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RESEARCH ARTICLE

Immune response at birth, long-term immune memory and 2 years follow-up after in-utero anti-HBV DNA immunization

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Infections occurring at the end of pregnancy, during birth or by breastfeeding are responsible for the high toll of death among first-week infants. In-utero DNA immunization has demonstrated the effectiveness in inducing specific immunity in newborns. A major contribution to infant immunization would be achieved if a vaccine proved able to be protective as early as at the birth, preventing the typical 'first-week infections'. To establish its potential for use in humans, in-utero DNA vaccination efficiency has to be evaluated for short- and long-term safety, protection at delivery, efficacy of boosts in adults and effective window/s for modulation of immune response during pregnancy, in an animal model suitable with human development. Here we show that a single intramuscular in-utero anti-HBV DNA immunization at two-thirds of pig gestation produces, at birth, antibody titers considered protective in humans. The boost of antibody titers in every animal following recall at 4 and 10 months demonstrates the establishment of immune memory. The safety of in-utero fetus manipulation is guaranteed by short-term (no fetus loss, lack of local alterations, at-term spontaneous delivery, breastfeeding) and long-term (2 years) monitoring. Treatment of fetuses closer to delivery results in immune ignorance without induction of tolerance. This result highlights the repercussion of selecting the appropriate time point when this approach is used to deliver therapeutic genes. All these findings illustrate the relevance of naked DNA-based vaccination technology in therapeutic efforts aimed to prevent the high toll of death among first-week infants.
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Introduction

Infections occurring at the end of pregnancy, during birth or by breastfeeding are responsible for the high toll of death among first-week infants. The World Health Organization estimated that 5 million infants died for infections during the first week of life in 1995. The most important pathogens include the well-known hepatitis B virus (HBV),[1] herpes simplex virus (HSV),[2] human immunodeficiency virus (HIV)[3] and the little-publicized group B streptococcus,[4] cytomegalovirus (CMV),[5] respiratory syncytial virus (RSV),[6] human papilloma virus (HPV),[7] hemophilus, and chlamydia.[8]

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Neonatal infection with hepatitis B is the result of neonate's contamination during delivery, but rarely may depend on a prior *in-utero* transmission of the virus.[9] HBV infection becomes chronic in 90% of infants perinatally infected, and 25% of these will die of related chronic liver disease as adults.[1] There are significant complications associated with perinatal HBV infection, ranging from fulminant HBV to chronic liver disease and to an increased risk for carcinoma.[10,11] In 1996, in the United States, an estimated 20 000 infants were born to women positive for hepatitis B surface antigen (HBsAg).[12] The transmission rate is expected at 90% when the mother is positive for viral DNA in her serum[13] or lacks antibodies to hepatitis B core antigen (HBcAb).[14]

To prevent these infections, newborns have been vaccinated early after birth[15] and mucosal immunity in newborns has been demonstrated following oral DNA immunization of the fetus in sheep model.[16] However, protective levels of antibodies are obtained only some

weeks after delivery. A major contribution to infant immunization would be achieved if a vaccine proved able to be protective as early as at birth, preventing the typical 'first-week infections', such as human RSV.

More significantly, early neonatal immune protection may find an important and life-saving application in hereditary or congenital diseases, like cystic fibrosis, asthma, congenital heart diseases, severely complicated by perinatal or early newborn infections, such as RSV.

DNA-based vaccination technology is at the frontline of therapeutic efforts in this field.[16,17] DNA vaccines may also enable to manipulate the immune system in situations where the response is inappropriate, redirecting the immune response.[17,18] *In-utero* DNA vaccination technology has great, but as yet significantly unrealized, extensive clinical applications. To establish its potential for use in humans, its efficiency has to be evaluated for early effectiveness, strong, long-lasting immune memory and safety.

Here we demonstrate that a single intramuscular *in-utero* anti-HBV DNA immunization at two-thirds of pig gestation induces significant levels of specific antibodies already at birth and for a long time after delivery. The boost of antibody titers after recall at 4 and 10 months demonstrates the establishment of a long-lasting immune memory. Treatment of fetuses near to delivery results in immune ignorance without induction of tolerance. The definite short- and long-term safety of the procedure, a major concern in developing approaches for *in-utero* gene delivery, was established by the absence of fetus pathology, spontaneous at-term delivery and 2 years follow-up after *in-utero* DNA immunization.

