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Engineering passive immunity in transgenic mice secreting virus-neutralizing antibodies in milk

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Protection against enteric infections can be provided by the oral administration of pathogen-neutralizing antibodies. To provide passive immunity, 18 lines of transgenic mice secreting a recombinant monoclonal antibody (Mab) neutralizing transmissible gastroenteritis coronavirus (TGEV) into the milk were generated. The genes encoding a chimeric Mab with the variable modules of the murine TGEV-specific Mab 6A.C3 and the constant modules of a human IgG_1 isotype Mab were expressed under the control of regulatory sequences derived from the whey acidic protein, which is an abundant milk protein. The Mab 6A.C3 binds to a highly conserved epitope present in coronaviruses of several species, which does not allow the selection of neutralization escape mutants. Antibody expression titers of 10^6 were obtained in the milk of transgenic mice that reduced TGEV infectivity 10^6-fold. The antibody was synthesized at high levels throughout lactation. Integration of matrix attachment region sequences with the antibody genes led to a 20- to 10,000-fold increase in the antibody titer in 50% of the transgenic animals. Antibody expression levels were transgene copy number independent and related to the site of integration. The generation of transgenic animals producing virus neutralizing antibodies in milk could provide an approach to protection against neonatal infections of the enteric tract.

Keywords: applied immunology, antibody engineering, mucosal immunity, coronavirus

The mucosal immune system and one of its predominant effectors, immunoglobulin, provide the initial immunologic barriers against most pathogens that invade the body at a mucosal surface[1]. This is especially true for viruses, and resistance to infection has been frequently correlated with the presence of specific antibodies in mucosal secretions[2]. Of particular interest is lactogenic immunity in which the antibodies in milk confer immunity to newborns[3-5]. This suggests that the oral administration of virus-neutralizing monoclonal antibodies (Mabs) may protect mucosal tissues from infection by viruses[5-7].

Transgenic technology has allowed the expression of novel proteins in milk under the control of abundant milk protein regulatory sequences, such as whey acidic protein (*WAP*) and β-lactoglobulin[8,9]. This approach could be extended to the expression of virus-neutralizing antibodies to provide protection against enteric infections[10].

Transmissible gastroenteritis is an important porcine disease that causes a mortality close to 100% in newborn animals[4,5]. This disease is caused by transmissible gastroenteritis coronavirus (TGEV) that infects enteric and respiratory tissues. Pathogenesis is induced following enteric tract infections, but respiratory infections generally are benign. The immune response to TGEV has been characterized[4,11], and full protection against this virus can be provided by passive immunity from immune sows. Nonetheless, TGEV is a major herd health problem[4]. Bottle feeding of newborn animals may target the antibody to epithelial surfaces providing protection against enteric virus infection[4,6,12], but this approach is not practical. Alternatively, transgenic animals secreting virus-neutralizing antibodies in their milk during lactation could protect piglets against infection. To explore this approach, the expression of a recombinant TGEV-neutralizing Mab with a human IgG_1 isotype ($rIgG_1$) under the control of *WAP* regulatory sequences was designed in order to target the expression of $rIgG_1$ to the mammary gland.

The major antigenic sites of TGEV involved in the induction of virus-neutralizing antibodies are located in the globular portion of the spike protein[13]. Studies on TGEV neutralization and genetic variability led to the identification of mouse Mab 6A.C3, which neutralized all TGEV isolates tested including TGEV-related coronaviruses infecting porcine, canine, and feline species[14,15]. As no neutralization escape mutants (*mar* mutants) were selected, it is likely that this Mab binds to an epitope essential for viral replication[15].

Matrix attachment regions (MARs) increase and normalize the control of transgene expression by flanking the transcription units and isolating them from the negative influences of surrounding regions[16] or by modifying the DNA[17,18]. Therefore, the effect of MARs on antibody gene expression was studied by microinjecting mouse embryos with expression constructs carrying the genes encoding light and heavy immunoglobulin chains alone or together with MAR elements.

We demonstrate the genetic engineering of mice that secrete high titers of virus-neutralizing Mabs into the milk of transgenic mice during lactation. This strategy may be of general utility in providing protection against many infections of the enteric tract.

