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Antibody Responses in Fetuses and Newborns

1. INTRODUCTION

The paradigm of neonatal unresponsiveness resulting from the immaturity of the neonatal immune system and the high susceptibility to tolerance of newborn lymphocytes was derived from an experiment carried out in mice. This experiment showed that newborn mice injected with high numbers of allogeneic hematopoietic cells failed to reject the allograft *(607)*.

This paradigm was challenged by findings generated from comparative immunology studies demonstrating substantial differences among mammalian species regarding the maturation of the immune system during fetal and postnatal life.

In the case of the antibody response, mature B cells were identified in the fetal liver during the last trimester of gestation and in the spleen and cord blood after birth in some mammalian species such as rabbits, lambs, swine, cattle, monkeys, and humans.

Accurate experimental and/or clinical studies demonstrated that in these species, both fetuses and neonates were able to mount responses induced by foreign antigens.

2. THE FETAL ANTIBODY RESPONSE IN MAMMALIAN SPECIES

The demonstration of induction of an antibody response after fetal immunization was determined in species in which the transfer of maternal antigens is impeded by placental structure. Simple surgical procedures permitted direct immunization of the fetus or injection of antigens into the amniotic fluid.

Because these maneuvers are not feasible or ethically acceptable in humans, the measurement of IgM and IgE antibodies, which do not cross the placenta, is used to ascertain the fetal origin of antibodies in newborns from infected mothers.

2.1. Antibody Responses in Fetal Lambs

The fetal lamb frequently has been used as a model to study early development of the immune response because the immune system reaches immuno-

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competence in the last trimester of gestation. The immune responses induced in fetal lambs after *in utero* immunization can be considered authentic primary immune responses because of the syndesmochondrial placentation structure that prevents transplacental transfer of immunoglobulins from ewe to lamb. In fetal lambs, immunization can be carried out either by direct injection of antigen into fetal muscle *(574,608)* or following administration of antigen into the amniotic fluid. It was shown that the latter route of immunization was particularly efficient for genetic immunization and the induction of mucosal immunity *(609)*

In utero immunization of fetal lambs by intramuscular injection as early as 66–70 d into the 150-d of gestation period showed that the fetus is able to synthesize large amounts of antibodies. The highest titers of antibodies were obtained following immunization with bacteriophages, and slightly weaker responses were observed after immunization with ferritin and ovalbumin. The magnitude of the antibody response was not significantly different in fetuses injected between d 70 and 120 of gestation. It is noteworthy that in this study no antibody response was elicited after *in utero* immunization of fetuses with *diphtheria toxoid*, *Salmonella thyphosa*, or Bacillus Calmette-Guèrin (BCG) *(574)*. In another study, Fahey and Morris showed that the antibody response against various T-cell-dependent and -independent antigens could be induced at various stages of gestation *(608)*. Thus, antibodies specific for ferritin were detected at d 64; against chicken red blood cells, polymerized flagellin, and ovalbumin at d 72–76; against monomeric flagellin and dinitrophenyl (DNP)-conjugate at d 78; and against chicken γ-globulin at d 82 of gestation. After 90 d of gestation, animals responded to all antigens tested, with the exception of the somatic antigen of *S. thyphosa*. Interestingly, the lack of antibody response induced by fetal immunization with the somatic antigen of *S. thyphosa* was observed in experiments carried out independently by two groups of investigators *(574,608)*.

Fetal immunization by oral genetic immunization of fetal lambs was studied using two model systems: (a) plasmids containing genes encoding hepatitis B surface antigen (HBsAg), or (b) a truncated form of glycoprotein D of bovine herpes virus-1 (BHV-1)—(gP) *(608,609)*.

Oral immunization using DNA that encodes the BHV-1 gP protein injected once into the amniotic fluid induced detectable amounts of gP-specific antibodies in 80% of newborn lambs. It is important to note that this type of immunization induced not only systemic immunity but also mucosal immunity as assessed by viral shedding in newborn lambs challenged with BHV-l. More importantly, this immunization method induced memory B cells, because a strong anamnestic response was evident in 3-mo-old lambs challenged with BHV-1 *(610)*. Similar results were obtained with HBsAg. In this model, the efficacy of intra-amniotic immunization with a plasmid encoding HBsAg was compared to immunization with a recombinant purified HB surface protein.

