracellular IFNγ and analyzed by flow cytometry. Data are shown as dot plots with numbers in quadrants representing the respective percent of the CD8+ cell population.

The production of IFNγ in culture supernatants correlated with intracellular cytokine FACS data (Figure 16, Table 7). The same groups of pooled splenocytes from DNA-immunized mice described in Figures 14 and 15 were cultured for 2 days with the $K^d$-restricted HA or NP peptides. Supernatants from duplicate wells were pooled and assessed for IFNγ by ELISA. IFNγ production was easily scored for all groups. Analysis of the ratios of IFNγ production per IFNγ-producing CD8+ cell revealed that g.g.-raised cells produced more IFNγ than i.m.-raised CD8+ cells ($p<0.001$; Table 7). This phenomenon was particularly marked for T cell responses to NP ($p<0.02$).

*Maternal antibody does not inhibit the generation of T helper responses.*

The presence of maternal antibody at the time of DNA immunization also did not affect the elicitation of long-term T helper responses (Figure 17). For CD4+ T cell assays, aliquots of the same splenocytes used for CD8+ cell assays were cultured for 2 days with an inactivated influenza virus. Supernatants were harvested, pooled and assayed for IFNγ and IL-5 by ELISA. Results are from a single experiment. As seen with CD8+ T cell responses, g.g. immunized mice had overall higher IFNγ production than the i.m. immunized mice ($p,0.0001$). For example, following g.g. immunization IFNγ responses ranged from 12,270 pg/ml to 50,645 pg/ml, whereas following i.m. immunization IFNγ responses ranged from 1265 pg/ml to 13,050 pg/ml. The same phenomenon was seen for IL-5 production ($p<0.0001$). IL-5 levels ranged between 880 pg/ml and 3960 pg/ml in g.g. DNA-immunized, and between 215 pg/ml and 835 pg/ml in i.m. immunized mice.

**Table 7.** CD8+ T cell responses following DNA immunization in the presence or absence of maternal antibody[a].

| Method | DNA | Stimulation | %CD8+CD69+IFNγ+[b] | | | | | IFNγ (pg/ml)[c] | | | | | Ratio[d] (x10³) |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | | 1 day[e] | | 12 weeks | | Geo Mean[f] | 1 day | | 12 weeks | | Geo Mean | |
| | | | Naïve | Flu | Naïve | Flu | | Naïve | Flu | Naïve | Flu | | |
| i.m. | HA | K^dHA | 0.06 | 0.12 | 0.08 | 0.30 | 0.11 (0.08, 0.16) | 3470 | 6775 | 808 | 9570 | 3668 (2125, 6344) | 33 |
| | NP | K^dNP | 0.59 | 0.37 | 0.16 | 0.60 | 0.38 (0.28, 0.52) | 24950 | 17621 | 85 | 15095 | 4973 (1258, 18869) | 13 |
| | HA+NP | K^dHA | 1.15 | 0.05 | 0.10 | 0.07 | 0.14 (0.07, 0.29) | 83760 | 310 | 7030 | 3170 | 4905 (1545, 15561) | 35 |
| | | K^dNP | 0.23 | 0.09 | 0.20 | 0.06 | 0.16 (0.13, 0.21) | 10900 | 100 | 4275 | 3730 | 2042 (727, 5738) | 13 |
| g.g. | HA | K^dHA | 0.73 | 0.11 | 0.76 | 0.54 | 0.43 (0.27, 0.68) | 40345 | 13090 | 31970 | 28960 | 26443 (20708, 33767) | 61 |
| | NP | K^dNP | 0.65 | 0.23 | 2.16 | ND | 0.69 (0.36, 1.31) | 26310 | 30700 | 219530 | 165765 | 73631 (42333, 128068) | 107 |
| | HA+NP | K^dHA | 0.19 | 1.41 | 0.21 | 0.39 | 0.38 (0.24, 0.61) | 10910 | 91805 | 45655 | 24700 | 32600 (20728, 51271) | 86 |
| | | K^dNP | 0.35 | 1.21 | 0.28 | 1.30 | 0.63 (0.42, 0.94) | 14940 | 155080 | 157275 | 151235 | 86160 (48043, 154518) | 137 |

[a] Mice were immunized and CD8+ T cell responses were analyzed by FACS or ELISA as given in Figures 14-16.
[b] Percentage of CD8+ T cells that were CD69+IFNγ+ following peptide stimulation.
[c] Mean IFNγ in cell culture supernatants following peptide stimulation (see also Figure 16).
[d] Ratio of (geometric meanIFNγ) / (geometric mean %CD8+CD69+IFNγ+).
[e] Age at time of immunization (1 day or 12 weeks) and immune status of mother (naïve or flu-immune).
[f] Geometric mean (95% confidence limits).