Results

Engineering a TGEV neutralizing recombinant antibody. A recombinant TGEV-neutralizing antibody was constructed by

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fusion of Mab 6A.C3 light chain (LC) or heavy chain (HC) variable modules to human constant modules. To verify the functionality of the chimeric $rIgG_1$ 6A.C3, COS-1 and murine Sp2/0 myeloma cells were transiently and stably transformed, respectively, with constructs encoding the chimeric H and L chains. Antibody expression levels ranging from 20 to 40 µg/ml were detected in the supernatant. The secreted chimeric immunoglobulins bound TGEV by radioimmunoassay (RIA) with titers up to 10^3 and neutralized viral infectivity in a plaque reduction assay approximately 10^4-fold (neutralization indices [NIs] around 4)[7] indicating that the recombinant Mab (rMab) had the expected biological activity.

Production of transgenic mice stably transmitting three transgenes. In order to generate transgenic mice expressing mouse-human $rIgG_1$ 6A.C3 in the milk, rMab 6A.C3 was expressed under the control of *WAP* gene regulatory sequences by inserting immunoglobulin gene cDNAs encoding LC and HC of rMab 6A.C3 into exon 1 of genomic *WAP* DNA. To study whether antibody expression under the control of *WAP* regulatory sequences was now hormone-inducible in epithelial cells, HC11 mouse mammary gland cells[19] were stably transformed with the WAP-immunoglobulin constructs. Selected transformed cells were stimulated with a combination of lactogenic hormones including prolactin, insulin, and hydrocortisone. High $rIgG_1$ titers (10^3) in the supernatant were detected by RIA after hormone induction (Fig. 1). Antibody expression was lactogenic hormone–dependent, with low levels of constitutive expression in the absence of hormone stimulation. As expected, no hormone induction was required when the antibody genes were cloned after SV-40 regulatory sequences. Mouse mammary gland epithelial cells synthesized, assembled and secreted functional recombinant antibodies.

WAP gene-based expression cassettes[20] encoding LC and HC were comicroinjected into the pronucleus[21]. A quantitative summary of the generation of transgenic mice with cDNA constructs microinjected in the presence or in the absence of MAR sequences is provided in Tables 1 and 2. In some cases, the expression cassettes were comicroinjected with an equimolar amount of MARs (Table 2). To identify founder animals carrying transgenes, genomic DNA isolated from tail biopsies of the 278 animals born was first screened for the presence of the WAP-LC and -HC transgenes by PCR using primer pairs that hybridized with the *WAP* gene (5′ primer) and with the immunoglobulin gene (3′ primer). The pronuclear injection of these chimeric constructs led to the generation of a total of 46 transgenic mice that had integrated at least one of the transgenes (Table 1). The majority of transgenic mice (35/46) had cointegrated the transgenes encoding both the LC and HC of $rIgG_1$ (Table 2). In most of the transgenic mice (33/46) the immunoglobulin genes were comicroinjected with MAR sequences. The majority of the transgenic mice that integrated both the LC and HC (27/33) also contained integrated MAR sequences (data not shown). Transgenic founder animals carrying both MAR sequences and HC and LC genes were bred for further analysis.

Nineteen out of 35 animals (i.e., 2/8 transgenic mice that did not integrated MAR sequences [MAR^-] plus 17/27 transgenic mice that had integrated MAR sequences [MAR^+] and that also had integrated both the heavy and light immunoglobulin gene chains) [Table 2] transmitted all of these genes to their progeny for at least two generations as determined by analyzing 4 to 10 offspring of each line (data not shown).

Expression of a functional coronavirus-neutralizing $rIgG_1$ in the milk of transgenic mice. Milk was collected from each G1 mouse that transmitted both transgenes. $rIgG_1$ was detected by RIA in the milk using TGEV as an antigen. Transgenic MAR^- or MAR^+ mice produced high titer TGEV-specific antibodies in the milk (Table 2 and Fig. 2). Two of the five MAR^- transgenic mice (Table 2) secreted TGEV-specific antibodies with titers around 1×10^2 as

