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Short Communication

Expression of a functional mouse-human chimeric anti-CD19 antibody in the milk of transgenic mice

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Abstract

Human B cell lymphomas are suitable targets for immunotherapy. Clinical trials with mouse-human chimeric B cell-specific monoclonal antibodies (mAbs) have already shown promising results. However, limitations for their use in clinical trials can be the lack of sufficient amounts and high production costs. Expression of mAbs in the mammary gland of transgenic animals provides an economically advantageous possibility for production of sufficient quantities of a promising antibody for clinical trials and beyond. In this paper, we show the feasibility of this approach, by generating transgenic mice expressing mouse-human chimeric anti-CD19 mAbs in their milk. Mouse anti-CD19 variable (V) region genes were combined with human IgG1 heavy (H) and kappa light (L) chain constant (C) region genes and fused to the bovine β-lactoglobulin (*BLG*) promoter in two separate expression cassettes. Co-injection resulted in five transgenic lines. In one of these lines completely assembled chimeric mAbs were secreted into the milk, at an approximate level of 0.5 mg/ml. These mAbs were able to bind specifically to the CD19 surface antigen on human B cells.

Abbreviations: ADCC–antibody-dependent cellular cytotoxicity; BLG–β-lactoglobulin; C-region–constant region; H-chain–heavy chain; kb–kilobase; kD–kilodalton; L-chain–light chain; mAbs–monoclonal antibodies; MW–molecular weight; V-region–variable region.

Introduction

Tumors of lymphoid origin are suitable targets for treatment with mAbs, because these tumors express a number of well-characterized antigens and are more accessible than solid tumors. In case of human B cell malignancies, therapy with mAbs can be directed against the tumor-specific surface immunoglobulin idiotype or against B cell restricted surface antigens like CD19, CD20 or CD22 (Link & Weiner, 1998). Effective treatment with unconjugated mAbs depends largely on their ability to recruit host effector mechanisms, such as complement-mediated lysis and antibody-dependent cellular cytotoxicity (ADCC). It has been shown that these effector functions can be improved by replacing the murine C-regions of the antibody with the corresponding human equivalents, resulting in mouse-human chimeric mAbs (Liu et al., 1987; Reff et al., 1994). In addition, it was found that chimeric mAbs have a prolonged survival in the human circulation and are less immunogenic (LoBuglio et al., 1989), allowing for repeated dosing strategies.

Recently, a large multicenter study has been completed in which 166 patients with B cell lymphoma have been treated with a mouse-human chimeric anti-

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CD20 antibody, resulting in an overall response rate of 48% (McLaughlin, 1998). However, a major limitation in clinical trials with promising mAbs can be the lack of sufficient quantities of these antibodies. Expression systems have been developed to achieve secretion of large amounts of mAbs in cell culture, but these systems appear to have a maximum expression level of 1 mg/ml (Reff, 1993), and are very expensive due to the high costs of serum, equipment and a specialized labor force. The mammary gland of living animals is able to produce high quantities of complex heterologous proteins and at a low cost, as has been amply demonstrated (Lee & de Boer, 1994; Wall et al., 1997). For example, expression levels of up to 10 mg/ml in milk of transgenic mice have been reported for complex human proteins such as fibrinogen (Prunkard et al., 1996), α1-antitrypsin (Archibald et al., 1990; Bischoff et al., 1992) and α-glucosidase (Bijvoet et al., 1998), and these proteins were shown to be biologically active. Here we report the expression in milk of transgenic mice of immunoglobulin H- and L-chain transgenes encoding a mouse-human chimeric IgG1/κ anti-CD19 mAb and show that it can bind specifically to its target on human B cells. If high expression levels of functional mAbs can be achieved in the milk of larger animals, then this method offers a possibility for large-scale, cost-effective production of mAbs that have great potential for use in treatment of human B cell malignancies.

Materials, results and discussion

To generate transgenic mice expressing mouse-human chimeric IgG1/κ anti-CD19 mAbs in the milk, two separate expression cassettes were constructed encoding the immunoglobulin H- and L-chain gene, under control of the bovine BLG promoter. The promoter was attached to the H- or L-chain V-region, which was constructed according to the method of Orlandi et al. (1989) and which contained a chimeric mouse-human anti-CD19 VDJ or VJ exon, respectively. In the H-chain expression cassette, the V-region was fused to the human IgG1 H-chain C-region (Takahashi et al., 1982), followed by a 2 kb 3′ flanking region of the bovine *BLG* gene (Alexander et al., 1993). For expression of the L-chain, the human kappa L-chain C-region (Hieter et al., 1980) with 3 kb of its 3′ flanking region was linked behind the V-region.