**A**



**B**



**C**



HA + NP
DNA

HA
DNA

NP
DNA
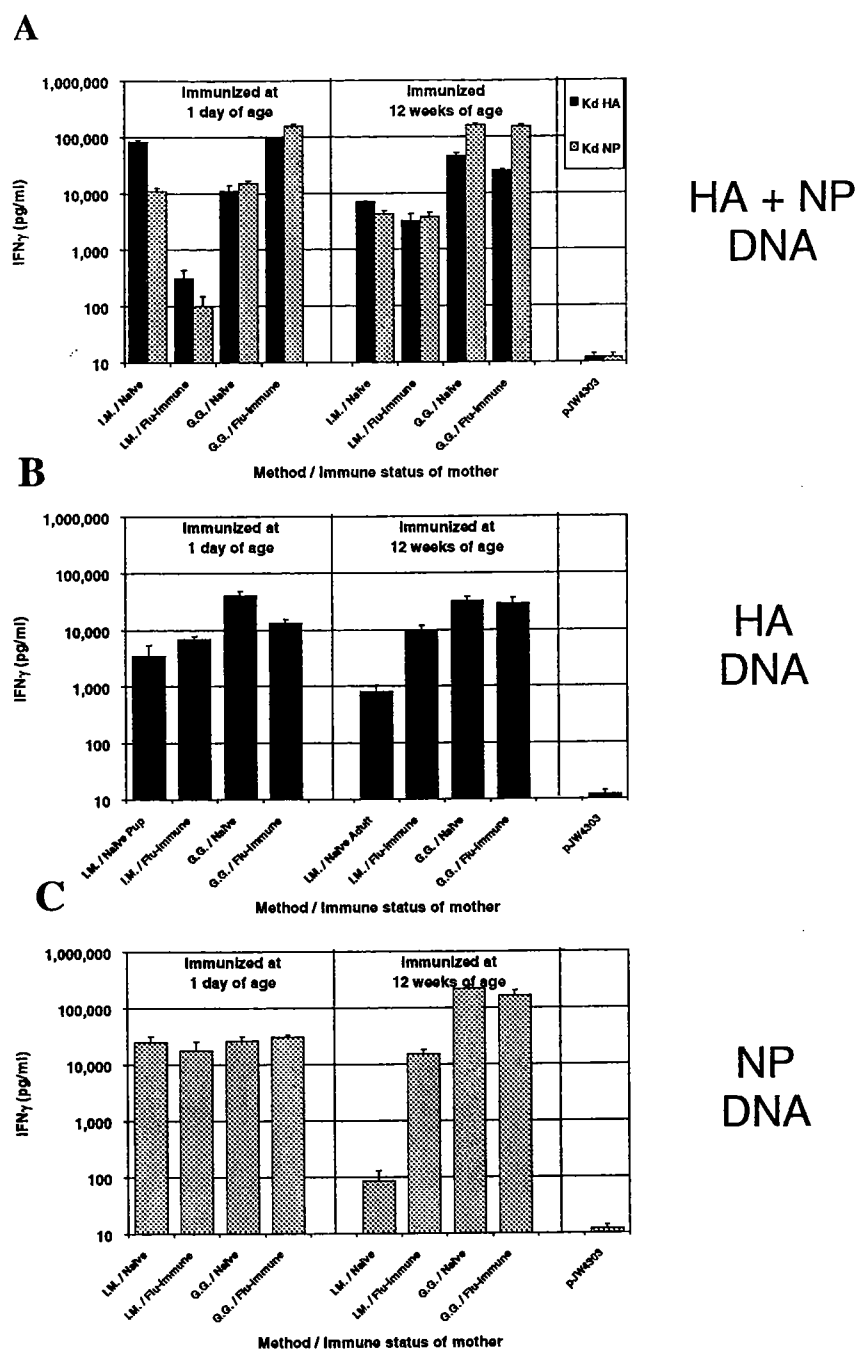
**Figure 16.** Effects of maternal antibody on the generation of DNA-raised CD8+ T cell responses, as determined by IFNγ production. Splenocytes from the same mice used in Figures 14-15 were cultured for 2 days with the appropriate $K^d$-restricted HA or NP peptide. Bulk culture supernatants were harvested, pooled and assayed for IFNγ production by ELISA. Results are shown as mean +/- SD levels of IFNγ of pooled supernatants from mice immunized with (A.) HA plus NP DNA, (B.) HA DNA or (C.) NP DNA.