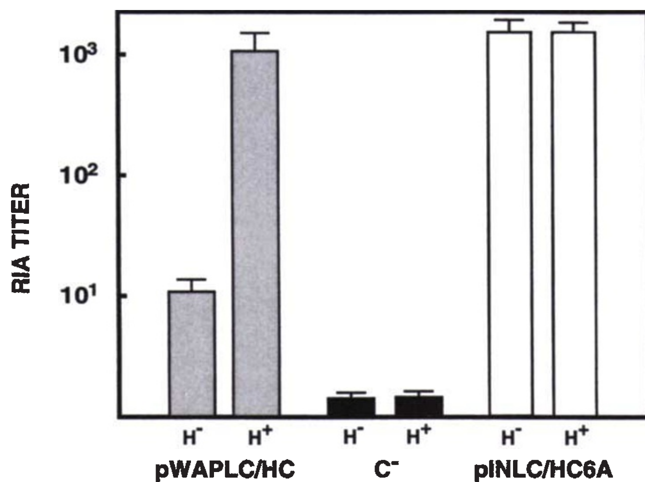

Figure 1. Analysis of the expression of recombinant IgG_1 under the control of the *WAP* promoter in transformed cells. Constructs with the chimeric immunoglobulin genes under the control of the *WAP* promoter were tested for stable expression in transformed HC11 cells. Antibody synthesis was evaluated by RIA against TGEV without (H^-) or after (H^+) induction with peptide and steroid hormones (prolactin, insulin, and hydrocortisone). pINLC/HC6A: chimeric immunoglobulin genes expressed under the control of the SV-40 promoter; C^-: supernatant from nontransfected cells. The data are the mean of the antibody titers secreted by the cell line producing optimum antibody levels. Bars indicate the standard deviation of three independent evaluations.

Table 1. Transgenic animals with cDNA constructs generated with or without MAR sequences.

MAR sequences	Microinjected embryos	Transfer embryos	Animals born	Animals weaned	Transgenics
MAR^-	1398	904 (65)	61 (7)	58 (95)	13 (22)
MAR^+	2039	1564 (77)	227 (15)	220 (97)	33 (15)

Numbers in brackets indicate the percentage of animals in relationship to the preceding step.

Table 2. Analysis of transgene integration, transmission, and expression.

Integration of MAR sequences	MAR^{-a}			MAR^{+a}		
Transgenic animals (G0)	13			33		
	LC	HC	LC+HC[b]	LC	HC	LC+HC[b]
Number of mice carrying transgene	2	3	8	3	3	27
Number of mice transmitting the transgene	0	3	4[c] 2[d] 2[e]	1	3	3[c] 7[d] 17[e]
Number of mice expressing rMab in milk		3	2	ND	3	16

[a]MAR^+ and MAR^- indicate the presence or absence of MAR sequences. [b]LC, HC, and LC+HC indicate the presence of light, heavy, and light and heavy chain transgenes, respectively as determined by PCR. [c]Number of transgenic mice that were sterile. [d]Number of transgenic mice not transmitting the transgene. [e]Number of transgenic mice transmitting both the light and the heavy chains.

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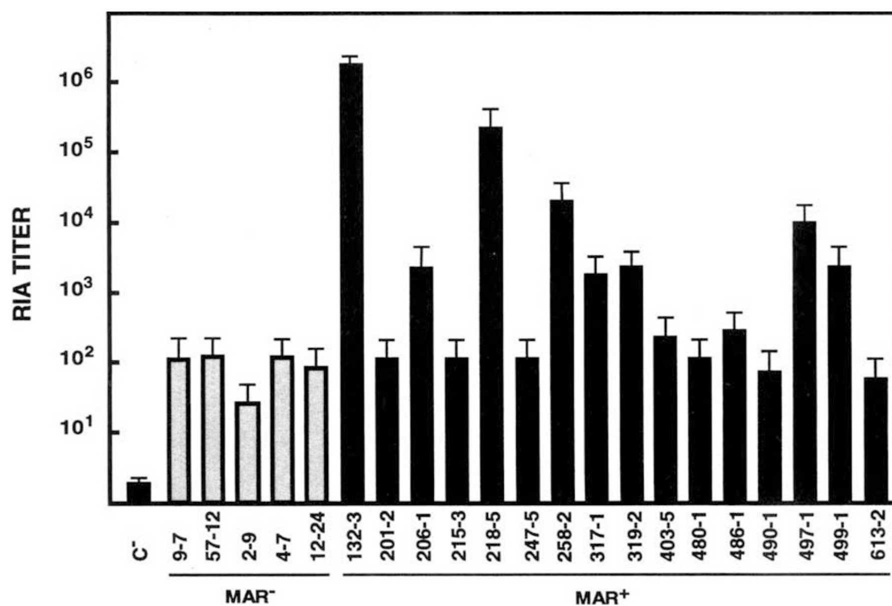