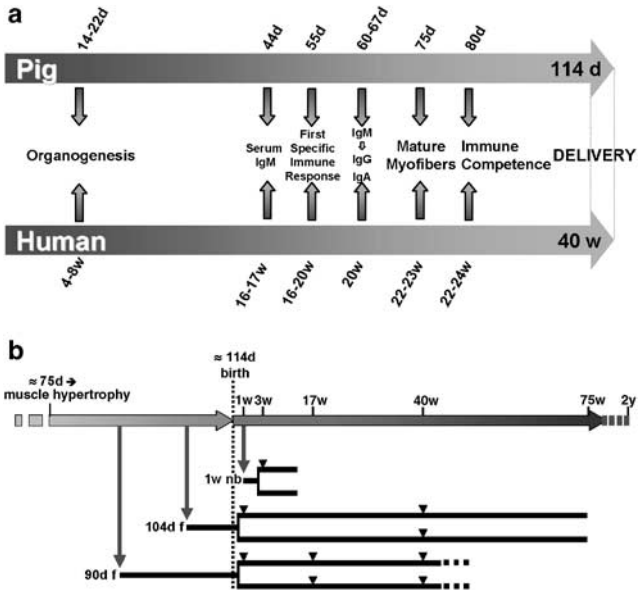

Figure 1 *Schemes of the experimental strategy. (a) Comparative evaluation of fetal development in pig and human. Gestational timing (horizontal arrows) is expressed as days and weeks, respectively, for pigs and humans. (b) Experimental design of in utero anti-HBV DNA immunization. A total of 75 days of swine pregnancy is the time indicating approximately the end of muscle cell proliferation and the development of mature myofibers. This time also marks the completion of the maturation of other tissues and organs. The horizontal dotted arrow indicates the fetal muscle hypertrophy starting at ≈75 days (75 d) of pregnancy; the birth after the gestation period of ≈114 days (114 d); the scheme of postnatal and late boosts (▼) at 1 week (1 w), 3 weeks (3 w), 17 weeks (17 w), and 40 weeks (40 w) after delivery. The vertical bold arrows indicate the first DNA injection performed in pig fetuses at 90 (90 d f) or 104 (104 d f) dp, or in newborn pigs 1 wad (1 w nb).*

Results and discussion

Experimental design of anti-HBV DNA immunization

In this study, we examine the early and late effectiveness and safety of intramuscular *in-utero* DNA vaccination. We choose pigs as an animal model to study fetuses whose dimensions resemble that of humans, in relatively large numbers per pregnancy, because a parallel exists between the development of muscle[19,20] and immune system[21] in man and pig (Figure 1a). The gestation period of the sow is approximately 114 ± 3 days. As a model antigen, the surface (S) antigen of HBV was chosen. A known correlation, always confirmed in experimental, clinical and epidemiological studies, exists in humans between circulating anti-HBs antibody levels and vaccine protective efficacy.[22,23] Anti-HBs antibody titer $\geqslant 10$ IU/l is the value considered to be protective in humans. Swines are not natural hosts for HBV, and there is no passive transplacental transfer of IgG in pigs.[21] These two facts together guarantee the absence of natural active or passive immunization of the fetuses. However, even if this animal model does not allow viral challenge, antibody levels may provide an acceptable validation of the vaccine efficacy. Accordingly, 27 untreated controls (seven fetuses, 15 newborns, and five pregnant sows) were found negative for anti-HBs. We could never detect even sporadic positive value in a time course for anti-HBs in any of the sows, following DNA immunization of their fetuses (data not shown). Thus, we can confirm that anti-HBs are not transferred from the mother to fetuses or spontaneously produced by pigs. Pigs have been chosen as experimental models for *in-utero* vaccinations by Binns,[24] showing that immunization in pig fetuses at 80 or 104 days of pregnancy (dp) was effective in inducing the rejection of skin graft later in life, and in preclinical studies, for evaluating the effectiveness and tolerability of DNA vaccine toward HBV, oriented to develop phase-1 clinical trials.[25] Therefore, we treated pig fetuses at 90 or 104 dp, which match the late 7th and 8th months of human pregnancy, respectively, or newborns 1 week after delivery (wad). Immunization and boosts were performed by intramuscular injection with pRc/CMV-HBs plasmid encoding the S antigen of HBV virus.[26] The overall scheme of injection and boosts is described in Figure 1b.