Whereas all lambs immunized at d 123 of gestation with plasmid demonstrated protective antibodies, by comparison, 55-d-old lambs immunized with recombinant protein exhibited only a low titer of specific antibody *(610)*.

A possible explanation for the efficient induction of mucosal immunity by oral vaccination with naked DNA might be related to reduced turnover of intestinal epithelial cells during gestation, which could allow for longer persistence of the plasmid. The induction of mucosal immunity by fetal or oral administration is an important observation relevant to vaccination against enterotropic viruses or viruses causing infection of the respiratory tract.

2.2. *Antibody Responses in Bovine Fetuses Infected With Leptospira*

The ability of bovine fetuses to produce antibody was studied by injection of an attenuated *Leptospira* strain into the placentome (composed of maternal caruncle and fetal cotyledon) of 8- to 9-mo-pregnant cows (gestation period: 280 d). In this experiment, dams inoculated at 110 or 134 d of pregnancy aborted their fetuses. However, fetuses of dams inoculated between d 134 and 168 of pregnancy survived. Fetuses, which survived the acute phase of infection, displayed agglutinating antibodies against *Leptospira saxkoebing*. In addition, plasma cells were found in fetuses examined 41 d after infection *(611)*. Because in the bovine, antibodies are not transmitted from mother to fetus, this observation suggests that infection of the fetus in the late stage of gestation may elicit a humoral antibody response.

2.3. **In Utero** *Naked DNA Gene Transfer in Fetal Piglets Induces Protective Immune Responses at Birth*

B-cell lymphopoiesis during fetal development of piglets occurs in the bone marrow at d 45 of gestation when in-frame V[D]J rearrangements reach 70% *(612)*. Similar to ovines and bovines, maternal immunoglobulins are not transferred to the fetus via the placenta. Fazio et al. recently showed that a single intramuscular injection of a plasmid encoding HBsAg led to the production of protective antibodies in 50% of 1-wk-old newborns and in 90% of 21-d-old piglets *(613)*. This result suggests that naked DNA can be transferred from mother to fetus and is capable of stimulating B cells to produce antibodies in the last trimester of pregnancy.

2.4. *Antibody Responses Induced by Fetal Immunization of Baboons*

Baboons exhibit close similarity to humans with respect to maternal–fetal interactions and, like humans, only IgG antibodies are transferred via the placenta. Watts et al. have shown that intramuscular injection of fetuses at d 90, 120, and 150 of gestation with Recombivax HBsAg induced the production of protective antibodies in the fetuses but not in the pregnant mother *(614)*. The

antibody response was maintained after birth up to age 40 mo. This response was significantly increased after virus challenge during neonatal life. This observation clearly demonstrated that fetal immunization primed B cells that were considerably expanded by neonatal challenge.

Experiments on fetal immunization with antigens or naked DNA in some mammalian species, including nonhuman primates, demonstrate three major findings. First, there are considerable differences among mammalian species regarding the maturation of B cells during gestation. In species that exhibit early maturation of the B-cell lineage, a fetal antibody response can be easily induced by injection of the antigen into the fetus or by oral immunization. Second, the early exposure to antigen during fetal life does not induce tolerance in the species mentioned above. Finally, these data may open new avenues for the improvement of vaccination in the case of vertically transmitted infectious diseases.

2.5. Antibody Responses in Human Fetuses Following Vertical Transmission of Infectious Agents

Vertically transmitted pathogens such as HIV, herpes virus, hepatitis B virus, *Treponema pallidum*, *Hemophilus sp. Chlamydia*, *plasmodium*, and *toxoplasma* are major causes of neonatal morbidity and mortality. However, the immune response in human fetuses after parenteral intrafetal immunization or infections with bacteria or viruses can not be studied because of ethical constraints. Currently, the presence of IgM or IgE antibodies in cord blood or serum is used to assess fetal immune responses in humans. In humans, neither IgM nor IgE cross the placenta.