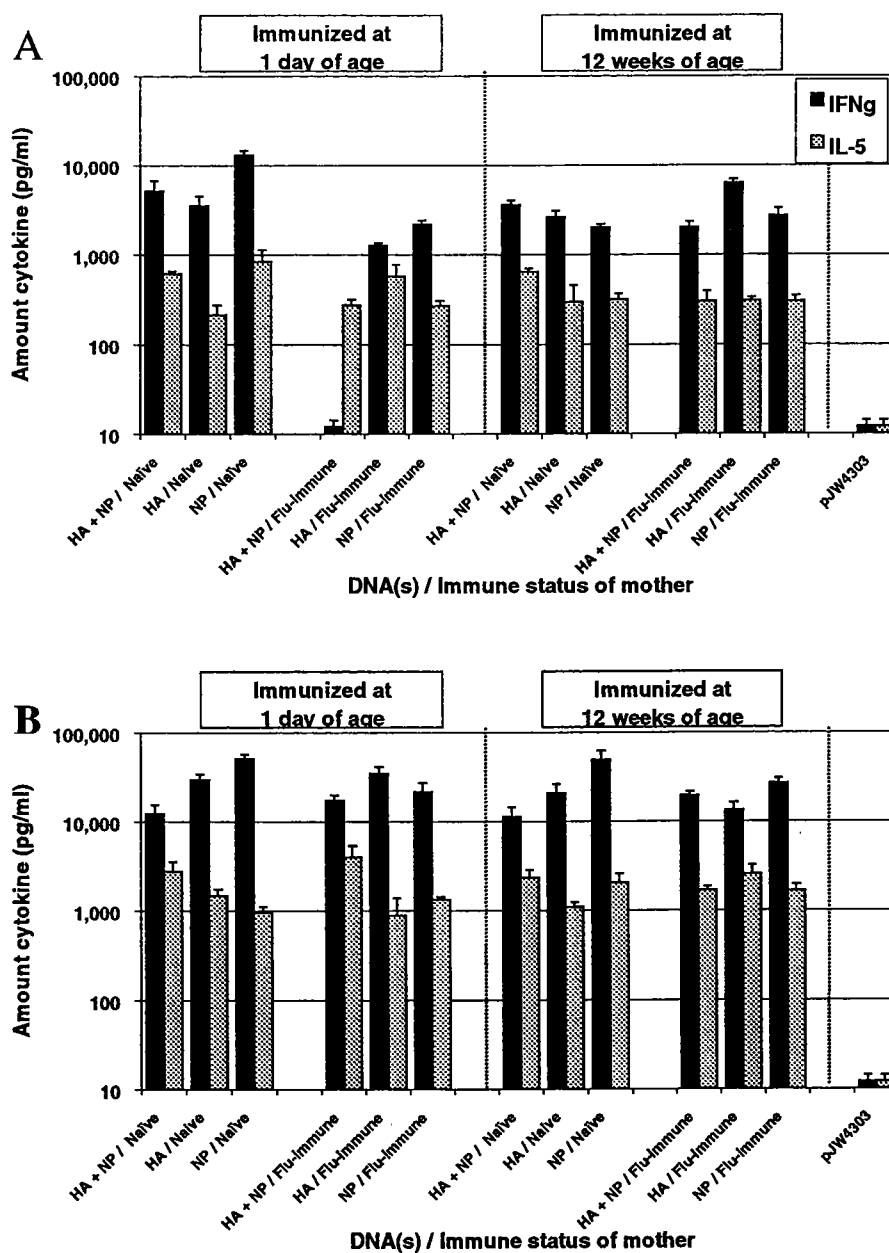
**Figure 17.** Effects of maternal antibody on the generation of DNA-raised CD4+ T cell responses. Splenocytes from the same mice used in Figure 14-15 were cultured for 2 days with inactivated influenza virus. Bulk culture supernatants were harvested and pooled and assayed for IFNγ and IL-5 production by ELISA. Results are shown as mean +/- SD IFNγ and IL-5 levels in supernatants from mice (A.) i.m. or (B.) g.g. immunized with HA and/or NP DNA.