Early effectiveness of intramuscular in-utero DNA immunization: antibody levels at birth considered protective in humans

To analyze immunity in the early days of life, we compared newborns immunized at 1 wad (1 wad group) with those immunized at 90 dp (90 dp group). Data are summarized in Figure 2a, b. Seven of eight untreated offsprings (negative for anti-HBs at birth) were immunized 1 wad. Measurable amounts of antibodies were detected 5 weeks after the injection, with the maximal response 9 weeks later (five of seven animals showing anti-HBs titers >10 IU/l; Figure 2a). In parallel studies, nine of 10 fetuses were immunized at 90 dp. Remarkably,

a Anti-HBs Response in 1 week newborn

Pig Number	1	⇩	3	⇩	6	10	11
	Time Post-Delivery (weeks)						
1	N	+	N	+	N	8	6
2	N	+	N	+	65	110	45
3	N	+	N	+	50	70	120
4	N	+	N	+	28	140	160
5	N	+	N	-	N	N	N
6	N	+	N	-	N	65	110
7	N	+	N	-	75	160	110
8 C⁻	N	-	N	-	N	N	N

b Anti-HBs Response to 90 Day Fetus I.M. DNA Vaccination

Pig Number	Birth	⇩	3	9	11	17	⇩	19	20	22	40	⇩	43
	Time Post-Delivery (weeks)												
1	N	+	10	140	150	500	+	4.700	2.800	1.600	900	+	50.000
2	N	+	80	50	26	25	+	30	110	120	70	+	17.000
3	28	+	N	N	8	25	+	110	150	130	140	+	500
4	30	+	15	130	55	18	+	130	120	140	125	+	130
5	N	+	25	40	12	40	+	34	38	18	35	+	50
6	150	-	35	40	22	18	+	150	130	80	190	+	170
7	18	-	50	150	100	120	+	180	190	160	95	+	1.500
8	N	-	14	42	18	N	+	28	130	160	160	+	150
9	38	-	65	120	120	130	+	200.000	60.000	1.600	1.200	+	NT
10 C⁻	N	-	N	N	N	N	-	N	N	N	N	-	N