The initial study suggesting a fetal immune response in humans was derived from the cytological analysis of spleens from fetuses with congenital syphilis or toxoplasmosis. The results of this study suggested that human fetuses are immunologically competent, showing precocious development of lymphoid organs and massive plasmocytosis *(615)*. The plasma cell represents the terminal differentiation endpoint of antigen-stimulated B cells. It should be mentioned that production of antibodies also was demonstrated in experimental models of congenital syphilis in rabbits and guinea pigs. In these experiments, it was shown that asymptomatic congenitally infected guinea pigs display, at the first day after birth, high levels of antitreponemal IgM *(616)*. Similarly, a humoral response was noted in congenital syphilis in rabbits infected with the Nicholas strain of *T. pallidum (617,618)*. Anti-rubella, *toxoplasma*, and cytomegalovirus (CMV)-specific IgM were found in babies born to mothers infected with these infectious agents.

Mumps virus infection of pregnant women may induce virus-specific antibodies and memory cells in the fetuses. This initially was shown in a study of 12 Eskimo children exposed to mumps virus during gestation. Exposure of the

fetus to mumps virus evoked an immune response that persisted into childhood, as assessed by induction of an anamnestic response *(619)*. This study is relevant because infection with mumps virus in the Eskimos population is very rare.

Acquired immune responses to the *Plasmodium falciparum* merozoite surface protein-1 (MSP-1) antigen were described in infants born in an area of stable malaria transmission in Kenya *(620)*. The sensitization of fetuses results from the accumulation of infected red blood cells at the interface between maternal and fetal circulation, resulting in the adherence and sequestration of infected red blood cells in the placenta *(621)*. The induction of a fetal immune response in congenital malaria may result either from the subsequent exposure of the fetus to the malaria parasite or to soluble malaria antigens.

IgM specific for the MSP-1-derived peptide MSP^{-11-19}, a vaccine candidate, was detected in the cord blood of 5.8% of newborns with congenital malaria. In addition, anti-MSP-1 IgM and IgG were identified in the culture medium of cord blood lymphocytes from 78% of newborns incubated with MSP-1-derived peptide. It is noteworthy that no antibodies specific for liver surface antigen LSA-1, an antigen expressed on the pre-erythrocyte hepatic form of malaria, were detected in these children. The results clearly show that some infants born in an area of coastal Kenya where malaria transmission is stable were primed to MSP-1 *in utero*.

In utero sensitization of fetuses also was described in infants born to pregnant women with helmintic infections such as Schistosomiasis and filariasis *(622)*. The priming of fetuses with antigens derived from these parasites was demonstrated by the presence of IgE antibodies specific for Schistosome and filarial antigens in the sera of newborns, as well as in the culture supernatant from cord blood lymphocytes stimulated with pokeweed mitogen, which is a T-dependent polyclonal B-cell mitogen.

Transplacental immunization of fetuses also was described in the case of pregnant women immunized with tetanus toxoid *(623)* and meningococcal serotype A and C vaccines *(624)*.

The capacity of the human fetus to produce antibody also was nicely illustrated in a rare case of *in utero* development of autoantibodies. This is the case of a few clinical observations of severe jaundice in newborns in which, during embryonic life, autoantibodies developed causing the hemolysis of red blood cells (RBCs) that was manifested by increased serum levels of bilirubin and reticulocytes, even without evidence of hemolysis *(625)*.

These results taken together strongly suggest that human fetuses, during the last trimester of gestation, have mature B cells that are capable of producing antibodies against antigens borne by infectious agents or that are transplacentally transmitted from mother to fetus. These findings may have practical implications leading to new ways of vaccination during pregnancy.

3. HUMORAL RESPONSES INDUCED IN NEWBORNS

The high degree of susceptibility to infection in newborns and protection against pathogens result from the presence of maternal antibodies. However, in addition to a protective effect, maternal antibodies may hamper responses early in ontogeny.

Previously, it was considered that the active immunization of newborns is affected by the immaturity of lymphocytes and, in the case of B cells, by defective or incomplete signaling following ligation of the BCR.

