Virus-Neutralizing Monoclonal Antibody Expressed in Milk of Transgenic Mice Provides Full Protection against Virus-Induced Encephalitis

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Neutralizing antibodies represent a major host defense mechanism against viral infections. In mammals, passive immunity is provided by neutralizing antibodies passed to the offspring via the placenta or the milk as immunoglobulin G and secreted immunoglobulin A. With the long-term goal of producing virus-resistant livestock, we have generated mice carrying transgenes that encode the light and heavy chains of an antibody that is able to neutralize the neurotropic JHM strain of murine hepatitis virus (MHV-JHM). MHV-JHM causes acute encephalitis and acute and chronic demyelination in susceptible strains of mice and rats. Transgene expression was targeted to the lactating mammary gland by using the ovine β-lactoglobulin promoter. Milk from these transgenic mice contained up to 0.7 mg of recombinant antibody/ml. In vitro analysis of milk derived from different transgenic lines revealed a linear correlation between antibody expression and virus-neutralizing activity, indicating that the recombinant antibody is the major determinant of MHV-JHM neutralization in murine milk. Offspring of transgenic and control mice were challenged with a lethal dose of MHV-JHM. Litters suckling nontransgenic dams succumbed to fatal encephalitis, whereas litters suckling transgenic dams were fully protected against challenge, irrespective of whether they were transgenic. This demonstrates that a single neutralizing antibody expressed in the milk of transgenic mice is sufficient to completely protect suckling offspring against MHV-JHM-induced encephalitis.

Coronaviruses are a group of enveloped viruses with a single-stranded RNA genome of positive polarity (37). They are frequently associated with respiratory and gastrointestinal disorders in both animals and humans. Many coronavirus infections are mild in adult animals, whereas they often cause severe and sometimes lethal diseases in neonates (9, 32). To a large extent, this is due to the immature immune system of the newborn host. Maternal antibodies supplied via the placenta and milk efficiently protect newborn animals against the fatal consequences of acute coronavirus infections during this critical phase (14, 15). Cross-fostering experiments have shown that milk-borne antibodies (immunoglobulin A [IgA] and IgG) are sufficient to completely protect newborn mice against lethal doses of murine hepatitis virus (MHV) (15).

Vaccination against coronavirus infections has been employed with various degrees of success (23, 25, 36). The vaccines are usually highly strain specific (16), but they are also dependent on specific routes of infection and often short-lived. Live-virus vaccines are also associated with the danger of in vivo recombination, leading to novel viruses with increased pathogenicity.

Neutralizing monoclonal antibodies generated in response to coronavirus infections have been isolated in many laboratories (12, 35, 42), and it has been shown that antibodies which inhibit virus entry into susceptible cells in vitro can also effectively prevent acute coronavirus-induced disease in vivo (26, 42). Coronavirus infections cause a high mortality only during a short time period (up to 20 days postpartum in mice), which largely coincides with the suckling period. We and others (3, 39) have therefore reasoned that the recombinant expression of neutralizing antibodies in the milk of transgenic animals may provide an effective strategy to protect animals during this critical phase. To provide a proof of principle, we have generated transgenic mice expressing a highly neutralizing monoclonal antibody directed against the neurotropic MHV strain JHM (MHV-JHM). The recombinant antibody was secreted into the milk at yields of up to 0.7 mg/ml. The biological activity of the milk-borne antibody was demonstrated by virus neutralization assays in vitro, and a linear correlation between antibody expression and neutralization was found. When litters suckling transgenic dams were infected with a lethal dose of MHV-JHM, they were completely protected against virus-induced disease, irrespective of whether the newborn mice were transgenic. These results provide the first example of transgene-mediated lactogenic immunity in vivo.