Figure 2. Analysis of rIgG$_1$ expression in the milk of transgenic mice. Milk from five transgenic mice generated by microinjection of immunoglobulin gene expression cassettes without the MAR sequences (MAR⁻) was evaluated for the presence of either TGEV binding antibodies or the HC of this recombinant Mab, using RIA or a double antibody sandwich technique, respectively[14,15]. Two of these mice (9-7 and 57-12) secreted TGEV specific antibody, and three of them (2-9, 4-7, and 12-24) secreted the HC of the same recombinant antibody. Milk from 16 transgenic lines that had integrated both the immunoglobulin genes and the MAR sequences (MAR⁺) were analyzed by RIA[14,15] using purified TGEV as antigen. Four to ten mice of each line were analyzed and the mean ± the standard deviation of the maximum titers obtained during lactation are shown. C⁻: milk from nontransgenic mice.

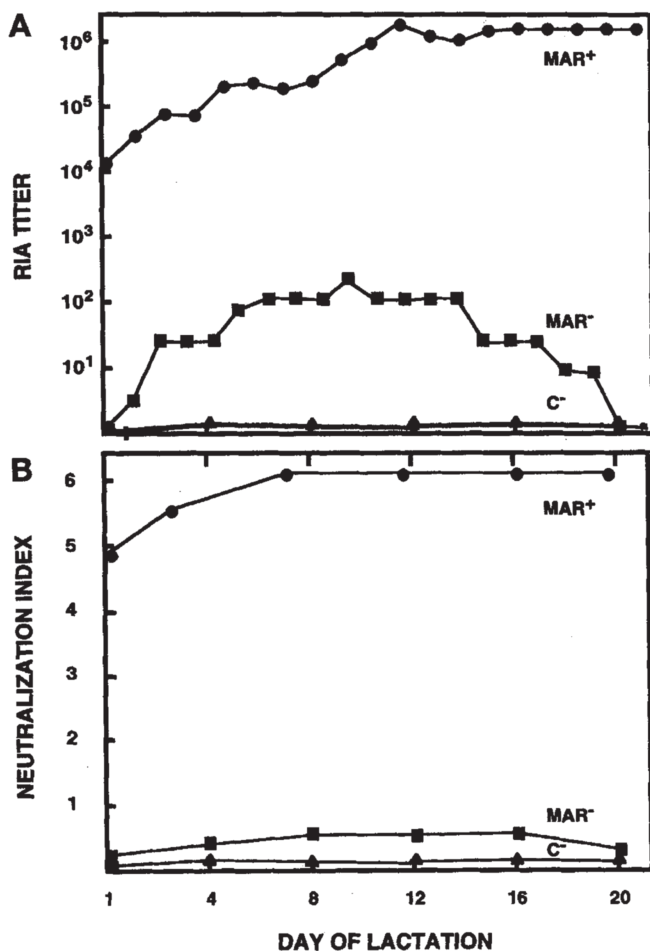

Figure 3. Expression of rIgG$_1$ in the milk throughout lactation. (A) Milk samples from two transgenic mice (57-12, MAR⁻; and 132-3, MAR⁺) were collected during lactation. Antibody expression levels were analyzed by RIA. C⁻: antibody titers of milk from nontransgenic mice. **(B)** Neutralization of TGEV by milk samples collected during lactation from transgenic mice 132-3 and 57-12. C⁻: neutralization index of milk from nontransgenic mice.

determined by RIA, and three produced the HC alone with titers between 10^1 and 10^2 as determined using a double antibody sandwich (DAS) technique (Fig. 2) indicating that the cointegration of MAR sequences was not an essential requirement for the expression of immunoglobulin genes. Interestingly, in the mice that had cointegrated MAR sequences, antibody titers in the milk ranged between 10^2 and 10^6 fold, and in 50% (8/16) of these mice antibody expression levels were increased 20- to 10,000-fold relative to the levels produced by the MAR⁻ mice.

