Effect of Passive Immunization or Maternally Transferred Immunity on the Antibody Response to a Genetic Vaccine to Rabies Virus

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A plasmid vector, termed pSG5rab.gp, expressing the glycoprotein of rabies virus was tested in young adult or neonatal mice in the presence of maternally transferred immunity or passively administered antibodies to rabies virus for induction of an antibody response. Mice born to rabies virus–immune dams developed an impaired antibody response to genetic immunization at 6 weeks of age, as had been previously observed upon vaccination with an inactivated viral vaccine. Similarly, mice passively immunized with hyperimmune serum showed an inhibited B-cell response upon vaccination with the pSG5rab.gp vector, resulting in both cases in vaccine failures upon challenge with a virulent strain of rabies virus. In contrast, the immune responses of mice vaccinated as neonates in the presence of maternal immunity or upon passive immunization to rabies virus with the pSG5rab.gp construct were only marginally affected.

Vaccines have been the most successful biomedical invention to prevent the morbidity and mortality of humans and animals caused by infectious diseases. Traditionally, vaccines have been based on protein or carbohydrate antigens presented either in the form of whole attenuated or inactivated pathogens or a structural part thereof. The surprising finding that vectors readily transfected cells in situ upon inoculation into skin or muscle tissue (by using either sophisticated propulsion devises or simple syringes), thus causing expression of the encoded protein and in consequence induction of a specific B- and T-cell-mediated immune response, led to the era of genetic vaccines (also commonly referred to as DNA vaccines) (28, 29, 33). Such vaccines, which are small circular pieces of DNA composed of a backbone for amplification and selection in bacteria and a transcriptional unit for translation of a pathogen’s gene in mammalian cells, have a number of advantages over more-traditional types of vaccines. One of the main advantages of vector vaccines, at least for experimenters, is the ease with which they can be constructed and manipulated. Immunologically, genetic vaccines seem to provide their own adjuvant in the form of CpG sequences present in the bacterial backbone (14, 16). Unlike inactivated vaccines, DNA vaccines cause de novo synthesis of proteins in transfected cells, leading to the association of antigenic peptides with major histocompatibility complex class I determinants and hence, the activation of cytolytic T cells (29). In addition, DNA vaccines do not elicit measurable immune responses to the carrier (i.e., the vector DNA [37]), thus allowing their repeated use. Furthermore, in general, plasmid vectors induce an immune response in neonates (3, 12, 30) that, due to the relative immaturity of their immune system, respond poorly to some of the traditional vaccines. Vaccination to many common childhood infections is therefore delayed, rendering young infants susceptible to infections. Neonates are partially protected against prevalent infections by maternally transferred immune effector mechanisms, most notably antibodies (9, 15, 18, 23). Notwithstanding, maternally transmitted immune effector mechanisms inhibit the offspring’s immune response to active immunization (1, 25, 34), providing further impetus to delay childhood vaccinations. This interference lasts well beyond the time span during which the offspring is reliably protected against infection by maternal antibodies (34), thus rendering the offspring highly susceptible to potentially fatal infectious diseases. Novel vaccines that induce a protective immune response in the presence of maternally transferred immune mechanisms in young individuals thus need to be developed. For example, dogs, the main vector in cases of human rabies, are not vaccinated until they are at least 3 months old in order to avoid vaccine failure due to maternally transferred immunity. Nevertheless, cases of human rabies, especially in children, are commonly caused by young dogs not yet eligible for rabies virus vaccination.

Rabies virus vaccination is generally initiated in humans after exposure to the virus by a single dose of hyperimmune serum, given locally to inactivate the virus and by a series of 4 to 12 shots of an inactivated rabies virus vaccine. Antibodies to rabies virus are known to affect the immune response to the viral vaccine (27), thus necessitating multiple active immunizations, an expensive and time-consuming endeavor. Although genetic vaccines are not currently considered for postexposure vaccination to rabies virus due to the slow kinetics of the developing antibody response that in mice requires up to 10 weeks to reach maximal titers (37), they might overcome the negative effect of passive immunization.

We conducted a series of experiments in either young adult or neonatal mice to test the effects of maternally transferred immunity and passively administered antibodies on genetic immunization of mice. Our results show that in adult mice, passively acquired immunity, either by maternal transfer or upon inoculation of hyperimmune serum, strongly reduces the B-cell response to the genetic vaccine. Surprisingly, this effect was much less pronounced upon immunization of neonates born to immune dams or inoculated with hyperimmune serum.