*Maternal antibody inhibits protection from influenza virus challenge.*

Maternal antibody inhibited the protective efficacy of DNA immunizations, with protection correlating with HA-specific IgG (Figure 18, Table 8). All mice DNA immunized as neonates in the presence of maternal antibody succumbed to intranasalinfluenza virus challenge as did mice immunized with the control DNA. In contrast, mice born to naïve mothers and immunized with HA or HA plus NP DNA were protected, with codelivery of HA and NP DNAs providing slightly better protection than HA DNA alone. NP DNA failed to protect any mice, regardless of the presence of NP-specific IgG, CD4+ and CD8+ T cell responses.

Young adult DNA-immunized mice born to influenza-immune mothers also were afforded less protection from influenza virus challenge than mice born to naïve mothers (Figure 18, Table 8). For instance, adult mice coimmunized with HA plus NP DNAs in the presence of residual maternal antibody had less than 30% survival; whereas adult mice immunized in the absence of maternal antibody had an 80% survival rate. The levels of protection directly correlated with the levels of anti-HA antibody (Figure 18).

## DISCUSSION

We have previously shown in Chapter 1 that immunization of neonatal mice with influenza HA DNA generates strong, long lasting, protective IgG responses (Pertmer and Robinson, 1999). In this chapter, we extend these studies to evaluate the effects of maternal antibody on the efficacy of neonatal DNA immunizations. Our results show that maternal antibody inhibits the magnitude of IgG responses to HA but not NP (Figure
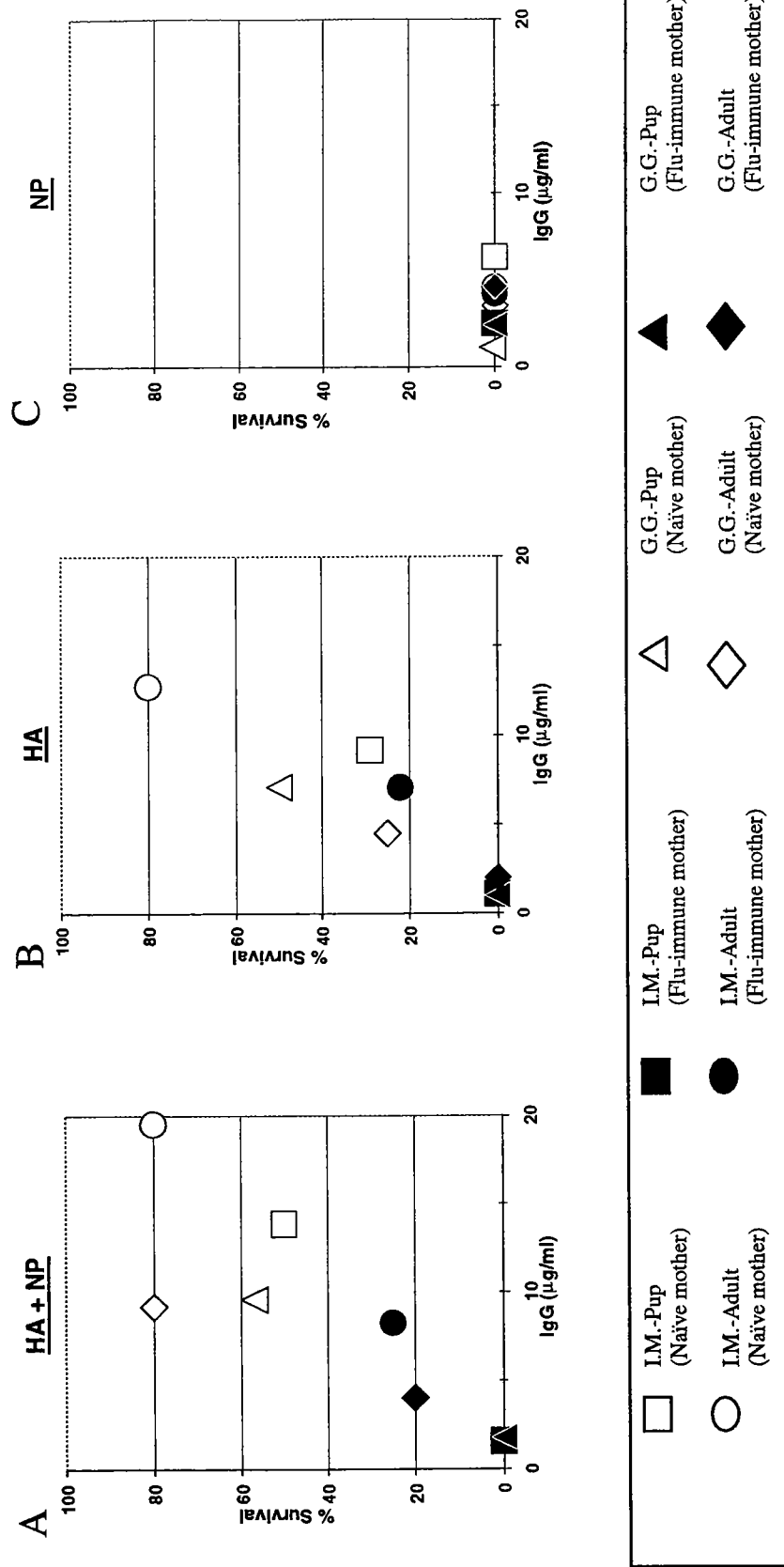
**Figure 18.** Protection correlates with antibody to HA. Mice born to naïve or influenza-immune mothers were immunized on the day of birth or in adulthood by i.m. or g.g. with (A.) HA plus NP, (B.) HA, or (C.) NP DNA. Thirty weeks later, groups of 4-8 mice were challenged i.n. with a lethal dose of influenza A/PR/8/34 and weighed daily. Mice were sacrificed following >20% loss of pre-challenge body weight. Results are shown as the percent survival at 7 days post-challenge versus the pre-challenge GMT of IgG.