The stability of antibody production in the milk was studied by analyzing four to ten mice for at least two generations in the 18 transgenic lines of mice secreting the antibody. In addition, two MAR⁻ mice and two MAR⁺ were analyzed for three generations. Within each line of transgenic mice, no significant variation in rIgG$_1$ expression levels was observed (Table 2). The lack of variation in the antibody production was probably due to the cointegration in the chromosome of both immunoglobulin genes and MAR sequences with a conserved gene arrangement.

In selected mice that had integrated MAR sequences and immunoglobulin genes antibody levels were already high (titers > 10^4 both by RIA and neutralization) on the first day of lactation (Fig. 3). In all cases (MAR⁻ and MAR⁺), maximum antibody titers in the milk were achieved by around day 10 of lactation (Fig. 3). At this time, rIgG$_1$ expressed in the milk of two mice (132-3 and 218-5) showed the highest neutralization indices (NI = 6), and reduced

Table 3. Relationship between transgene copy number and antibody expression levels.

Transgenic mice		Transgene copy number		Expression*
		Light chain	Heavy chain	
MAR⁻	9-7 57-12	10	40	$1–5 \times 10^2$
MAR⁺	132-3	1	3	$1–8 \times 10^6$
	218-5	3	12	$1–5 \times 10^5$
	317-1	2	3	$1–5 \times 10^3$
	258-2	2	2	$1–5 \times 10^4$

*Titer, highest dilution giving a binding threefold the background.

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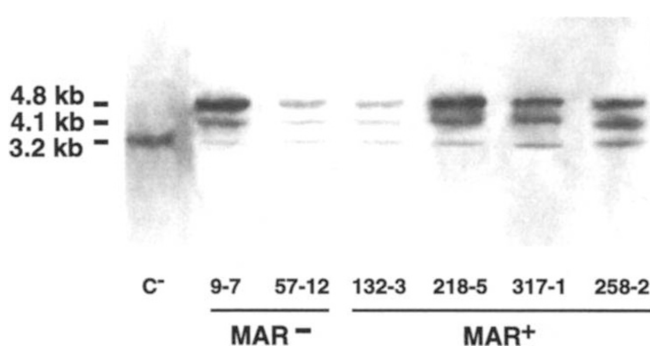

Figure 4. Relationship between transgene copy number and antibody expression levels. Southern blot analysis of WAP-immunoglobulin transgene integration. Genomic DNA from transgenic mice was hybridized with a *WAP*-specific probe. The bands shown correspond to WAP-HC transgene (4.8 kb), WAP-LC transgene (4.1 kb) and endogenous *WAP* gene (3.2 kb) sequences. MAR$^-$ and MAR$^+$ indicates the absence or the presence of MAR sequences. C$^-$: nontransgenic mice.

virus infectivity 10^6-fold. The rIgG$_1$ concentration in the milk was estimated by RIA using a standard curve generated with known amounts of purified rIgG$_1$ 6A.C3. Recombinant IgG$_1$ production ranged from 0.005 mg/ml to approximately 5 mg/ml in different mice (Figs. 2 and 3).

To determine whether antibody expression levels were transgene copy number dependent, transgenic mice carrying rIgG$_1$ genes were analyzed by Southern blot hybridization using a probe specific for a promoter sequence present in the expression cassette for the LC and HC genes and in the endogenous *WAP* gene. DNA extracted from the tail of transgenic mice was digested with restriction endonuclease BglII to identify fragments of 4.8, 4.1, and 3.2 kb (Fig. 4), which corresponded to the genes encoding the heavy and light rIgG$_1$ chains and the endogenous *WAP* gene, respectively. The intensity of the endogenous *WAP* gene band was taken as the reference to estimate the integrated immunoglobulin gene copy number[22]. Expression levels were independent of transgene copy number (Table 3), and the highest expression levels with titers of 10^6 were obtained with MAR$^+$ mice that had integrated one and three copies of the LC and the HC rIgG$_1$ genes, respectively. No apparent adverse phenotype was associated with the expression of high concentrations of mouse-human rIgG$_1$ in the milk of the transgenic mice and no major effect of recombinant antibody expression on other immunoglobulin levels in the milk was observed by Western blot analysis, although the impact on the overall protein production in the milk has not been determined.