More recent data showed that newborn infants and animals are able to produce IgM and, in certain cases, IgG and IgA upon antigen exposure. Thus, the view of low competence or immune incompetence of newborns has gradually changed, in spite of the fact that the antibody response may be low. Antibody responses can be induced in neonates in certain conditions where there are notable differences among mammalian species with respect to maturity of the immune system during ontogeny.

3.1. Antibody Responses Induced in Newborn Mice

The ability of newborn mice to mount an antibody response depends on the type of antigen used. The response to T-dependent antigens requires the presentation of peptides derived from the processing of antigens by antigen-presenting cells (APCs), cognitive recognition of the major histocompatibility complex (MHC)-peptide complex by T cells, recognition of the antigen by B cells, and collaboration between T and B cells. The antibody response induced by T-independent antigens TI-1 and TI-2 does not require MHC-class-restricted presentation of antigen to T cells. However, the cytokines produced by T cells or APCs may influence the magnitude of the antibody response in neonates.

The antibody responses of newborn mice are genetically programmed with the sequential activation of clones specific for various antigens seen during postnatal life.

Howard and Hale were among the first to show that, in certain conditions, adult mice injected with small amounts of bacterial polysaccharides as newborns can mount an antibody response to that antigen *(604)*.

Antibody responses against some T-independent antigens such as galactan, levan, and lipopolysaccharide (LPS) can be induced after immunization of 1-d-old mice. However, the magnitude of neonatal antibody responses elicited by these antigens is 30–50% lower than the responses induced in adult mice *(586)* (*see* Table 22). Antibody responses specific for phosphoryl choline and T-dependent antigens such as phenyl arsenate and DNP and TNP conjugates, can be induced only in 5- to 9-d-old mice *(580–582,595)*. The unresponsiveness of neonates to TI-2 antigens can be restored by exogenous cytokine administration. Thus, Snapper et al. showed that highly purified neonatal B

Table 22
Effect of the Parenteral Administration of Anti-Idiotype Antibodies in 1-d-old Newborn Balb/c Mice

Amount of antibody given (μg)	Levan-specific PFC/spleen	%A48Id$^+$ PFC
Saline	3600±0.125 (3977)[a]	6±3
0.01	3508± 0.123 (3218)	46±14
0.1	2855±0.218 (717)	65±17
1	2960± 0.127 (913)	73±20
10	2999±0.183 (997)	73±19

[a]Mean ± SEM for $\log_{10}$ plaque-forming cell (PFC)/spleen, the geometric mean is in parentheses.
Five mice were studied for each group. Mice were immunized with bacterial levan at age 5 wk of age and the PFC response was measured 5 d after immunization.
Adapted from ref. *634*.

cells that are defective for IgM secretion, in response to stimulation by anti-Ig dextran conjugates, can synthesize IgM upon in vitro addition of CD40 ligand or polyclonal activators such as a recombinant protein-Osp or *E. coli* LPS *(626)*. Neonatal B cells, compared to adult B cells, show a relative enhancement in IgE and IgA synthesis. These results suggest that neonatal B cells are competent to synthesize Ig in response to TI-2 antigens if adequate stimuli are provided. Another report demonstrated that neonatal B cells are able to mount an adult-like antibody response to TNP-Ficoll, a TI-2 antigen, after the addition of interleukin (IL)-1 and/or IL-6. Anti-TNP antibodies that are secreted by neonatal B cells stimulated with TNP-Ficoll, IL-1, and IL-6 exhibit an avidity similar to antibodies produced by adult B cells *(627)*.

In vivo antibody responses specific for TNP-Ficoll *(590)* or type II pneumococcus *(628)* can be elicited only in 2- to 3-wk-old mice. However, the immunization of 1-d-old mice with these antigens must somehow prime the neonatal B cells, because stimulation with LPS or monophosphoryl Lipid A can overcome neonatal unresponsiveness and induce the differentiation of neonatal B cells into antibody-secreting cells *(629,630)*.