MATERIALS AND METHODS

Mice. Male and female C3H/He mice were purchased from Jackson Laboratory, Bar Harbor, Maine. They were bred by housing 2 females with one male at the Animal Facility of The Wistar Institute. Mice were separated once pregnancies were established. Pups were separated from their dams according to sex at 4 weeks of age. Mice of both sexes equally distributed between the different groups were used for the experiments. Experiments were done two to four times using fairly large groups of genetically immunized mice. The number of mice for
the presented experiments is given in the figure legends. Mice of each experiment were bled several times in monthly intervals to ensure that potential differences were not a reflection of a shift in kinetics.

Cells. Baby hamster kidney BHK-21 cells and 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum as described previously (30).

Viruses. Rabies virus of the Evelyn Rokitniki Abelseth (ERA) strain was grown on BHK-21 cells, purified, and inactivated with betapropiolactone (BPL) (hereafter referred to as ERA-BPL) (31). Rabies virus of the challenge virus strain CVS-24 was propagated in the brain of suckling ICR virus and titrated in adult C3H/He mice by intramuscular (i.m.) inoculation (32). CVS-11 virus was grown and titrated on BHK-21 cells. The E1-deleted adenovirus recombinant expressing the rabies virus glycoprotein of the ERA strain, termed Adrab.gp, was grown and titrated on E1-transfected 293 cells as described previously (39).

Plasmid vector. The pSG5rab.gp vector, which expresses the rabies virus glycoprotein of the ERA strain under the control of the simian virus 40 promoter, was propagated in Escherichia coli DH5a and purified by using kits from Promega or Qiagen according to the manufacturer's specifications. The vector was quantitated by agarose gel electrophoresis against a known standard. Details about construction and testing of this plasmid have been described elsewhere (5, 37, 38).

Immunization and challenge of mice. Adult female mice were immunized two or three times with 5 μg of ERA-BPL virus given i.m. or with 10^6 PFU of Adbrab.gp virus injected subcutaneously (s.c.) prior to mating. Male and female pups were immunized either within 48 h of birth or at 5 to 7 weeks of age with 5 μg of ERA-BPL virus or 50 μg of the pSGrab.gp vector. For booster immunization, mice were injected with 10^6 PFU of Adbrab.gp virus given s.c. Neonates were immunized s.c. with 25 μl or i.m. with 10 μl applied by a Hamilton syringe, adult mice were inoculated i.m. into the quadriceps muscle with 50 μl of vector containing saline. For passive immunization, mice were injected intraperitoneally (i.p.) with 30 μl (neonatal mice) or 100 μl (adult mice) of hyperimmune serum to rabies virus containing 100 IU (per ml) of virus-neutralizing antibodies (VNA) (determined by titration against a National Institutes of Health (NIH) reference serum to rabies virus). Control mice were inoculated with the same amount of a syngeneic normal mouse serum. Experiments were conducted with groups of 4 to 15 mice.

Mice were challenged with 10 mean lethal doses of CVS-24 virus given i.m. Mice were observed daily starting 7 days later. Mice were euthanized once or three times with 5 μg of ERA-BPL virus given i.m. or with 10^6 PFU of Adbrab.gp virus injected s.c. Rabies virus of the Evelyn Rokitniki Abelseth (ERA) strain was grown on BHK-21 cells infected with CVS-11 virus pretreated with serial dilution of heat-inactivated sera as described previously (34). An NIH reference serum to rabies virus was tested at 10 IU for comparison. Data, expressed as international units, were calculated by dividing the VNA titer of the experimental serum by that of the reference serum and multiplying the result by 10.

RESULTS

Effect of maternally transferred immunity on the efficacy of a genetic vaccine in adult mice. We had shown previously that mouse pups born to rabies-virus-immune dams develop impaired B and T helper cell responses upon immunization with an inactivated rabies virus vaccine (34). This inhibition, which is seen in experimental animals as well as in human infants to a number of different infectious agents and vaccines (1, 8, 11), is long lasting and exceeds the time that maternal immunity provides reliable protection to infections of the offspring. Although the mechanism of this inhibitory effect of maternal immunity is poorly understood, maternal antibodies have been implicated to reduce the efficacy of active immunization by neutralization of the vaccine and/or targeting of antigen to immature antigen-presenting cells. Genetic vaccines do not express proteins until de novo synthesis is initiated in transfected cells. Upon inoculation, they are, at the initial stage, not susceptible to neutralization or reactivity by antibodies and might thus potentially provide an avenue to overcome maternal interference.