Discussion

To engineer passive immunity in order to protect against mucosal infections we have used as a model TGEV, which produces high mortality in suckling piglets. To this end, transgenic mice expressing a TGEV-neutralizing Mab in the milk with high titers under the *WAP* promoter have been constructed. This Mab neutralizes all known TGEV strains and does not lead to the selection of neutralization escape mutants[13].

Ninety-five percent (18/19) of the transgenic mice transmitting the genes for both LC and HC (WAP-LC and WAP-HC) secreted the antibody in milk. The amount of functional rIgG$_1$ estimated in the milk of two transgenic mice (5 mg/ml) represents a 250-fold increase over that obtained using the same constructs in stably transformed mammalian cell lines[7], and it is one of the highest concentrations of a polymeric recombinant protein observed in mammalian expression systems[8].

The results of viral binding and neutralization obtained with the antibody present in the milk of transgenic animals showed functional behavior identical to that secreted by the original hybridoma. This indicates that epithelial cells of the mouse mammary gland (a non-B-lineage cell) perform adequate post-translational processing required for the correct expression and assembly of antibody molecules. The reduction in TGEV infectivity, higher than 10^6-fold, observed with milk samples of selected transgenic animals, suggested that the antibody secreted could be sufficient to protect offspring against TGEV infection, similar to the protection provided by lactogenic immunity using immune sows[4,6]. Using the same *WAP* regulatory sequences, protein C has been successfully expressed in pigs[23,24]. These results suggest that the expression of rIgG$_1$ under the *WAP* promoter probably will work in pigs. High levels of rIgG$_1$ were detected in the milk of the transgenic mice from the first day of lactation, which supports the possibility of an effective protection of newborn offspring against TGEV infection. The kinetics of antibody production during lactation showed no reduction in the antibody synthesis after midlactation in transgenic mouse 132-3. Identical results were observed after two gestation periods of the same transgenic mice, but in others, a reduction in antibody synthesis after midlactation was detected (data not shown).

The change in milk composition caused by the production of the recombinant antibody in the transgenic mice was not accompanied by any apparent side effect during their normal lifespan. In addition, no significant weight loss or perinatal mortality was observed in the pups suckling milk containing rMabs. These data indicate that the changes introduced in milk composition did not affect normal mammalian development.

In order to optimize the number of transgenic animals efficiently expressing the antibody, MAR sequences were coinjected with the rIgG$_1$ genes[25]. MAR sequences have been shown to increase the frequency of transgene expression by flanking the transcription units and isolating transgenes from negative regulatory signals present in the flanking chromatin[16,25]. Once the isolation is accomplished, expression levels proportional to transgene copy number may be expected[26,27]. In our experimental model system the transgenic mice that were not comicroinjected with MAR sequences also expressed rIgG$_1$. Consequently, we could not assert the effect of MAR sequence inclusion on the frequency of antibody expressing mice. Among the mice that cointegrated MAR sequences with the rIgG$_1$ genes, no direct relationship between transgene copy number and rIgG$_1$ expression levels in the milk was found. This suggests that transgene integration site determines transcriptional levels, as previously reported[28]. Interestingly, the highest expression levels were obtained only when MAR sequences were cointegrated with the antibody genes. The RIA titers for immunoglobulin production in the five MAR$^-$ mice were always below 10^2, while 8/16 MAR$^+$ mice secreted antibodies with significantly higher titers. Nevertheless, the integration of MARs did not guarantee an increase in antibody production.

The transgenic animals secreting neutralizing rMabs have been designed to provide neonates with resistance to infections of the enteric tract by coronavirus. Other potential applications of the generation of transgenic animals by introducing beneficial genes to improve health and disease resistance have been described based on specific immunity[29–33] and on generic resistance to infectious agents[34]. Previous publications have reported immunoglobulin expression in lymphoid cells of transgenic animals[31]; however, the association of the transgenic immunoglobulin chains with endogenous immunoglobulins led to heterologous immunoglobulins that lost their expected specificity. The production of chimeric antibodies in milk has also been described[35], but in this case, antibodies were not directed against an infectious agent and resistance to disease was not pursued.