**Table 8.** Effect of maternal antibody on protection from influenza challenge.

| Age at time of immunization | Immune status of mother | DNA | # of survivors / total tested | | Protection (%) |
| --- | --- | --- | --- | --- | --- |
| | | | i.m. | g.g. | |
| 1 day | Flu-immune | HA+NP | 0 / 7 | 0 / 4 | 0 |
| | | HA | 0 / 8 | 0 / 4 | 0 |
| | | NP | 0 / 7 | 0 / 4 | 0 |
| | Naive | HA+NP | 3 / 6 | 4 / 7 | 54 |
| | | HA | 2 / 7 | 2 / 4 | 36 |
| | | NP | 0 / 6 | 0 / 4 | 0 |
| 12 weeks | Flu-immune | HA+NP | 1 / 4 | 1 / 5 | 22 |
| | | HA | 1 / 4 | 0 / 4 | 13 |
| | | NP | 0 / 4 | 0 / 4 | 0 |
| | Naive | HA+NP | 4 / 5 | 4 / 5 | 80 |
| | | HA | 4 / 5 | 1 / 4 | 56 |
| | | NP | 0 / 4 | 0 / 5 | 0 |

10, Table 5). They also show that maternal antibody does not interfere with the raising of CD4+ or CD8+ T cell responses (Figures 14-17, Table 7). Finally, we show that protection against a lethal challenge correlates with the presence of antibody to HA (Table 8, Figure 18). Cellular immune responses in the absence of antibody to HA failed to protect against influenza challenge (Table 8, Figure 18).

### *Maternal antibody inhibits IgG responses to HA but not NP*

Interestingly, the presence of maternal antibody at the time of DNA immunization inhibited the generation of antibody responses to HA but not NP (Figure 10, Table 5). DNA-raised IgG responses to HA were reduced by at least 17-fold, whereas antibody responses to NP were not significantly affected by the presence of maternal antibody (Table 5). This was true both for g.g. and i.m. deliveries of DNA. When expressed in a DNA vaccine, the influenza HA localizes to the plasma membrane, while the influenza NP is intracellular. This suggests that maternal antibody inhibits the generation of IgG responses to plasma membrane but not intracellular proteins.

The ability of maternal antibody to inhibit DNA-raised IgG responses to plasma membrane, but not intracellular proteins, is consistent with some, but not all, prior literature (Table 9). The one other study of a DNA-expressed intracellular protein, LCMV NP, is consistent with our findings with influenza NP (Hassett, Zhang, and Whitton, 1997; Table 5). Thus, in general, intracellular proteins may be protected against the blocking activity of maternal antibody. Five other studies have been done on the

**Table 9.** Studies of neonatal DNA immunization in the presence of maternal antibody.

| Type of protein | Gene | Virus | Model | Ab | CTL | Th | Reference |
|---|---|---|---|---|---|---|---|
| Internal | NP | LCMV | Mouse | + | + | ND[a] | (Hassett, Zhang, and Whitton, 1997) |
| | NP | Influenza | Mouse | + | + | + | This study |
| Plasma membrane | HA | Measles | Mouse | - | + | + | (Siegrist et al., 1998a) |
| | HA | Influenza | Mouse | - | + | + | This study |
| | gD | Pseudorabies | Pig | - | ND | ND | (Le Potier et al., 1997; Monteil et al., 1997) |
| | gp | Rabies | Mouse | + | ND | ND | (Wang et al., 1998) |
| | gD | Bovine Herpes | Mouse, Sheep | + | ND | + | (Lewis et al., 1997; Van Drunen Littel-van den Hurk et al., 1999) |
| | gB | Herpes | Mouse | + | ND | + | (Manickan, Yu, and Rouse, 1997) |
| Secreted | C fragment | Tetanus toxin | Mouse | - | + | + | (Siegrist et al., 1998a) |

[a] ND, not done.

ability of maternal antibody to block humoral responses to DNA-expressed plasma membrane proteins (Table 9). These varied in the ability to raise IgG in the presence of maternal antibody. Similar to our findings with influenza HA, maternal antibody blocked DNA-raised IgG responses to measles HA and pseudorabies gD. In contrast, maternal antibody failed to block DNA raised IgG responses to herpes simplex gB, bovine herpes gD or rabies glycoprotein (Lewis *et al.*, 1997; Manickan, Yu, and Rouse, 1997; Van Drunen Littel-van den Hurk *et al.*, 1999; Wang *et al.*, 1998; Table 9). The differences in the ability of plasma membrane proteins to raise antibody may be due to differences in the levels of maternal antibody in the different model systems. This is suggested in the rabies studies where the level of passive antibody affected the extent of blocking (Wang *et al.*, 1998). A second, more interesting possibility would be that plasma membrane proteins differ in how antibody affects their interactions with the immune system. If this is so, plasma membrane proteins that are sensitive to blocking by maternal antibody may be able to be engineered for resistance.

In contrast to antibody responses, all DNA-expressed proteins have raised cell-mediated immunity in the presence of maternal antibody (Figures 14-17, Table 7, Table 9). This ability to raise cellular immunity is independent of the ability to raise antibody (Table 9). These findings imply that maternal antibody does not block processing and presentation of DNA-expressed antigens.