To test this hypothesis, female C3H/He mice were vaccinated twice with an inactivated rabies virus vaccine of the ERA strain. Control mice were inoculated with saline. Both groups of females were mated with syngeneic males 2 weeks after the second immunization. Pups were vaccinated with either 5 μg of ERA-BPL virus given s.c. or 50 μg of the pSG5rab.gp vector given i.m. when they were ~6 weeks old when maternal antibodies had declined. Mice were bled 6 weeks later, and serum antibody titers were tested by an ELISA on plates coated with inactivated rabies virus. As shown in Fig. 1, upon immunization with either vaccine, pups from rabies virus-immune dams developed reduced antibody titers in comparison to pups from sham-vaccinated dams. The impairment of the immune response to the genetic vaccine was in several experiments slightly less pronounced than that to the viral vaccine; nevertheless, maternally transferred immunity clearly dampened the antibody response to the rabies virus antigen as expressed by the plasmid vector. The pSG5rab.gp vector stimulates a nonspecific response to the viral glycoprotein, the sole target antigen of rabies virus VNA which are the main immune correlates of protection (35). The rabies virus vaccine, on the other hand, induces antibodies to a number of viral proteins, most notably the nucleoprotein in addition to the viral glycoprotein. The same batch of sera tested by ELISA as shown in Fig. 1 was next tested for VNA titers to rabies virus. The results of the biological assay confirmed those of the ELISA; sera of pups from rabies virus-immune dams had reduced antibody titers upon immunization with either of the two vaccines compared to the sera of control pups. Nevertheless, VNA titers were higher in either group of pSG5rab.gp-vaccinated mice than in mice immunized with inactivated rabies virus which gave rise to measurable, albeit low, titers in pups from naive dams but not in pups from immune dams (Fig. 2A). Pups immunized with the genetic vaccine were next, i.e., 8 weeks after immunization, challenged with the mouse adopted viru-
lent CVS-24 strain of rabies virus which is antigenically closely related to the ERA strain. Protection paralleled VNA titers as expected, all of the pSG5rab.gp-vaccinated pups from naive dams remained symptom-free, while 20% of DNA-vaccinated pups from immune dams succumbed to the infection. VNA titers in surviving pSG5rab.gp-vaccinated mice were tested 2 weeks after challenge. Injection of live virus had a clear booster effect, indicating that the vaccine had not induced sterilizing immunity in either group. Again, postchallenge titers were higher in pups born to naive dams than in pups from rabies virus-immune dams (Fig. 2A).

To further ascertain that maternal immunity to the rabies virus glycoprotein impaired the offspring’s B-cell response to the pSG5rab.gp vaccine, 6-week-old pups born to Adrab.gp virus-immune dams were vaccinated with the pSG5rab.gp construct. The Adrab.gp virus is a recombinant that, similar to the genetic vaccine, induces a monospecific response to the glycoprotein of rabies virus as well as responses to the adenovirus antigens (39). Other mice born to the same set of Adrab.gp virus-immune dams were vaccinated with ERA-BPL virus. For comparison, mice from sham-vaccinated dams were inoculated either with the pSG5rab.gp vector or with ERA-BPL virus. Mice were bled 6 weeks later, and serum antibody titers to rabies virus were determined by an ELISA (Fig. 3). Mice born to Adrab.gp-immune dams immunized with either vaccine showed a strongly reduced antibody response which was below the level of detectability in pups vaccinated with the vector. A neutralization assay conducted with the same set of sera confirmed these results; pups born to immune dams vaccinated with either construct developed VNA titers of 1:15 which are at the lowest level of reliable detectability, while pups from naive dams vaccinated with the viral vaccine or the vector had VNA titers of 1:135 and 1:405, respectively.

**Effect of passive immunization on the immune response to the DNA vaccine.** To test if passively transferred antibodies directly affect the efficacy of the genetic vaccine, we tested the immune response to the pSG5rab.gp vector in mice inoculated with hyperimmune serum to rabies virus. Groups of adult C3H/He mice were injected i.p. with 100 μl of a syngeneic hyperimmun serum derived from ERA-BPL virus-immune mice. This serum contained 100 IU of VNA to rabies virus per ml. Control mice were inoculated with an equivalent dose of normal C3H/He mouse serum. Resulting serum VNA titers were determined the following day; mice inoculated with the hyperimmune serum had 3 IU of circulating VNA, and control mice had no VNA. Four days following passive immunization, mice were vaccinated either with 50 μg of the pSG5rab.gp vector given i.m. or with 10 μg of ERA-BPL virus given s.c. Serum antibody titers to rabies virus were tested 6 weeks later by an ELISA. As shown in Fig. 4, mice inoculated with serum to rabies virus developed an impaired antibody response upon vaccination with the inactivated viral vaccine as described previously (34). Inhibition was also seen upon genetic vaccination, confirming the results obtained in mice born to rabies virus-immune dams. Upon challenge with CVS-24 virus, all of the passively immunized mice vaccinated with the pSG5rab.gp construct succumbed to infection, while genetically vaccinated control animals were completely protected.