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The modular approach used to obtain recombinant antibodies by linking variable to constant immunoglobulin modules could easily be applied to antibodies with different therapeutic purposes. As there is a large number of known Mabs that neutralize enteric pathogens, this approach could be extended to the generation of transgenic animals resistant to a variety of enteric infections of the newborn.

Experimental protocol

Virus and antibody evaluation. Strain PUR46-MAD of TGEV was used in all experiments[14,15] and was grown and titrated using swine testis cells[14,15]. Viral NIs were determined using a plaque reduction assay by incubating 10-fold dilutions of the virus with one volume of 10-fold dilutions of the antibody-containing supernatant or milk samples. Dilutions were performed in phosphate buffered saline with 2% fetal calf serum, and the virus-antibody mixtures were incubated at 37°C for 30 min and titrated[14,15]. NIs were defined as the ratio between the virus titer in the presence of a control or the indicated antibody and were expressed as the $\log_{10}$ of this ratio. Antibody titers were determined by RIA using purified TGEV as the antigen[14,15], with the RIA titer defined as the inverse of the highest dilution giving a binding to purified TGEV virus threefold higher than the background. Immunoglobulin chain titers were determined by a DAS similar to the RIA. Briefly, the presence of the HC was detected by coating the wells with protein A saturated with rabbit antihuman antibody HC to capture the recombinant HC. Milk dilutions were added, and the plates were incubated at 37°C for 1 h. The assay was developed using rabbit antiserum specific for human immunoglobulins and ^{125}I-labeled protein A, as in the RIA above.

Construction of transgenes. The genes encoding recombinant TGEV-neutralizing Mab were expressed using the regulatory sequences from the *WAP* gene. These sequences were cloned in the vector pWAP7K kindly provided by L. Hennighausen (NIH, Bethesda, MD), a Bluescript-based plasmid containing 2397 nucleotides of the mouse *WAP* promoter region, four exons, and nucleotides 5172 to 6765 of the 3′ noncoding end of the gene[20] (EMBL GenBank # U38816). The first 114 nucleotides derived from *WAP* gene exon 1 incorporated in the expression plasmid are not translated because the initiation codon has been mutated. The immunoglobulin sequences were inserted in an EcoRV cloning site introduced after nucleotide 24 of *WAP* exon 1. The remaining portion of exon 1 and exons 2, 3, and 4 of the *WAP* gene are not expressed because transcription stop signals were introduced after the immunoglobulin genes. The genes encoding the LC and HC of Mab 6A.C3 and the characteristics of this Mab have been reported[7,13]. Briefly, a chimeric mouse-human LC expression cassette was engineered by modifying the human constant module from vector pING2016E-gpt (refs. 36, 37) by PCR mutagenesis to introduce ClaI and SacI restriction sites[7]. The variable fragment from LC was amplified by RT-PCR from mRNA extracted from a mouse hybridoma secreting Mab 6A.C3[13]. This fragment was subcloned into the SalI-ClaI site of the expression plasmid carrying the constant module, resulting in the plasmid designated pINLC6A. The chimeric mouse-human HC expression cassette was engineered by ligating the BamHI-ApaI V_H fragment (amplified by RT-PCR from mouse Mab 6A.C3 mRNA) to the ApaI-BamHI fragment from human IgG_1 constant module isolated from plasmid pGMH6 (ref. 37), and cloning the resulting cDNA into the expression plasmid pING2003E-neo[36,37] previously digested with BamHI. The resulting plasmid was designated pINHC6A. The two cDNA fragments encoding the chimeric LC and HC of $rIgG_1$ were blunt ended and inserted separately at the unique EcoRV site of the plasmid pWAP7K carrying the *WAP* gene.