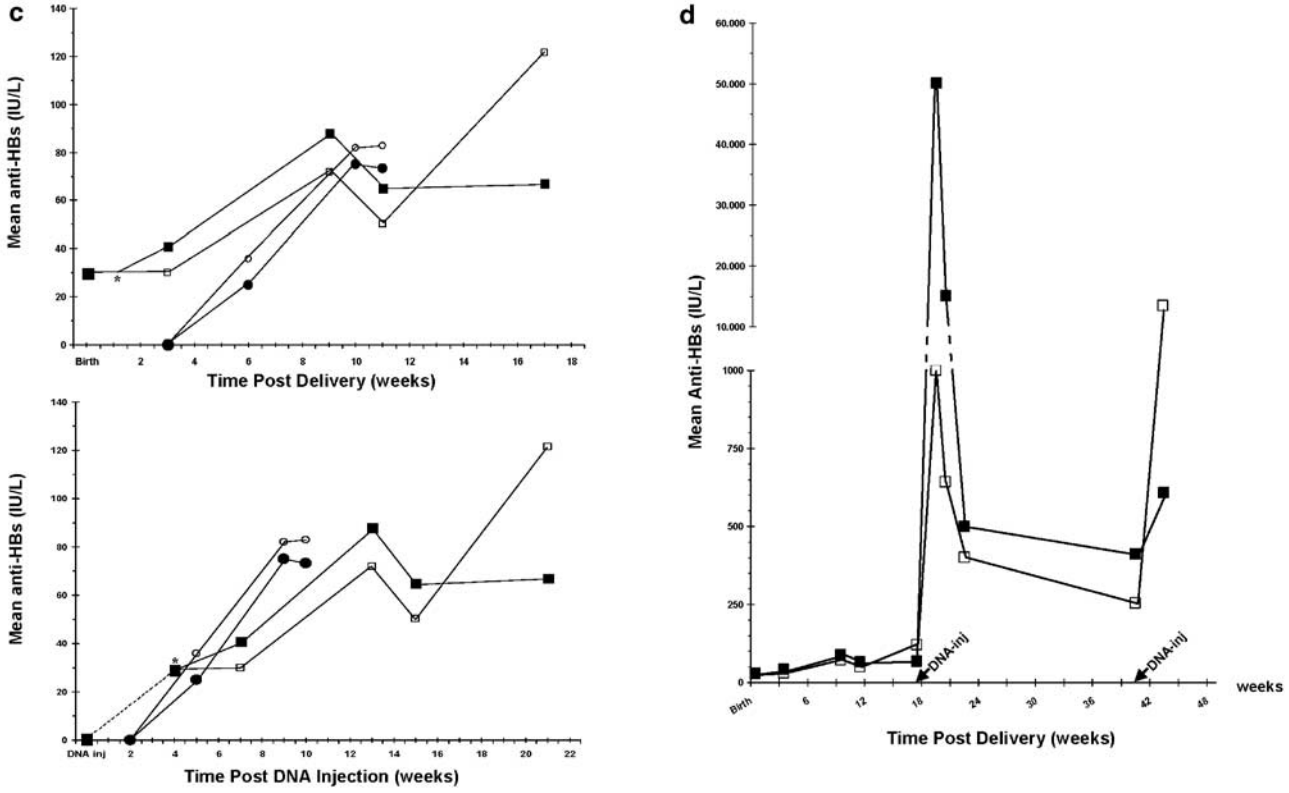

Figure 2 *Anti-HBs response to 1-week newborn (a) and 90-day fetus (b) intramuscular DNA immunization. Anti-HBs values are expressed in IU/l and were measured at the indicated time (weeks) postdelivery. N: negative value; ⇓: immunization or boosts performed in injected (+) with respect to not-injected (−) animals; C⁻: noninjected negative control. (c) Comparative anti-HBs titers of newborns immunized 1 wad (●) and boosted 2 weeks later (○) with fetuses immunized at 90 dp (■) and boosted (*) 1 week after birth (□). The data are represented both as time postdelivery (upper panel) and as time post-DNA injection (lower panel). (d) A 43-weeks follow-up of levels of anti-HBs in pigs immunized at 90 dp (■), and boosted at birth (□). The following DNA injections, performed in both postnatal boosted and nonboosted animals at 17 and 40 weeks after delivery, are indicated with arrows (↙ DNA-inj).*

five of nine (55%) animals had already significant levels[22] of anti-HBs at birth (approximately 4 weeks after *naked* DNA injection), and 3 weeks after delivery eight of nine (89%) newborns developed anti-HBs responses at levels considered protective in humans (Figure 2b). This represents a crucial improvement in terms of prevention of disease transmission during delivery or early breast-feeding.[27] One fetus of this litter (fetus no. 10) had not been injected during pregnancy and did not develop anti-HBs throughout the experimental period (43 weeks). Interestingly, the interval time required for antibody response to develop quite overlapped in the 90 dp group and in the 1 wad group (Figure 2c). Half of the newborns of both groups were boosted with a second *naked* DNA injection 1 week after birth (90 dp group) or 3 weeks after delivery (1 wad group). In both groups, this second injection had no relevant effect (Figure 2). The antibody titers in boosted and nonboosted animals overlapped. Thus, the maximal immune effect that can be obtained early in life is the one that is already achieved by a single intramuscular *naked* DNA injection at 90 dp. Moreover, the 90 dp nonboosted group maintained high levels of antibodies up to 17 weeks.

Long-term effectiveness of intramuscular in-utero DNA immunization: induction of immune memory

We next examined the long-term effect of *in-utero* vaccination on immune memory. Pigs belonging to the 90 dp group received a boosting injection at 17 weeks of life. Eight of nine (89%) animals had an increase of their anti-HBs up to 10-fold within 3 weeks after boost,

unrelated to the early boost (Figure 2b, d). Although the titer declined subsequently, 23 weeks after the boost injection, it was ~3-fold greater than that before. At this point, pigs received again a *naked* plasmid DNA injection and again a boost effect was observed.

Anti-HBs response following intramuscular DNA immunization in 104-day gestational age fetuses

In a new vaccination schedule, seven fetuses were immunized at 104 dp (104 dp group), approximately 1 week before delivery. None among the seven animals had developed anti-HBs at birth, and borderline detectable antibody levels were found only in one by week 4 after delivery (Figure 3a). Three hypotheses can explain our result: immune response may require a longer time in this group to become measurable; *naked* DNA injection at that time may have generated tolerance instead of immunity;[28] this litter belongs to a 'nonresponder' group, a condition that occurs in about 10% of humans receiving subunit vaccination for HBsAg.

To address the first point, three out of seven pigs of the 104 dp group did not receive any further boost until week 40 after birth, and anti-HBs were measured at various time points. These pigs failed to produce amounts of anti-HBs comparable to those produced by pigs belonging to the 90 dp and 1 wad groups within 9 weeks after a single intramuscular DNA injection. No HBsAg could be detected in the sera of these animals. Nevertheless, the erratic appearance of low levels of anti-HBs (that never appears in untreated pigs) rules out the possibility that no DNA had been injected for technical reasons.