MATERIALS AND METHODS

DNA cloning. Monoclonal antibody (MAb) A1 was selected for these studies because it is highly potent with regard to virus neutralization and inhibition of virus-induced cell-to-cell fusion (42). The isolation and cloning of cDNAs encoding the variable regions of MAB A1 have been described previously (21). In brief, mRNA was isolated from the A1 hybridoma cell line and reverse transcribed. The resulting vx and vy cDNAs were amplified by PCR, using primers which bind in the framework of the variable regions (21). The variable region-
The chimeric antibody carrying the human constant regions is easily identified against the background of murine antibodies in murine milk by immunological analysis of the ovine β-LG gene. The vector also includes the transcriptional initiation site and polyadenylation signal at the end of exon 7 of the β-LG gene, but it lacks the β-LG translational initiation codon (Fig. 1). The first initiation codon in the chimeric mRNA is therefore provided by the inserted AIL or AIH gene sequences (Fig. 1).

Animals. Transgenic mice (F1, CBA x C57BL/6) were produced by pronuclear injection at the animal facility of the Roslin Institute as described previously (4, 43). The plasmids pBJ41-AIL and pBJ41-AIH and the genomic β-LG vector pSS1tgXS (1) were all linearized by digestion with SfiI-XbaI and microinjected at a 1:1:3 ratio. pSS1tgXS carries the entire β-LG coding region, with 4.3 kb of 5′ flanking region and 1.9 kb of 3′ flanking region. An excess of genomic β-LG vector over the expression vector biases for increased frequency of transgene expression (4). Transgenic mice were identified by PCR and Southern blot analysis of genomic DNA. The pBJ41-AIH transgene was detected by PCR, using the primers pBJup (5′-AGC CCT GTC TCA GCC CT 3′) and AIHsp (5′-TGC ATG TGA TGG ACA GCC-3′). The primer pair gives rise to a 264-bp product. The pBJ41-AIL transgene was detected by Southern blot analysis of BsmHI-digested genomic DNA. The blot was hybridized with a pBJ41-AI specific 281-bp PCR product amplified with the primer pair pBJup and AIHsp (5′-CTA AGG TTT TTG CAT TA-3′), using plasmid DNA as template. Transgene copy number was determined by Southern blot analysis on liver DNA from G1 mice. Aliquots (20 μg) of DNA were digested with BamHI, separated on a 1% agarose gel, and blotted to a nylon membrane. The blot was subsequently probed with a 1.6-kb BamHI fragment of β-LG cDNA which is present in all three constructs. Eleven transgenic founder mice were generated, eight of which transmitted the transgenes. These eight lines were used for further analysis.

The virus challenge experiments were carried out with pathogen-free C57BL/6 (B6) mice obtained from the National Cancer Institute (Bethesda, Md.). These mice were seronegative for MHV-JHM. Transgenic females were crossed back with B6 males for five generations and offspring were screened for the presence of the transgenes as described above. For cross-fostering experiments, suckling mice born to B6 females mated with B6 males and transgenic females mated with B6 males were switched within 24 h of delivery.

Protein analysis. Western blot analyses were done essentially as described previously (20). The milk of transgenic mice (isolated at peak lactation, day 10 postpartum) was diluted 1:5 with water and centrifuged at 14,000 x g for 5 min. Three phases were separated: a layer of fat above an aqueous phase and a pellet. The aqueous phase containing the whey fraction was isolated, and aliquots were mixed with reducing and denaturing sample buffer and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The electrophoretically separated proteins were transferred to a nitrocellulose membrane by semidry electrophotography. The constant regions of the chimeric A1 antibody were detected by using a rabbit anti-human IgG-horseradish peroxidase (HRP)-linked antiserum (DianoNova). The results were quantitated by densitometric scanning on a Molecular Dynamics Densitometer.

Cells and viruses. DBT (delayed brain tumor) cells (24) were cultivated at 37°C in minimum essential medium (Life Technologies) supplemented with 10% fetal bovine serum (Sigma), nonessential amino acids, glutamine, and antibiotics. The MHV-JHM strain used for the in vitro experiments was described previously (35). Virus neutralization was quantified in syncytium focus reduction assays. MHV-JHM infections lead to extensive cell-to-cell fusion without inducing cytopathic effects in the first 24 h postinfection. However, syncytium formation (as assessed by syncytia containing more than 100 DBT cells) typically occurs about 48 h postinfection. Thus, the results were quantitated by densitometric scanning on a Molecular Dynamics Densitometer.