Functional analysis of transgenes. To study whether the plasmids carrying the immunoglobulin genes under the control of *WAP* regulatory sequences were functional and hormone inducible, HC11 mouse mammary gland epithelial cells[19] were used. Plasmids encoding LC and HC under *WAP* gene control (Fig. 1) were linearized at the EcoRI restriction site and were transfected together with plasmid pSV2-neo providing resistance to the antibiotic Geneticin into 2×10^7 HC11 cells by electroporation[38]. Cells were seeded in an M-24 well microplate (4×10^5 cell per well). Transformants were selected in the presence of Geneticin (0.8 mg/ml). The expression of immunoglobulin genes under the control of the SV-40 promoter and polyadenylation signals and the intronic enhancer of IgM immunoglobulin was performed using expression plasmids pING2016E-gpt and pING2003E-neo[36,37] in which the variable immunoglobulin domains from Mab 6A.C3 were introduced as described above[7]. Antibody expression levels from selected HC11 cells were analyzed in the supernatant by RIA after induction with hydrocortisone (5 μg/ml), insulin (5 μg/ml), and prolactin (2 μg/ml) (Fig. 2). A total of 24 cloned cell lines secreting TGEV-neutralizing antibodies were established. The data shown represent the result obtained with the transformed cell line expressing the highest antibody levels.

Generation of transgenic mice. Transgenic mice were generated essentially as described[21]. The WAP-LC and WAP-HC transgenes were excised from the plasmid vectors with restriction endonuclease EcoRI leading to DNA fragments of 7.5 kb and 8 kb, respectively. The MAR sequences were obtained by cleavage of the pBSKA plasmid[39] with XbaI and BamHI restriction endonucleases. All DNA constructs were purified using sodium chloride gradients[40]. DNA for microinjection was resuspended in TE (10 mM Tris, pH 7.4, 0.1 mM EDTA), at a final concentration of 2 to 6 μg/ml. Two sets of microinjections into pronuclear stage ova obtained from superovulated (C57BL/6 × CBA) F1 females were performed. The first set (MAR^-) corresponded to the comicroinjection of WAP-LC and WAP-HC constructs in an equimolar ratio in the absence of MAR sequences. The second set (MAR^+) corresponded to the comicroinjection of WAP-LC and WAP-HC constructs and also included the MAR sequences at the same molar ratio. Transgenic lines were propagated by backcrossing to the (C57BL/6 × CBA) F1 hybrid mice.

Screening of founders. DNA from tail biopsies was prepared as described[21]. The presence of WAP-immunoglobulin transgenes and MAR sequences was identified by a PCR assay using primers specific for the *WAP* promoter, the immunoglobulin variable modules, and MAR sequences. The primers used were: *WAP* 5′-CAAAGTCTTCCTCCTGTGGGTC-3′; V_L 5′-CCGTCCCA-GATCCACTGCC-3′; V_H 5′-GGCCTTGCCCTGGAACTTCGGG-3′; MAR_1 5′-GGCAGTTGGGCACTGCACTGCC-3′; and MAR_2 5′-CTGTGGGCTTTGTCTCCC-3′. PCR amplified products of 359, 355, and 400 nucleotides corresponding to WAP-LC, WAP-HC, and MAR fragments, respectively, were obtained. To determine transgene copy number, PCR-positive animals were examined by Southern blot analysis[41] using a probe that detected the expression cassette for the LC and HC immunoglobulin genes and also the single copy endogenous *WAP* gene. Genomic DNA was digested with BglII, and three fragments corresponding to endogenous *WAP*, WAP-LC, and WAP-HC genes were detected by hybridization. The radiolabeled DNA fragment used to probe the blot was specific to the common *WAP* promoter region (nucleotides 888–997), allowing a comparison of the transgene copy number in each animal on the same Southern blot. Copy number was determined by densitometry (Molecular Analyst; Bio-Rad, Hercules, CA).

Characterization and functional activity of the recombinant antibody in mouse milk. Milk from 4- to 6-month-old transgenic mice and from nontransgenic mice with the same characteristics (C57BL/6 × CBA) F1 were collected from lactating females as described[42], diluted 1:10 with Tris buffered saline (25 mM Tris, pH 7.4; 100 mM NaCl), and centrifuged at 14,000 × G for 10 min at 4°C. Whey phase was removed from the pelleted casein avoiding the fat supernatant. TGEV binding and neutralization assays[14] were used to study the functional activity of the recombinant antibody. To estimate chimeric immunoglobulin levels, purified $rIgG_1$ (ref. 7) was diluted to different concentrations in nontransgenic mouse milk and a standard curve was generated relating protein antibody and titer in RIA. Rabbit antihuman IgG (Cappel, Turnhout, Belgium) was used to detect recombinant immunoglobulins, and the RIA was performed using purified TGEV as antigen[14]. DAS-RIA was performed as described above.

Acknowledgments

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