*Protection correlates with antibody titers to HA*

In agreement with prior studies HA DNA immunization afforded protection whereas NP DNA immunization failed to protect against influenza challenge (Figure 18, Robinson *et al.*, 1997a). The ability of HA to protect correlated with the level of anti-HA antibody (Figure 18). Anti-HA antibody differs from anti-NP antibody or cell-mediated responses to HA or NP in that it can block virus entry. Thus antibody to HA can limit the incoming infection. By contrast, CTL to HA and NP are unable to block infection, but can play a role in the recovery from influenza virus infection. Other groups have shown protection using NP DNA immunizations (Fu *et al.*, 1999; Ulmer *et al.*, 1993). These studies differed from ours in using multiple booster immunizations and challenging with a relatively low dose of the more attenuated A/HK/68 (H3N2) influenza virus (Ulmer *et al.*, 1993; Montgomery *et al.*, 1993). In agreement with prior studies, coimmunization with HA plus NP DNAs had a synergistic effect in enhancing protective immunity (Table 8, Figure 18, (Bot, Bot, and Bona, 1998; Donnelly *et al.*, 1995).

## CONCLUSION

We tested the ability of intramuscular and gene gun immunization with DNA expressing influenza HA and NP to raise protective humoral and cellular responses in the presence and absence of maternal antibody. Neonatal mice born to influenza-immune mothers raised full antibody responses to NP but failed to generate antibody responses to HA. In contrast, the presence of maternal antibody did not affect the generation of long-lived CD4+ and CD8+ T cell responses to both HA and NP. Thus, maternal antibody did

not affect cell-mediated responses, but rather it limited humoral responses, with the

ability to limit the antibody response correlating with whether the DNA-expressed

immunogen was localized in the plasma membrane or within the cell.

# SUMMARY,

# OVERALL DISCUSSION

# AND

# FUTURE DIRECTIONS

## SUMMARY

When this study was initiated, there were three conflicting studies published regarding DNA immunization of neonatal mice. Two of the studies, in which DNA expressing measles N (Fooks *et al.*, 1996) or influenza NP (Bot *et al.*, 1996) was inoculated i.m., had success in eliciting antibody responses. The third study showed long-term tolerance following i.m. inoculation at birth with DNA expressing the *Plasmodium yoelli* (mouse malaria) circumsporozoite protein (Mor *et al.*, 1996). Due to the importance for vaccination in early life, it was important to determine the effectiveness of these novel DNA-based vaccines in a neonatal animal model. We wished to investigate whether tolerance was an exception or the rule in neonatal DNA vaccination. Further, if DNA vaccination was not tolerizing, it was critical to better characterize the associated DNA-raised immune responses.

The results of this dissertation revealed several important insights regarding i.m. and g.g. immunization of neonatal mice with HA and/or NP DNAs.:

1.) Newborn mice developed stable, long-lived, protective IgG responses similar to those in adult-immunized mice.

2.) Unlike adult mice, which developed polarized IgG2a and IgG1 responses following i.m. and g.g. immunizations, respectively, neonatal mice elicited a variety of IgG1-, IgG2a- and mixed IgG1/IgG2a responses regardless of the inoculation method.

3.)   Newborn mice were not able to generate IgG responses following protein subunit immunization.

4.)   Both responsiveness to protein immunization and development of polarized IgG responses appeared by 2 weeks of age.

5.)   Codelivery of Th1 genetic adjuvants was capable of shifting the mixed isotype profiles elicited following neonatal DNA immunization to IgG2a-biased responses.

6.)   Codelivery of Th1 genetic adjuvants in neonates caused a marked shift towards production of IFN$\gamma$.

7.)   Maternal antibody inhibited DNA raised IgG responses following HA, but not NP, DNA immunization.

8.)   Maternal antibody did not inhibit DNA-raised T help or CTL.

9.)   Protection from influenza virus challenge following immunization with HA and/or NP DNAs was dependent on anti-HA-specific IgG and was independent of cellular immune responses.

## OVERALL DISCUSSION

*Hypothesis #1:* *DNA vaccines are effective in eliciting protective IgG responses in neonatal mice and can circumvent the Th2-bias normally associated with neonatal immune responses to other vaccine preparations.*

In Chapter 1, we found that neonatal mice were capable of generating strong, long-lived, protective IgG responses that were similar in magnitude to those in adult

DNA-immunized mice. However, in contrast to the strongly polarized subtype profiles seen following adult i.m. and g.g. DNA immunization, neonatal mice generated a variety of IgG1, IgG2a and 'mixed' IgG1/IgG2a responses, regardless of immunization method. Several groups have since shown that DNA immunization with various DNA vaccine antigens are also capable of eliciting IgG responses (Table 10), with results regarding isotype patterns being varied (Table 11). Importantly, it is clear that DNA vaccines do not cause a predominant IgG1 profile (indicative of a Th2 type response). Further, temporal studies revealed that development of adult polarized patterns of DNA-raised IgG subtype responses occurred in mice immunized after 2 weeks of age. These results were confirmed by another group (Brazolot Millan *et al.*, 1998).