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respectively). Mice were monitored daily for mortality and morbidity.

The resulting chimeric open reading frames were inserted into cell fusion. We have isolated the variable regions of MAb A1 described here, the MAb A1 variable regions were linked to the generation of transgenic animals. 

In all, eight lines of transgenic mice were generated. The expression vectors pBJ41-A1L and pBJ41-A1H were microinjected together with the expression vectors pBJ41-A1L and pBJ41-A1H were microinjected into fertilized oocytes at equimolar levels. However, the transgene copy numbers seem to indicate that consistently more copies of the light chain were incorporated into the host genome. The reason for this is unknown, but it may be due to a leaky expression of the transgenes during early developmental stages which, in turn, leads to a cytotoxic overproduction of heavy chain protein and the subsequent loss of embryos. 

In all, eight lines of transgenic mice were generated. The expression vectors pBJ41-A1L and pBJ41-A1H were microinjected together with the β-LG expression construct pSS1tgXS (1) at a ratio of 1:1:3. This vector is expressed in a copy number-dependent manner in transgenic animals (43) and has been shown to increase the expression of coinked transgenes (4). The molecular basis of this “transgene rescue effect” is not known, but it may be due to a leaky expression of the transgenes during early developmental stages which escape the silencing effect of neighboring chromatin structures (4).

Defatted milk of transgenic mothers was analyzed for the light chain protein and the subsequent loss of embryos. The concentration of heavy and light chain protein as quantified by densitometric scanning of Western blots (as described in Materials and Methods).

![Image](https://via.placeholder.com/150)

**FIG. 2.** Western blot analysis of milk samples. Aliquots of defatted milk samples (corresponding to 1 μl of milk) isolated from mice of six transgenic lines were separated by 15% polyacrylamide gel electrophoresis alongside a human IgG standard (1,000, 300, and 100 ng of IgG) and blotted onto nitrocellulose. The human IgG portion of the chimeric recombinant antibody was detected by using an HRP-linked rabbit anti-human IgG antiserum. The blot was developed using a chemiluminescent detection system (Pierce). The positions of the antibody chains and the sizes of the molecular marker proteins are indicated.
No correlation between the transgene copy numbers and the levels of antibody expression could be detected (Table 1). We interpret this to indicate that the site of transgene integration had a dominant effect on gene expression. Although expression of the light chain protein was consistently higher than expression of the heavy chain protein, this did not directly correlate with transgene copy number. Additionally, no correlation could be detected between the levels of β-LG protein expression and antibody expression (Table 1).

The concentrations of the heavy and light chain proteins were quantified by densitometry in comparison to IgG standards (Beriglobin; pooled human immunoglobulin; Behringwerke). Maximum levels of 0.5 and 2.9 mg/ml, respectively, of heavy and light chain proteins were obtained in transgenic line HEP50. The heavy chain, which comprises 69% of the total molecular weight of an IgG molecule, can only be secreted from cells as part of a complete antibody. In contrast, the light chain protein can be secreted individually. Therefore the 0.5 mg of heavy chain protein per ml measured in the milk of transgenic line HEP50 corresponds to a total recombinant IgG concentration of 0.7 mg/ml. The heavy chain, which comprises 69% of the total molecular weight of an IgG molecule, can only be secreted from cells as part of a complete antibody. In contrast, the light chain protein can be secreted individually. Therefore the 0.5 mg of heavy chain protein per ml measured in the milk of transgenic line HEP50 corresponds to a total recombinant IgG concentration of 0.7 mg/ml.