**Effect of maternal immunity on the immune response of neonatal mice to genetic immunization.** The pSG5rab.gp vector has been shown previously to induce an immune response upon injection into neonatal mice (30). The immune system is in several aspects immature at birth (4, 10), and a vaccine given...
before immunological maturation might be affected differently by maternal immunity than one given thereafter. We therefore tested the effect of maternal immunity on the B-cell response upon genetic vaccination of neonatal mice. Pups born to ERA-BPL virus or sham-vaccinated C3H/He dams were inoculated with 50 μg of the pSG5rab.gp vector given s.c. within 48 h of birth. Control mice received an equivalent dose of a control serum preparation. Four days later, mice were vaccinated with 10 μg of ERA-BPL virus or 50 μg of pSG5rab.gp vector. Antibody titers were determined by an ELISA 6 weeks later using a normal mouse serum for comparison. The genetic vaccine pSG5rab.gp and inactivated rabies virus vaccine ERA-BPL were used. NMS, transfer of normal mouse serum; HIS, transfer of hyperimmune serum; OD (405 nm), optical density at 405 nm.

Effect of maternal immunity on the isotype profile of the antibody response to genetic vaccination. The isotype profiles before immunological maturation might be affected differently by maternal immunity than one given thereafter. We therefore tested the effect of maternal immunity on the B-cell response upon genetic vaccination of neonatal mice. Pups born to ERA-BPL virus or sham-vaccinated C3H/He dams were inoculated with 50 μg of the pSG5rab.gp vector given s.c. within 48 h of birth. The ERA-BPL virus, which induces a poor immune response in neonatal mice, was not included in this set of experiments. At the earliest time point tested, i.e., 1 month after immunization, antibody titers were much higher in pups born to rabies virus-immune dams, which is most likely a reflection of residual maternal antibodies. These antibodies decreased 2 months after vaccination but were still detectable. Later on, at 4, 6, and 8 months of age, the antibody titers of pups from immune dams eventually declined below those of pups from naive dams (Fig. 5A); nevertheless, the differences in titers were marginal compared to that seen upon immunization of 6-week-old pups from naive or rabies virus-immune dams or upon passive transfer of antibodies prior to genetic immunization of adult mice. To ensure that the slight difference observed in pups from immune dams was not within the limits of natural variability (which is rather high upon genetic immunization), 10-week-old mice were given booster immunizations of low doses (i.e., 10⁵ pfu) of an E1-deleted adenovirus recombinant that we have previously reported to boost the immune response to the pSG5rab.gp vaccine (36). As shown in Fig. 5B, both groups of mice rapidly developed an anamnestic B-cell response to the rabies virus antigen that was clearly superior in mice born to naive dams.

Effect of passive immunization on the immune response of neonatal mice to genetic immunization. To further evaluate the effect of preexisting antibodies on the immune response of mice inoculated as neonates with the pSG5rab.gp vaccine, groups of C3H/He mice were injected within 48 h of birth with 1 IU of a hyperimmune serum to rabies virus or an equivalent dose of a normal mouse serum. Both sera were derived from syngeneic donors. Mice were then vaccinated with 50 μg of the pSG5rab.gp vaccine. Antibody titers to rabies virus were tested 3 and 6 months later by an ELISA. As shown in Fig. 6, at both time points, titers from pups vaccinated in the presence of antibodies to rabies virus or a normal serum preparation were indistinguishable, which is in stark contrast to the results obtained upon genetic vaccination of passively immunized adult mice.