*Hypothesis #2:* *The disparity between adult and neonatal DNA-raised IgG responses is due to a deficiency in Th1-type cytokines at birth, and thus, it is possible to overcome this deficiency by codelivering Th1 genetic adjuvants at the time of DNA immunization.*

In Chapter 2, we found that codelivery of HA and IL-12 or IFNγ DNA in neonates elicits predominantly IgG2a responses, while codelivery in adults has no effect on IgG subtype patterns. Our results also show that codelivery of IL-12- or IFNγ -DNA in neonates causes a marked shift towards production of IFNγ, whereas codelivery of IL-12 DNA has a much stronger effect than codelivery of IFNγ DNA in adults. Very recently, one other group has assessed the effect of Th1 cytokine DNA codelivery on neonatal DNA-raised T cell responses (Kovarik *et al.*, 2000). This study showed that

**Table 10.** Studies on neonatal DNA immunizations in mice.

| Protein | Method | Ab | Th | CTL | Protection | Reference |
|---|---|---|---|---|---|---|
| Murine leukemia virus gag, pol, env | i.m. | ND[a] | + | + | + | (Butts *et al.*, 1998; Sarzotti *et al.*, 1997) |
| Herpes simplex virus gB | i.m. | + | ND | + | ND | (Manickan, Yu, and Rouse, 1997) |
| Influenza NP | i.m., g.g. | + | + | + | - | Chapter 3, (Bot *et al.*, 1997) |
| HA | i.m., g.g. | + | + | + | + | Chapter 3, (Bot *et al.*, 1997; Pertmer and Robinson, 1999) |
| LCMV NP | i.m. | + | ND | + | + | (Hassett, Zhang, and Whitton, 1997) |
| Measles N | i.m. | + | ND | ND | + | (Fooks *et al.*, 1996) |
| H | i.m. | + | + | + | ND | (Martinez *et al.*, 1997) |
| Rabies virus gp | i.m. | + | + | ND | + | (Wang *et al.*, 1997; Wang *et al.*, 1998) |
| Sendai virus NP | i.m. | ND | ND | + | ND | (Martinez *et al.*, 1997) |
| Tetanus toxin C fragment | i.m. | + | + | ND | ND | (Martinez *et al.*, 1997) |

[a]ND, not done

**Table 11.** Overview of IgG subtype profiles following neonatal DNA immunization.

| Protein | Method | IgG profile[a] | Reference |
|---|---|---|---|
| Herpes simplex virus gB | i.m. | IgG1/IgG2a | (Manickan, Yu, and Rouse, 1997) |
| Hepatitis B virus surface antigen | i.m. | IgG1/IgG2a | (Brazolot Millan *et al.*, 1998) |
| Influenza HA (WSN strain) | i.m. | IgG1/IgG2a | (Bot *et al.*, 1997) |
| Influenza HA (A/PR/8/34) | i.m., g.g. | IgG1/IgG2a | Chapter 1, 2 |
| Influenza NP (A/PR/8/34) | i.m., g.g. | IgG1/IgG2a | Chapter 1, 2 |
| Tetanus toxin C fragment | i.m. | IgG1/IgG2a | (Martinez *et al.*, 1997) |
| Rabies virus gp | i.m. | IgG2a | (Wang *et al.*, 1997) |
| Measles virus HA | i.m. | IgG2a | (Martinez *et al.*, 1997) |
| Sendai virus NP | i.m. | IgG2a | (Martinez *et al.*, 1997) |

[a]Predominant IgG subtype response; IgG1/IgG2a indicates a "mixed" response.

codelivery of IL-12 DNA with measles HA caused an increase in IFNγ production and a decrease in IL-5 production, similar to the results presented in Chapter 2. Thus, it may be possible to supplement neonatal deficiencies with cytokine-expressing DNAs in an effort to tailor immune responses to DNA vaccine antigens.

### *Hypothesis #3: DNA vaccines are effective in generating both humoral and cellular immune responses in the presence of maternal antibody.*

In Chapter 3, we showed that maternal antibody does not interfere with the raising of CD4+ or CD8+ T cell responses. We also showed that maternal antibody inhibits the magnitude of IgG responses to HA but not NP. The ability of maternal antibody to inhibit DNA-raised IgG responses to plasma membrane, but not intracellular proteins, is consistent with some, but not all, prior literature (Table 9). The one other study of a DNA-expressed intracellular protein, LCMV NP, is consistent with our findings with influenza NP (Hassett, Zhang, and Whitton, 1997). Thus, in general, intracellular proteins may be protected against the blocking activity of maternal antibody. Similar to our findings with influenza HA, maternal antibody blocked DNA-raised IgG responses to measles HA and pseudorabies gD. In contrast, maternal antibody failed to block DNA raised IgG responses to herpes simplex gB, bovine herpes gD or rabies glycoprotein (Lewis *et al.*, 1997; Manickan, Yu, and Rouse, 1997; Van Drunen Littel-van den Hurk *et al.*, 1999; Wang *et al.*, 1998; see Table 9).