The antibody responses induced by the vast majority of proteins, with the exception of flagellin, are T-dependent and cannot be induced in newborns. However, immunization of animals with some proteins in the first weeks of postnatal life can elicit an antibody response. An antibody response against hen egg lysozyme (HEL) was elicited by the immunization of 7-d-old mice with HEL in FCA. These mice displayed 1013±303 HEL-specific PFC compared to 4717±2936 plaque-forming cells (PFCs) in adults. The expression of idiotypes of HEL antibodies (IdXE), which is characteristic of the adult response, also was present as early as age 10 d *(459)*. Protective antibody

responses against influenza virus type A also were induced by immunization with purified hemagglutinin (HA) and neuraminidase (NA) coinjected with IL-12 on the first day after birth. The mice immunized simultaneously with soluble HA, NA, and IL-12 exhibited 100% survival after lethal challenge with infectious influenza virus compared to those immunized with the antigens alone. These mice produced higher levels of IgG1 and IgG2a antibodies. The higher protection observed in mice immunized with soluble antigens and IL-12 at birth was antibody-mediated, as demonstrated by the lack of a protective response in mice with B-cell deficiency resulting from a disrupted IgM gene *(631)*. In contrast, newborn mice immunized with the WSN strain of influenza virus exhibited a long-lasting unresponsiveness manifested by a very low concentration of anti-HA antibodies at 30 and 90 d before and after challenge with the virus *(632)*. These observations suggest that cytokines such as IL-12 exhibit an adjuvant effect on the response of neonates against influenza viral antigens.

The BCRs of neonatal B cells express idiotypes, which are the phenotypic markers of V genes that encoding the antigen specificity of the BCR as well as that of secreted antibodies.

Neonatal treatment with high amounts of anti-idiotypic antibodies induces the suppression of clones bearing corresponding idiotypes (reviewed in ref. *49*). However, neonatal treatment with minute amounts of idiotypic or anti-idiotypic antibodies can select and expand silent clones, which are not expressed during the immune response of adult mice.

Exposure of newborn mice to antibodies specific for Schisostoma soluble egg antigen (SEA) led to expression of IdX at age 9 wk. These mice produced significant SEA-specific IgG antibodies and showed prolonged survival after infection with *Schisostoma mansoni*. In the serum of mice injected with IdX anti-SEA antibodies as newborns, both idiotypes and anti-idiotypes were detected *(633)*. High levels of protective IdX antibody were explained by the induction of anti-idiotype antibodies bearing an internal image of the antigen, which led to expansion of the clones that shared similar idiotypes early in the neonatal life. This explanation is strongly supported by demonstration of the expansion of a silent clone following treatment with low doses of anti-idiotypic antibodies. In these experiments, 1-d-old mice were injected with various amounts of anti-A48 IdX antibodies. The A48 idiotype is expressed on the ABPC48 levan-binding myloma protein but is not expressed on the antilevan antibody produced by adult mice. The data presented in Table 22 show that injection with 0.01–10 μg anti-A48IdX antibody after birth induced a strong antilevan PFC response and that 46–73% of B cells that produce antilevan antibodies displayed A48IdX. This response was levan-specific because the injection of 1-d-old mice with anti-M384 IdX antibodies that recognized an idiotype expressed on an LPS-binding myeloma protein did not induce an

Table 23
Concentration of Anti-HA Antibodies Produced by Mice Immunizedas Adult or Newborn With a Plasmid Containing WSN Influenza Virus Hemagglutinin

Age of mice	Immunization	Anti-HA antibodies Before boost	7 d after boost
Newborn			
	Saline	<0.1[a] (30 d)	42±10
	WSN virus	0.1±0.1 (30 d)	2.2±0.3
	pC[b]	<0.1 (30 d)	48±12
	pHA[c]	0.5±0.18 (30 d)	55±17.5
	WSN virus	0.2±0.05 (90 d)	3.3±0.06
	pHA	2.0±1.2 (90 d)	30±27
Adult			
	Saline	<0.1	35.6±11.4
	WSN virus	33.6±11.4 (30 d)	273±21.6
	pC	0.2±0.3 (30 d)	51±28
	pHA	9.8±3.9 (30d)	118±58
	pHA	0.28±03 (90 d)	266±28.8

[a](µg anti-HA antibodies/mL; in parentheses the day of bleeding after completion of the immunization.