Four newborns were boosted with *naked* DNA injection 1 week after birth to establish if the 104 dp group was not

a

Anti-HBs Response to 104 Day Fetus I.M. DNA Vaccination

Time Post-Delivery (weeks)

Pig Number	1	⇩	4	8	10	12	17	29	40	⇩	44	50	74
1	N	+	N	62	38	45	180	230	130	+	13.000	2.000	380
2	N	+	N	N	N	N	15	50	25	+	110	85	32
3	N	+	N	N	28	130	65	170	90	+	1.700	150	160
4	N	+	N	25	45	250	300	500	380	+	35.000	14.000	2.200
5	N	-	10	N	N	N	32	12	9	+	320	650	1.800
6	N	-	N	N	N	12	N	14	10	+	90	70	120
7	N	-	N	20	70	N	N	N	N	+	50	450	1.400

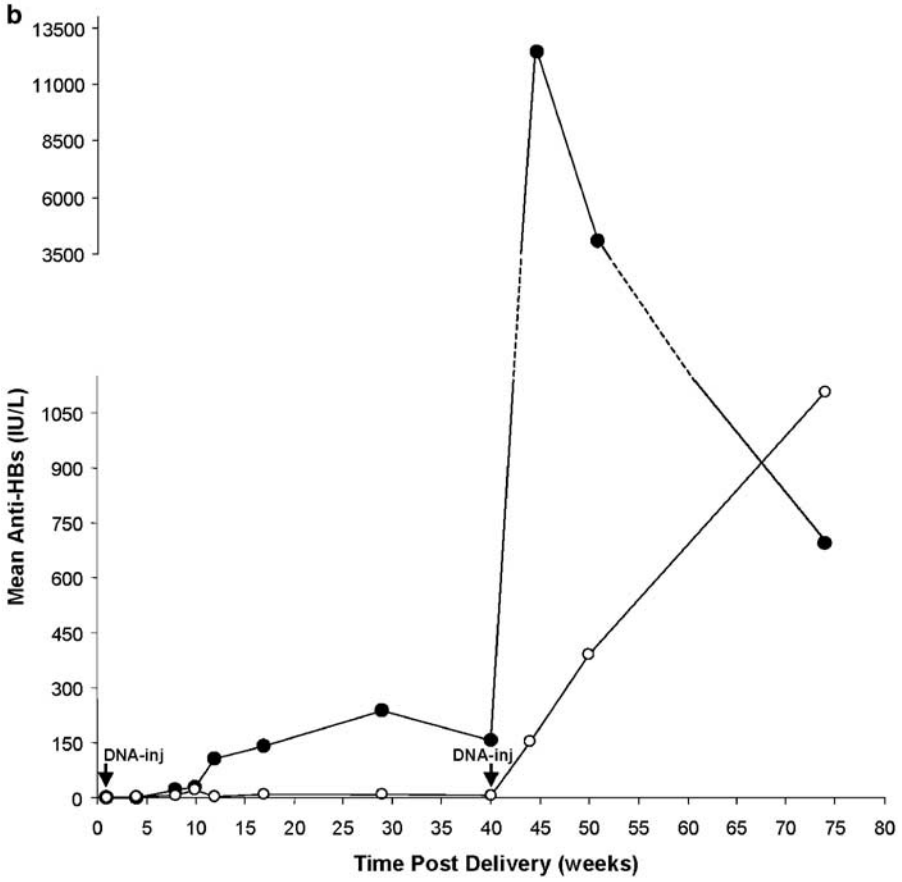

Figure 3 *Anti-HBs response to 104-day fetus intramuscular DNA immunization. Anti-HBs values are expressed in IU/l, and were measured at the indicated time (weeks) postdelivery. (a) N: negative value. ⇓: boosts performed in injected (+) with respect to noninjected (−) animals. (b) A 74-weeks follow-up of levels of anti-HBs in animals immunized at 104 dp (○), and boosted at birth (●). The DNA injections, performed at birth and 40 weeks after delivery, are indicated with* arrows (↙ *DNA-inj.).*

able to respond to HBsAg because of genetic reasons or induction of tolerance. Results show that all of these animals developed an anti-HBs response when challenged 1 wad, although in a longer time with respect to 1 wad group immunized only at birth. This result excludes the possibility that this litter was a 'nonresponder' group for genetic reasons. It also demonstrates that no permanent tolerance is achieved by antigen injection just before the delivery, but some sort of 'reduction' in the ability to respond to an early challenge can be observed.