## **FUTURE DIRECTIONS**

The work presented in this dissertation will be relevant to both the fields of DNA

vaccination as well as neonatal immunology in general. Both fields are extremely

complex and many of the very basic studies still need to be done. The complete picture

of the immaturity of the newborn immune system remains to be elucidated. Mouse

models will only be of limited utility in that mice develop at a much more rapid rate than

humans. To add to the complexity, the basic mechanisms behind how DNA vaccines

work are still unclear, although many groups have been working on the problem for more

than 7 years. The results presented in this dissertation, as well as other recent studies,

suggest DNA vaccines are capable of being used for infant immunization. Although

further studies need to be conducted on the ability to immunize in the presence of

maternal antibody, this work will provide rationale for preclinical testing of DNA-based

vaccination for various pathogens in larger animal models, such as monkeys.


While several studies have shown the efficacy of neonatal DNA vaccination, only

a handful of groups have addressed the ability of DNA to vaccinate in the presence of

maternal antibody (Table 9). The ability to immunize in the presence of maternal

antibody will be necessary to vaccinate effectively in a clinical setting. As described

previously, maternal antibody interferes with active immunization, and vaccination

protocols are often delayed several months, putting the infant at risk for severe bacterial

and viral infections. Based on results presented here, together with results from other

groups, it appears that some antigens are more affected by the presence of maternal

antibody than others. For instance, vaccination with DNAs encoding the plasma membrane proteins of herpes simplex virus gB, bovine herpes virus gD, and rabies virus gp appear to elicit IgG responses in the presence of maternal antibody; whereas influenza virus HA and measles virus HA do not (Table 9).

It is possible that the DNA-expressed plasma membrane proteins may differ in how antibody affects their interaction with the immune system. One possibility is that HA proteins differ, with respect to the other mentioned proteins, in the ability to undergo antibody-mediated capping. Capping involves the unipolar modulation of surface plasma membrane proteins. Early studies showed that measles virus plasma membrane proteins on the cell surface of infected human cells redistributed into polar aggregates following exposure to measles bivalent antibody in vitro (Joseph and Oldstone, 1975), and further stripped viral antigen from the cell surface (Oldstone *et al.*, 1983). Later studies showed that, like measles virus HA, influenza HA on the infected cells also capped in the presence of anti-influenza antibody in vitro (Lydy, Basak, and Compans, 1990), and further, the capping was due to intrinsic properties of the surface glycoprotein itself (Lydy, Basak, and Compans, 1990). One can visualize a similar scenario occurring in vivo. If true, anti-influenza (or -measles) antibody would cap and further strip DNA-expressed HA from the cell-surface, thereby limiting access of HA to naïve B cells.

The aforementioned studies indicate that influenza HA, measles HA, as well as other capping-susceptible vaccine plasma membrane proteins (e.g., vesicular stomatitis

virus G protein or parainfluenza type 3 F protein (Lydy and Compans, 1993)) may be engineered in the cytoplasmic domain-coding regions using recombinant DNA technology. It has been suggested that the primary reason why glycoproteins have differences in the ability to cap relates to how these proteins are anchored within the cell membrane. For instance, some glycoproteins may be unable to effectively interact with host cell components, such as the cytokeleton, via the cytoplasmic domain, thereby increasing lateral mobility within the plasma membrane (Lydy and Compans, 1993).

There are two possible ways to apply this theory to potentially overcome the maternal antibody-inhibiting effect of HA DNA vaccines. First, cloned HA genes can be truncated to various extents using oligonucleotide-directed mutagenesis via polymerase chain reaction. Similar cloning strategies have been previously designed to generate recombinant vaccinia virus vectors expressing a truncated influenza HA (Lydy and Compans, 1993). This group showed that the truncated HA was expressed at normal levels at the cell surface but was not capped, thus this strategy provides a reasonable expectation of success. In a second experiment, cloned HA genes can be engineered to "swap" cytoplasmic and/or transmembane domains of non-capping viral glycoproteins e.g., those of herpes gD or rabies glycoprotein to create a chimeric molecule. This can also be done using standard molecular biology techniques, such as polymerase chain reaction. Since analogous studies have not previously been attempted, it will be necessary to optimize the DNA coding region in terms of each part of the chimeric molecule. For instance, it may be advantageous to generate DNA that expresses many

variations of chimeric HA proteins with progressive deletions from the cytoplasmic

domain through the transmembrane anchor domain of HA. These DNAs can then be

cloned in-frame with DNA encoding progressive substitutions from the transmembrane

domain through the cytoplasmic domain of the secondary non-capping glycoprotein.

Prior to using the truncated HA construct or the chimeric HA construct as a vaccine,

mobility patterns must be checked in vitro in transfected cells using e.g.,

immunofluorescence to determine if, and to what extent, mobility patterns will be altered

in the presence of antigen-specific antibody. Furthermore, expression studies using the

truncated or chimeric HA-expressing DNAs must be completed to determine both the

extent of expression and the integrity of the chimeric proteins, which can be also be

achieved using transfected cells in vitro. Once in vitro studies confirm potential vaccine

candidates for use in the presence of antibody, the DNA(s) can be taken into a

preliminary vaccine trial in vivo. Together, these two experimental strategies provide

guidance in designing an alternative HA DNA vaccine that may be able to be used in the

presence of maternal antibody.

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