[b]Empty plasmid (control).

[c]Plasmid expressing WSN influenza virus hemagglutinin under the control of SV40 promoter.

Immunization was carried out as follows: i.p with 10 µg purified WSN virus; i.m. with 100 µg/mouse pC or pHA three times on d 1, 3, and 6 after births in the case of newborn mice and on d 0, 21, and 42 in the case of adult mice.

Adapted from ref. *632.*

A48IdX$^+$ antilevan antibody response *(634)*. Similarly, injection of neonates with a monoclonal anti-idiotypic antibody specific for the idiotype of a myeloma protein induced protection against myeloma cell growth that lasted until adulthood *(635)*.

These observations indicate that anti-idiotypic antibodies can function as surrogate antigens and can stimulate the expansion and expression of clones bearing a BCR that expresses the corresponding idiotypes. Long-lasting responses into adulthood suggest the induction of memory cells.

3.2. Antibody Responses in Newborn Rabbits

In rabbits, immune competence develops gradually over the first weeks of postnatal life. However, splenic B cells from 1-d-old mice are strongly stimu-

lated by antiallotype antibodies and are able to produce immunoglobulins upon in vitro stimulation with NWSM, a polyclonal B-cell mitogen *(273)*. The level of NWSM-induced synthesis of immunoglobulin by 1-d-old splenic B cells is 25% of that produced by 12-mo-old animals *(274)*. This result is in agreement with other observations that show anti-DNP IgG production within 12 h of birth. Isoelectric focusing analysis of anti-DNP antibodies obtained 8 d after immunization of neonatal rabbits with DNP-bovine gamma globulin (BGG) conjugates in saline, Freund's incomplete adjuvant (FIA), or FCA showed unique monoclonal or pauciclonal pattern differences in individual rabbits. This pattern was maintained for several weeks until a boost with antigen was performed that caused a more heterogeneous response *(636)*. These data indicate that newborn rabbits possess a large set of *V* genes that encode DNP-specific antibodies but that neonates differ from adults in their capacity to express a complete genetic repertoire for antibody diversity.

3.3. Antibody Response Development in Newborn Piglets

The immune responses of newborn piglets differ from other species, because piglets receive no maternal antibodies, and, therefore, at birth they are free from maternal protective factors. Butler et al. carried out interesting studies aimed at testing whether precosial newborn piglets can respond to a T-dependent (TD) antigen, such as FL-KLH, or a TI-2 antigen, such as TNP-Ficoll *(637)*. The results of this study suggest that bacterial colonization of the gastrointestinal tract results in a substantial increase of follicles in Peyer's patches and is associated with an increase of antibodies specific for the two antigens studied. The amount of antibodies generated depended on the nature of the colonizing bacteria.

Colonized piglets immunized at d 3 with FL-KLH exhibited a modest primary response by d 10, which was considerably increased 1 wk after challenge. No increase in anti-TNP antibodies was noted after booster immunization with TNP-Ficoll at age 4 wk *(637)*. The antibody response observed only after bacterial colonization of piglets is probably because of production of costimulatory molecules by macrophages and dendritic cells; by ligation of Toll-like receptors such as TollR2 and TollR4 by LPS, lipoprotein, and peptidoglycan; or by ligation of TollR9 by bacterial DNA that is rich in CpG motifs.

3.4. Infant B-Cell Responses

At birth, human newborns have a full repertoire of antigen-specific B cells in the bone marrow. However, the maturation of B-cell responses occurs gradually during the first years of life. The human newborn is able to produce IgM and even IgG and IgA at low concentrations. In vitro polyclonal activation of neonatal B cells with *Staphylococcus aureus* Cowan I or Epstein–Barr virus

induces the synthesis of small amounts of IgM. Stimulation with pokeweed mitrogen (PKM) induces the production of IgM but not IgG, in spite of the fact that PKM is a T-dependent polyclonal activator *(638)*. In contrast to B cells from adults, peripheral blood lymphocytes (PBL) from cord blood did not differentiate into antibody forming cells upon culturing with type 4 pneumococcal polysaccharide *(639)*.