The defatted milk of transgenic line HEP50 was subsequently analyzed in a virus neutralization assay. A 10⁻⁴ dilution of defatted milk reduced MHV-JHM infectivity by 90% (Fig. 3A), which corresponds to 125 pg of IgG being required to neutralize 1 PFU of MHV-JHM. This is consistent with results we have obtained in cell culture, where 100 pg of IgG was required to neutralize 1 PFU of MHV-JHM (21). Milk isolated from nontransgenic mice did not show any neutralizing effect against the MHV-JHM infection (Fig. 3A). This confirms the fact that the breeding colony of mice was seronegative for MHV-JHM and also that the recombinant antibody is the decisive virus-neutralizing factor in milk. Defatted milk samples from four other transgenic lines were also analyzed by neutralization assays. All of the samples neutralized MHV infectivity, albeit with different levels of efficacy (Fig. 3B). The dilutions at which MHV-JHM infectivity was reduced by 90 and 50% were correlated with expression of the heavy chain (which is the limiting factor for antibody formation) (Fig. 3C). The linear correlation observed confirms that the concentration of the recombinant antibody in the milk of these transgenic animals is the critical factor determining neutralization activity.

Finally, the ability of recombinant antibody secreted in breast milk to protect suckling mice in vivo was determined. Transgenic mice and their nontransgenic littermates were infected intranasally with virulent MHV-JHM. Under these conditions (31), 100% of naive mice succumb to acute, fatal encephalitis by 5 to 7 days postinoculation. However, as shown in Table 2, 23 of 23 suckling mice nursed by transgenic dams did not develop acute encephalitis, whereas 6 of 6 suckling mice nursed by transgene-negative animals died by 7 days postinfection (Table 2). Infected mice nursed by transgenic dams also grew at the same rate as uninfected mice nursed by transgenic
TABLE 2. MHV-specific antibody in breast milk protects mice from acute encephalitis

<table>
<thead>
<tr>
<th>Dams (litters)</th>
<th>Offspring (no.)</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEP50[+] (5)</td>
<td>HEP50[+] × B6 (23)</td>
<td>0</td>
</tr>
<tr>
<td>HEP50[−] (1)</td>
<td>HEP50[−] × B6 (6)</td>
<td>100</td>
</tr>
<tr>
<td>HEP36[+] (3)</td>
<td>HEP36[+] × B6 (20)</td>
<td>0</td>
</tr>
<tr>
<td>HEP36[+] (2)</td>
<td>HEP36[−] × B6 (8)</td>
<td>100</td>
</tr>
</tbody>
</table>

*Transgenic mice are designated HEP50[+] and HEP36[+]. B6 mice are nontransgenic control mice. Nontransgenic litters of HEP50[+] mice are designated HEP50[−]. Offspring from transgenic mice (HEP50[+] and HEP36[+]) females mated with B6 males, nontransgenic mice (HEP50[−] females mated with B6 males) and naive B6 mice were challenged intranasally with MHV-JHM as described in Materials and Methods. Mortality was monitored in the indicated number of litters of suckling mice nurshed by transgenic (HEP50[+]) or nontransgenic (HEP50[−]) dams.

Two features distinguish transgene-mediated lactogenic immunity from the natural process of passive immunization by maternal antibodies in the milk of transgenic animals. Milk-borne antibodies have been shown to be an efficient means of preventing infectious disease in mammals (5, 6, 8, 11, 17, 18). Synthetic maternal neutralizing antibodies can be induced by vaccines or natural infections. In some instances (e.g., if vaccination is ineffective or if infection with live pathogens results in high mortality), alternative routes have to be employed to provide passive immunization to mammalian offspring. Purified neutralizing MAbs can be injected into pregnant or lactating host animals (5) or directly administered to neonates (2, 38). In order to generate milk which contains antibodies of therapeutic value, animals which are not the natural host (preferably ruminants, which yield large amounts of milk) can be immunized with live pathogens (7, 17).

We have analyzed whether expressing a neutralizing antibody as a recombinant protein in the milk of transgenic animals may provide an alternative to these procedures. This transgenic approach is particularly useful if (i) no useful vaccines are available, (ii) neutralizing antibodies are a major component of the host’s defense against the pathogen in question, and (iii) the period during which the infection is life-threatening is limited. The use of transgenic animals offers the additional advantage that the most potent antibodies can be selected by in vitro analyses before transgenes are established from the respective hybridoma cell lines. Moreover, no live pathogens have to be introduced into an animal colony. Coronavirus, which cause diseases of economic importance in animals and humans, fulfill all of the above-mentioned criteria. Therefore, transgenic animals that express neutralizing antibodies in the milk may, in the long term, provide a strategy to protect animals during the suckling period. The experiments described here demonstrate that this approach is practicable. However, antibody isotypes mediating lactogenic immunity vary between different species (27). There are also profound differences in the intestinal uptake of immunoglobulins (27). Transgene-mediated strategies manipulating lactogenic immunity in livestock will therefore have to be adapted to the requirements of individual species in terms of immunoglobulin isotype and expression strategy. In that respect, the transgenic mouse-MHV model provides an excellent opportunity to determine the critical factors for successful immune protection through in vivo challenge experiments.