All pigs belonging to the 104 dp litter received a new *naked* DNA injection at week 40 after birth. In this case, the responses were different in the group that had also been rechallenged soon after birth, with respect to those pigs that had received only the injection *in-utero* (Figure 3a, b). The first group responded to this late injection with a boost of their anti-HBs response that showed the same kinetic and degree as the 90 dp group. The effect of this late boost on the group that had been injected *in-utero* only was different, with a long-lasting raise of antibody titer, still keeping on 34 weeks after the boost.

It is known that delivery is characterized by suppression of immune responses of the fetus, and this inability has also been invoked to explain at least in part the severe outcome of infections acquired during delivery. A major role in inducing this 'stupor' of the immune system may be played by prostaglandin (mainly PGE) and corticosteroid produced in proximity of delivery.[29] Plasma cortisol levels in the pig fetus, which are relatively constant between days 70 and 100 of pregnancy, increase rapidly at the end of pregnancy until delivery.[30] This modulation of the cortisol level reproduces that in human pregnancy.[31] These changes may have a potent suppressant action on several cells involved in immune response, and in particular on the survival of immature lymphocytes and activated T cells. All these data imply that negative regulation of immune competence starts some time before delivery. These results have implications for modulating immune tolerance as well as to enhance vaccine efficiency,[32] and push for further investigations.

Tolerance for some DNA-vaccine-encoded antigens had previously been demonstrated in newborns during the first week of life.[28] We found a relative ignorance in the 104 dp immunized litter, with delayed response to an early boost but with prompt and strong response to a delayed boost. 104 dp immunized animals that received only the late boost (40 weeks) developed a slow but significant response to this challenge. Thus, the ability to respond to vaccine injection later in life does not appear to be hindered in any of the injection protocols.

Safety of in-utero fetus manipulation

In *in-utero* DNA vaccination protocols, safety has to be supplementary assessed in terms of lack of adverse reaction in the fetuses and effect on long-term responsiveness. Injected animals were kept under accurate observation up to 2 years of age, to test any early or late adverse effect. All fetuses immunized with plasmid DNA displayed normal fetal development. No fetal loss was ever reported. Fetuses subjected to intramuscular injection on either day 90 or day 104 of gestation had a 100% survival rate. All fetuses were spontaneously delivered at term, and were born alive and without assistance. The newborns' weight and vitality at the birth were in the norm and no malformation, abnormalities or deficiency were detected by veterinary inspection (Figure 4a). Thus, *in-utero* intramuscular DNA immunization did not appear to affect fetal development or neonatal viability. None of the pups showed clinical signs of distress or illness following the surgical procedure. The infants were breastfed and growth patterns and behavioural development were in the norm. No adverse reactions (fever, local erythema, detectable wheal or bleb, etc) or alterations in feeding behavior were observed after boost immunizations. In no case we observed the dwarfism of the newborn, as reported in fetuses immunized with cell suspensions[24] (Figure 4b).

In a previous study, we have evaluated direct intramuscular *naked* DNA transfer at different gestational ages, for systemic or local delivery of recombinant proteins.[33] We have reported the expression of various genes in almost all the fetuses several days after injection, both before and following delivery. A confined step gradient of expression in the site of injection was observed, and no transgene expression was detected in the controlateral muscle. These results are in agreement with those reported by Everett *et al*,[34] indicating the localized plasmid expression close to the site of injection. The precise control of the area interested by *in-utero* DNA-immunization procedure represents a further safety margin, with respect to other *in-utero* gene-delivery approaches.[16,27] A conclusive summary of safety considerations included in our experimental design is incorporated in Table 1. In the present work, any experimental procedure that might impair the normal development and behavior of pig fetuses, newborns and adults, in this long-term follow-up, was carefully avoided, including extensive blood samples and biopsies.

Our results can be integrated with the current knowledge of the limited spreading of effective *naked* plasmid DNA outside the injection area,[35,36] and of the extremely rare integration of plasmid DNA into muscle genomic DNA (the calculated rate of integration is 3000 times lower than the accepted rate of spontaneous mutation for mammalian genome).[37] Even if a very low potential risk of plasmid DNA integration is documented, particular caution must be taken into account, especially if gene transfer is to be performed at such an early stage of human life. The reported observation that the immune reaction itself limits the temporal expression of antigenic plasmid vaccines represents a further safety margin.[38] Klinman and co-workers have specifically demonstrated that repeated DNA injections did not alter the onset or