Because of ethical considerations, there is no information in human newborns and infants on antibody responses induced by immunization with foreign antigens, except the responses elicited by vaccines. This information will be presented and analyzed in Chapter 12.

4. ANTIBODY RESPONSE OF NEONATES ELICITED BY SOMATIC TRANSGENE IMMUNIZATION WITH PLASMIDS

Genetic immunization represents a new and appealing approach to induce antibody responses in newborns and infants. It based on two important findings: (a) the demonstration that a reporter gene (β-galactosidase) engineered into a plasmid is transcribed and translated in tissues at the site of injection *(640)*, and (b) injection of mice with a plasmid containing the *bovine growth factor hormone* gene resulted in the production of antihormone antibody *(641)*.

We first demonstrated that, in contrast to the inability of an inactivated influenza vaccine to induce a cytotoxic T lymphocyte (CTL) response in neonates, immunization of 1-d-old mice with a plasmid containing the influenza virus nucleoprotein (NP) gene (bearing epitopes recognized by T cells in association with MHC class I molecules) generated CTL activity comparable to that of adult mice injected with the same dose of plasmid. The cytotoxic activity was related to an expansion of CTL precursors as assessed by measuring pCTL frequency 1 mo after immunization *(642)*.

Our pioneering study stirred interest in the use of genetic immunization for the induction of humoral immune responses in newborns. Thus, it was shown that the immunization of mice after birth with a plasmid that encodes the full-length rabies virus glycoprotein (gP) under control of the SV40 promoter developed higher antibody responses compared to mice immunized with the empty plasmid. The majority of anti-gP antibodies were of the IgG2a isotype, indicating the participation of T cells known to be required for Ig class switching *(643)*. Induction of antibody responses in newborn mice induced by genetic immunization with a plasmid containing *influenza virus HA gene (pHA)* also was demonstrated.

Antibodies against HA and NA have been shown to confer protection against influenza virus. Anti-HA antibodies prevent the HA-mediated binding of the virus to the sialoprotein receptor of host cells and subsequent fusion of the

virion with the plasma membrane. Meanwhile, anti-NA antibodies inhibit the enzymatic activity of NA, thereby preventing the cell-to-cell spread of virus.

HA-specific neutralizing antibodies are thought to play the major role in immunity to influenza virus. The induction of the HA-specific antibody response was studied in newborn Balb/c mice injected with an empty plasmid (pC) or with a plasmid containing the HA of influenza virus WSN strain (pHA). The antibody response was assessed by measuring the hemagglutination inhibition (HI) titer and by radioimmunoassay (RIA).

The majority of adult mice immunized with pHA displayed high HI titers at 1 and 3 mo after immunization and no detectable antibodies after 9 mo, corresponding to clearance of the plasmid from the site of injection.

In the case of mice immunized with pHA as newborns, high HI titers were observed in 75% of mice at 1 and 3 mo after immunization. Newborn mice immunized with pC and then challenged with WSN virus showed titers comparable to adult primary responses, whereas mice immunized with pHA exhibited titers characteristic of a secondary response (Table 23). These results demonstrated that, in contrast to adult mice immunized with WSN or with pHA that developed vigorous primary and secondary responses, mice immunized as neonates failed to mount an anti-HA antibody response after challenge with WSN virus. Strikingly, neonates immunized with pHA developed a weak primary response but exhibited a strong secondary response subsequent to challenge with virus *(644)*.

These findings suggest that the immunization of neonates with pHA primed the B cells and induced B-cell memory. This concept was supported by two additional groups of findings: (a) the isotype pattern of the secondary response of mice that were immunized as neonates and adults was quite similar and was characterized by the predominance of IgG2a and IgA antibodies, with the exception of an increased concentration of IgG1 anti-HA antibodies in mice that were immunized as neonates with pHA; and (b) the percentage of survival of mice immunized with pHA as neonates or adults and challenged with a lethal dose of live virus was similar *(644)*.

Analysis of the reactivity pattern of HA-specific clonotypes for six different strains of influenza (H1N1) that were obtained by spleen focus assay from mice immunized as neonates or adults with WSN virus or pHA showed very interesting results.