Effect of maternal immunity on the isotype profile of the antibody response to genetic vaccination. The isotype profiles...
of antibodies to rabies virus from mice immunized as neonates with the pSG5rab.gp vaccine were determined to establish if the presence of maternally transferred immunity had shifted the type of the response. Sera harvested from pups born to naive or rabies virus-immune dams vaccinated as neonates with the pSG5rab.gp construct were tested by an ELISA on ERA-BPL-coated plates 5 and 7 months later for the distribution of isotypes of antibodies. As shown in Fig. 7, both groups of mice had the same antibody isotype profile to rabies virus, with immunoglobulin G2a (IgG2a) being clearly predominant and indicative of a Th1 type response.

**DISCUSSION**

Several studies using different pathogens have shown that passive immunization, either by iatrogenic inoculation of hyperimmune sera or monoclonal antibodies or by maternal transfer of immune effector mechanisms, impairs the immune response to active immunization (8, 34). The pathways through which passively acquired immunity impedes stimulation of a primary immune response are currently still ill defined. Antibodies are thought to play a pivotal role by neutralizing the antigen or by forming immunocomplexes on specific, naive B cells, thus causing their tolerization. In addition, upon processing of the antibody-antigen complexes, such naive B cells might present antigen fragments to virgin T cells, resulting in their tolerance (17). Immune dams transmit antibodies and lymphocytes to their offspring through the placenta as well as by lactation. In inbred mice, such lymphocytes might conceivably persist for extended periods of time affecting the pup’s primary immune response. Additional mechanisms, such as induction of regulatory T cells in the offspring by maternal immune effector mechanisms, as has been suggested in a mouse malaria model (11), have been postulated.

Genetic vaccines (that at least upon immunization of young adult mice induce an impaired immune response in the presence of maternal antibodies or a passively administered hyperimmune serum) do not express the antigen until de novo synthesis is initiated in transfected cells. The vaccine itself can thus not be affected by maternally or passively transferred antibodies. Passively transferred antibodies might bind to the surface-expressed protein and mask B-cell epitopes. Alternatively, these antibodies might eliminate antigen-expressing cells by triggering complement-mediated cytolysis or antibody-dependent cellular cytotoxicity (ADCC), thus reducing the antigenic load and the duration of antigen expression, two parameters that are presumably crucial for the efficacy of a DNA vaccine. Upon genetic immunization given i.m., the majority of antigen is expressed by non-antigen-presenting cells such as muscle cells. Bone marrow-derived cells, such as dendritic cells, are required to initiate the immune response upon genetic immunization (6). Either of these cells can be transfected by the vector DNA, or they can conceivably reprocess antigen derived from other transfected cells. Reprocessing of cleaved antigen could be affected by antibodies, but considering that the majority of B-cell epitopes of the rabies virus glycoprotein are highly conformation dependent, interactions between antibodies and cleaved fragments of the rabies virus glycoprotein are unlikely to have a major impact.

Surprisingly, the interference of induction of a primary antibody response by maternally or passively administered antibodies was much less pronounced upon genetic vaccination of neonates. Neonates born to rabies virus-immune dams or injected shortly after birth with a fairly high dose of hyperimmune serum to rabies virus developed upon vaccination with...
the pSG5rab gp vector antibody titers to rabies virus that were comparable to those of control mice. It is unclear why young adult mice and neonatal mice responded differently to the genetic vaccine given in the presence of passively acquired immunity. We have shown previously that immunization of neonates with the pSG5rab gp vector resulted in a T helper cell response and antibodies to the rabies virus glycoprotein that were indistinguishable from those seen in adult mice (30). Others have reported comparable results demonstrating induction of cytolytic T cells and protective immunity upon genetic vaccination of newborn mice (3, 12). Nevertheless, data presented here clearly indicate a qualitative difference in the effect of preexisting antibodies on genetic vaccination.

The neonatal immune system is anatomically in place, yet immature in several aspects. Neonates are relatively deficient in production of some cytokines such as gamma interferon (4). Immunoglobulin-negative pre-B cells that are more susceptible to tolerization are commonly found in neonates (21); nevertheless, neonates also have mature B cells that secrete specific antibodies upon activation. Cytolytic T effector cells show variably decreased effector functions (10, 22). Functionally, the neonatal immune system is incapable of generating antibody responses to polysaccharide antigens due to a developmental delay in maturation of the corresponding B-cell subset (20). Alloantigens expressed on lymphoid cells can induce tolerance in some mouse strain combinations, thus allowing successful skin transplantations later on (2, 13). The induction of tolerance to alloantigen can be circumvented by presenting such antigens to T cells with concomitant activation of B cells, resulting in viral persistence (22). We assume that the inability of passively transferred immunity to protect against subsequent viral challenge in mice born to immune mothers is due to the same mechanisms.

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