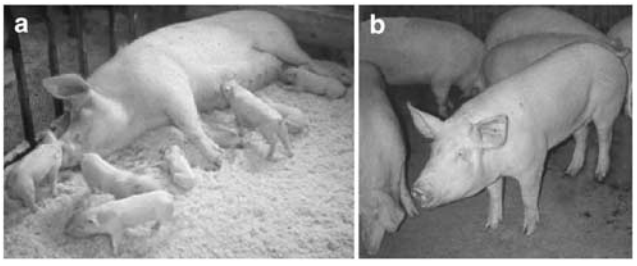

Figure 4 *Follow-up of 2 years of in-utero vaccinated pigs. The injected animals were kept under accurate monitoring just after birth (a), during growth into adulthood (b), up to 2 years following in utero anti-HBV DNA immunization.*

Table 1 Clinical safety parameters evaluated following *in-utero* DNA immunization

Short-term safety *Perinatal monitoring*	*Long-term safety* *Two years follow-up*
At-term spontaneous delivery	All *in-utero* immunized animals have developed protective levels of anti-HBs and have responded to boost DNA immunizations
No fetal loss	
No malformations	No antigen tolerization
Normal fetal development	In no case dwarfism
Normal newborn development	Normal growth patterns and behavioral development
Normal newborn behavior	No alterations in feeding behavior, following boost immunizations
Newborn breastfeeding by the own mother	No detectable adverse reactions (fever, local erythema, detectable wheal or bleb, etc), following boost immunizations

course of disease in vaccinated animals, and that DNA vaccines neither initiate nor accelerate the development of systemic autoimmunity not inducing the production of anti-DNA or antimuscle cell autoantibodies.[39] To our knowledge, meta-analysis of preclinical and clinical trials did not provide any consistent evidence of autoimmune reaction against DNA vaccination itself. The safety margin will be further improved in humans by ultrasound-guided injection of plasmid DNA, as already demonstrated.[40]

In conclusion, we demonstrate that intramuscular DNA vaccination *in-utero* induces safely high levels of anti-HBs already at birth and for a long time after delivery, with a single injection at the appropriate time of pregnancy. Antibody response in our DNA vaccination strategy matches with that obtained in adult humans trials. Antibody levels in the course of standard anti-HBV vaccination protocols in human newborns and young child result in great variations, including high responders and nonresponders at the two extremes.[41] Indeed, pig newborns may be comparable with humans for their body weight and immune development. The relatively low titer of antibody levels in pig newborns that are not the natural host for HBV is always higher than the lowest anti-HBs value considered protective in human newborns.

This protocol also produces a long-lasting immune memory. The data strengthen the clinical relevance of recent reports about short term effects of vaccination *in-utero*.[16,27,42] We extend these observations with a long-term follow-up and an effective and potent 10 months boost immunization. A previous report based on three *in-utero* direct immunizations of the baboon fetus with conventional recombinant hepatitis B surface antigen ascertained detectable antibody responses in 75% (five of eight) of newborn baboons.[42] Not all infants responded to later vaccinations with hepatitis B surface antigen, and follow-up was limited to 190 days. In our protocol, a single *in-utero* DNA immunization produces levels of anti-HBs considered protective in humans in five of nine animals already at birth, and in eight of nine newborns at 3 weeks after delivery. All *in-utero* immunized animals responded to boost immunizations in the course of 2 years follow-up.

Plasmid DNA intramuscular injection will further yield relevant informations by studying the delivery in the fetus of immune-modulating and/or therapeutic molecules,[43,44] in light of the fact that, as we also observe, maternal and fetal factors negatively modulate immune responses in the proximity of delivery.[28]

The present investigation evaluated several clinical safety parameters, including the risk of preterm delivery as a result of the procedure. The absence of premature delivery following *in-utero* DNA immunization at a gestational period corresponding to the human 28–32 weeks support the feasibility of such a technique for the prevention of infections with perinatal morbidity and mortality, such as RSV disease. More extensive data and appropriate experimental design may further analyze this important point for human application. This approach may be a safe, efficient and cost-saving vaccination strategy, generally applicable via ultrasound guidance. Several reports in the current literature have always confirmed that the risk of hepatitis B transmission to the fetus during amniocentesis is low, making this approach feasible in HBV-positive pregnant women.[9,45] This observation corroborates the safety margin of *in-utero* DNA vaccine injection, but needs further investigations in more appropriate animal models.