The immunization of adults with virus or pHA increased the frequency of B-cell clonotypes with a broad reactivity pattern. In sharp contrast, whereas the immunization of neonates with live virus induced a long-lasting unresponsiveness and the few clones stimulated in vitro produced only antibody specific for WSN, immunization with pHA led to the occurrence of clonotypes displaying an adult-like pattern of reactivity *(632,644)*.

The most striking observation comes from studies of neonatal DNA immunization and consists of an early shift of the neonatal repertoire toward the adult repertoire, as assessed by a broader reactivity pattern of B-cell clonotypes.

This is surprising, because it is known that the restricted neonatal B-cell repertoire results from position-dependent utilization of the J-proximal V_H gene family, exhibits shorter CDR3 length, lacks N-region diversity, and has a low rate of somatic mutation compared to adults. This may be related to increased receptor editing or revision. An antibody response was induced in newborn mice immunized with plasmids containing measles virus HA, Sendai virus NP, and tetanus toxoid C fragment *(645)*.

Monteil et al. have studied IgG-specific antibody responses following immunization of 1-d-old piglets with a plasmid containing the gP gene of pseudorabies virus followed by boosting on day 42 *(646)*. After the boost, the piglets developed medium levels of IgD-specific antibodies that exhibited virus-neutralizing activity in vitro. Furthermore, they developed an anamnestic response after challenge at day 115. However, in spite of the fact that the animals produced antibodies, no protection was observed after challenge. This may be related to the low expression of gP protein after intramuscular injection of plasmid.

Successful humoral responses were elicited by genetic immunization of newborn nonhuman primates. Thus, in chimpanzees immunized at birth with a plasmid containing the hepatitis B surface antigen, a transient increase of antibody titer was observed between d 12 and 20 after immunization. An increase in antibody titer, which was long lasting, was observed after challenge at 33 wk with 100 CID_{50} of Hepatitis B virus (HBV) *(647)*. From this study, it was concluded that DNA-based genetic immunization was able to induce a protective anti-HBV response in newborn chimpanzees. Significant levels of antibodies also were detected in chimpanzees immunized after birth with a plasmid containing the HIV gag/pol genes. Antibody responses were evident as early as 4 wk after intramuscular and intravaginal delivery of plasmid. It is noteworthy that the serum titers of antibodies in infant animals were comparable to the serum level of antibodies of adult animals immunized with the same construct *(648)*.

Similar results were obtained after neonatal immunization of baboons with a plasmid that encoded the type A influenza virus HA. HA-specific antibodies were detected by ELISA at 28 d after immunization, and by both ELISA and HI at 2 and 3 mo after immunization. It is important to note that the immunization of newborn baboons with 50μg UV-inactivated virus did not elicit antibody production *(649)*. The induction of memory cells was demonstrated by challenge of 18-mo-old baboons immunized with virus as neonates , resulting in an increased titer of IgG1 anti-HA antibodies. This finding demonstrated that genetic immunization of newborn baboons triggered long-lasting immune memory that persisted beyond infancy *(650)*.

These findings question the paradigm of neonatal tolerance because of the immaturity of B cells, which are more susceptible to antigen deletion or anergy.

Several factors may explain neonatal immune responsiveness to genetic immunization. First, transfected cells secrete small amounts of antigen. It is well known that the induction of peripheral tolerance in neonates requires large amounts of antigen ("high-dose tolerance") and that central tolerance (deletion) requires the recognition by B cells of antigen expressed at the surface of somatic cells, including the antigen-presenting cells. Second, genetic immunization results in the long persistence of antigen. This ensures the priming of newly emerging cells from the bone marrow and eventually the generation of memory cells, as illustrated by good anamnestic responses after challenge. Finally, neonatal immune responses may result from the intrinsic adjuvant activity of plasmids that are rich in CpG motifs. The binding of CpG-rich DNA to TollR9 may trigger signaling pathways, thereby circumventing the limiting number and immaturity of B cells. This also can increase the synthesis of GM-CSF, IL-12, and interferon types I and II, which have an adjuvant effect, and enhance the reactivity and maturation of B cells in neonates (reviewed in ref. *651*).