Two features distinguish transgene-mediated lactogenic immunity from the natural process of passive immunization by
maternal antibodies. In the natural situation, newborn animals are provided with a polyclonal mixture of antibodies against a particular pathogen. The transgenic dams described here only provide a single, albeit highly neutralizing, antibody to their offspring. As has been shown previously, the β-LG-based expression system is able to supply immunoglobulin at a high concentration throughout the entire lactation period (39). In mammals, the total immunoglobulin concentration in colostrum ranges between 5 mg/ml (rat) and 250 mg/ml (cow). Lower immunoglobulin levels, ranging from 1 mg/ml (human) up to 10 mg/ml (pig), are found in milk (27, 41). Therefore, the concentration of recombinantly expressed neutralizing antibody of 0.7 mg/ml exceeds the concentration of any single monospecific antibody present in milk by some orders of magnitude. Nevertheless, there appeared to be no selection of virus escape mutants, which could overcome the immune protection and lead to overt disease within the time frame of the experiment. This indicates that production of a single MHV-JHM neutralizing antibody in milk is sufficient to provide full protection during the suckling period.

Milk proteins and their derivatives have been shown to have antibacterial and antiviral effects (29, 34, 44). In our experimental model, the neutralizing effect of the mouse milk samples was dependent on the concentration of the recombinant antibody. This suggests that no other natural components of milk provide a protective effect against MHV-JHM infections.

The levels of antibody production and secretion into milk are critical factors for the establishment of transgene-mediated virus resistance. The concentration of 0.7 mg of mature IgG per ml is at the lower end of expression levels that have been previously reported for other recombinant antibodies expressed in the milk of transgenic mice (0.4 mg/ml [28], 0.8 mg/ml [30], 4 mg/ml [13], 5 mg/ml [3], and 6 mg/ml [39]). Nevertheless, complete protection of litters suckling transgenic dams could be obtained at these expression levels. The comparably low expression level is at least in part due to the failure to produce equimolar amounts of heavy and light chain protein (compare Table 1 and Fig. 2). This confirms our previous experiences with cell culture (21) and data published by others (33, 44). The consistent excess of light chain protein over heavy chain protein is due to the cytotoxicity of unpaired heavy chains (19), which leads to the death of cells in which the heavy chain is overexpressed. Additionally, we found that the MAb A1 heavy chain transgene was present at a lower copy number than the MAb A1 light chain construct in all strains of mice analyzed. This is consistent with the findings of Sola and colleagues (39), who used a β-LG-based expression system to express a recombinant antibody that neutralizes the porcine coronavirus transmissible gastroenteritis virus in the milk of transgenic mice. In contrast, when an expression system based on the whey acidic protein (WAP) promoter was used to express IgG-encoding genes, most of the transgenic lines generated carried an excess of heavy chain expression constructs (3). One possible explanation is that the β-LG promoter is active at an early stage of development and causes the loss of embryos in which the (cytotoxic) heavy chain is overexpressed. Transgenic animals in which the two antibody chains are expressed in equimolar amounts can be generated by microinjection (33) but may require the screening of a larger number of transgenic lines.

In conclusion, we have demonstrated that high levels of a virus-neutralizing antibody can be generated in the milk of transgenic mice and that full protection against virus-induced disease can be accomplished in newborn animals via this route. Thus, in the murine system, we have established a proof of principle, and the application of this technology to generate virus-resistant animals can now be pursued. Additionally, it now seems feasible that ruminants could be modified by transgenic methodology to produce milk containing neutralizing antibodies directed against pathogens responsible for major human infant diseases.

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