These data, in view of the USA National Institutes of Health recommendations on vaccine research for the devastating diseases in newborns, may be of clinical relevance for potential vaccines, offering society benefits both by preventing deaths and general suffering, as well as by saving on health-care costs.

Materials and methods

Plasmid vector

We used plasmid pRc/CMV-HBs(S)[26] which express the hepatitis B surface antigen (small, or S, protein) under the control of the CMV immediate-early promoter. This plasmid was used in several experiments both for adult and newborn immunization, and is proposed as a standard vector for validating DNA-based immunization methods (http://www.dnavaccine.com).

'Naked' plasmid DNA preparation

DNA was prepared using Qiagen Endotoxin-free Mega-prep kit (Qiagen Inc., GmbH, Hilden, Germany), according to the manufacturer's protocol. DNA preparations had an A260/A280 ratio in the range of 1.8–2.0. All plasmid preparations were free of detectable chromosomal DNA, RNA, and protein impurities, and contained highly enriched covalently closed circular plasmid DNA,

as checked by gel electrophoresis. Prior to intramuscular injection, DNA was ethanol precipitated, aseptically resuspended in sterile, endotoxin-free, 225 mM NaCl, and stored at −78°C until use.

Animals, surgery, and fetal immunization

Naturally mated sows (Azienda Agricola Rossi, Aranova (Rm), Italy) were maintained under GLP conditions at the 'Stabilimento di Allevamento e di Utilizzazione Animali U.C. S.C.'. Animal experimental protocols were approved by the Italian Ministry of Health.

At 90 or 104 dp, the sows underwent a surgical intervention under general anesthesia. Premedication included ketamine (5 mg/kg bw, Sigma-Aldrich), atropine (7 mg/kg bw, Sigma-Aldrich), and droperidol (17 mg/kg bw, Sigma-Aldrich). Induction of anesthesia was achieved with alothane in oxygen and nitrous oxide (1:1). After positioning an i.v. line, the airway was secured by a cuffed orotracheal tube. Intraoperative monitoring included an EKG trace and continuous clinical evaluation. Intravenous supplementation of anesthesia consisted of vecuronium bromide (0.08 mg/kg bw) for muscle relaxation, and morfine sulfate (0.01 mg/kg bw, Sigma-Aldrich) for intra- and postoperative analgesia. Before surgery, the pregnant pig received a prophylactic dose of 2 g of ampicillin (Sigma-Aldrich) intravenously. The gravid uterus of the pig was exposed and the position of fetuses in each pregnant horn was determined and registered. Each fetus was administered 200 μg of supercoiled plasmid vector resuspended in 150 μl of sterile 225 mM NaCl. DNA injection was performed with a sterile 24G needle through the uterine wall into the right thigh muscles, on the center of the midline between the tail attachment and the hip joint. Following injections, the abdominal wall was closed in two layers with silk sutures. At the termination of the surgical procedure, muscle relaxation was reversed by prostigmine and atropine. Beta-mimetic drugs (5 mg/kg bw die isoxysupryne hydrochlorate, ICN Biomedicals) were administered on the day of surgery and the following day. The animal was allowed to recover after surgery and maintained with free access to food and water, under clinical veterinary monitoring.

Postnatal and boost immunizations

Newborns (7-days old) immunization was performed i.m. with 200 μg of plasmid DNA. Half of the newborns of each group underwent an early postnatal boost with 200 μg of DNA, and all of the newborns that had received *in-utero* intramuscular injection had also one or two rounds of late boosts with 400 μg of DNA. All newborns were bled at delivery, before any boost injection and at several time points for up to 75 weeks after delivery. Continuous veterinary control was performed during the entire experimental time course up to 2 years after birth.

Hepatitis B antibody and antigen detection

The antibody against hepatitis B surface antigen (anti-HBs) was detected using commercially available ELISA test (Ausab EIA, Abbott, Divisione Diagnostici, Roma, Italy). The first incubation step of serum samples and controls with HBsAg-coated beads was performed overnight at room temperature; after washing, HBs peroxidase conjugate was added for 1 h at 40°C and color development was obtained using OPD substrate solution. Absorbance of controls and specimens was determined at 492 nm.

The quantitative measurement was performed using a reference curve obtained by testing a panel of specimens containing known amounts of anti-HBs.

Specimens were tested blindly at least in double, and intra-assay and interassay variations were less than 10%.

Hepatitis B surface antigen (HBs) was detected using commercially available ELISA test (Auszyme, Abbott, Divisione Diagnostici, Roma, Italy). To increase the sensitivity of the assay, the double-incubation procedure was used.

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