The 7.9 kb H-chain and 7.7 kb L-chain expression cassettes were co-injected in a 1:1 molar ratio into fertilized mouse eggs. Analysis of tail DNA of 61 mice by PCR and Southern blotting showed that six mice, one male and five females, carried both transgenes. No mice bearing only one transgene were found. The six founder animals (numbered 1–6) were used to generate five transgenic lines. Female founder number four did not transmit the transgenes to the offspring, indicating germline chimerism. The transgenic offspring of the other founders were all double transgenics, indicating that the co-injection had resulted in co-integration of the transgenes at a single chromosomal site. By Southern blotting it was shown that the number of integrated transgene copies per cell was the same for both transgenes in all founders, and that in all lines the integrated transgenes were intact. The copy numbers that were found were: 1 copy per cell for line 2, 3 and 6, 5–10 copies per cell for line 1 and more than 10 copies per cell for line 5.

Total RNA was isolated from mammary gland tissue of two transgenic females per line at mid-lactation (days 11–13) and expression of the transgenes was determined by northern blotting. Transgene-derived transcripts of the correct sizes were only found in line 2, in which the L-chain transgene was expressed at a much higher level than the H-chain transgene (data not shown). There was no relationship between the transgene copy number and mRNA levels, indicating that expression was integration site dependent.

The higher expression level of the L-chain transgene in line 2 is probably not due to a difference in copy number between the two transgenes, because the Southern blot of tail DNA indicated that only one copy of both transgenes was integrated. Both transgenes are driven by the same promoter, but the H-chain expression cassette was constructed with a 3′ *BLG* flank, while for the L-chain expression cassette its own 3′ flank was used. Therefore, the difference in expression level might be caused by differences in transcription rate of the transgenes or by differences in stability of the respective mRNAs. It could also be that this effect is caused by neighboring genomic sequences at the integration site. More transgenic lines have to be made before a conclusion can be drawn about the difference in expression levels.

The chimeric antibody expression levels were measured in milk samples collected at mid-lactation from two female mice of line 2. Equal amounts of diluted milk were separated on SDS/PAGE under reducing and nonreducing conditions, followed by western blotting. On the blot of a reduced gel, a mouse anti-human kappa L-chain mAb that cross-

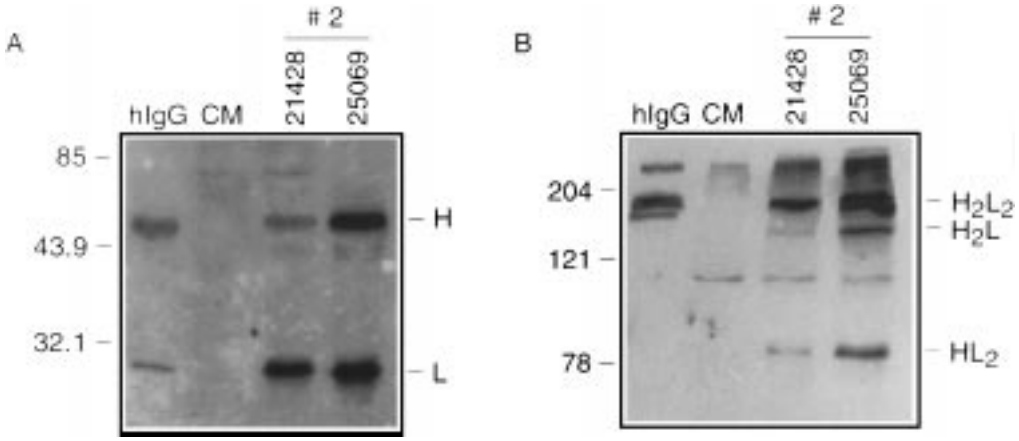

Figure 1. Western blot analysis of milk from two female transgenic mice of line 2. Equal amounts (2 µl) of diluted milk (1:10) were fractionated on 10% (reducing conditions, panel A) and 7.5% (nonreducing conditions, panel B) SDS–polyacrylamide gels, followed by western blotting. Milk samples were collected at mid-lactation (days 10–12) from two transgenic females of line 2 (mouse 21428, 10 dL; mouse 25069, 12 dL) and from a female non-transgenic littermate as negative control (CM, 12dL). Purified human IgG1/κ was used as positive control (hIgG). Blots were probed with a mouse anti-human kappa L-chain antibody, cross-reacting with the human IgG1 H-chain. The position of the H-chain (H), the L-chain (L) and assembled H- and L-chain complexes (H_2L_2, H_2L and HL_2) are indicated. Molecular weight markers are shown on the left in kD.

reacts with the IgG1 H-chain was used to show the presence of the human H-chain and L-chain in the milk samples of line 2. A single band was found for the H-chain and the L-chain, with the expected molecular weights (MW) of 50 kD and 25 kD, respectively (Figure 1A). By comparing the intensities of the single H- and L-chain bands in the transgenic milk samples, it can be seen that the amount of human L-chain protein is higher than the amount of human H-chain (Figure 1A).

Analysis of the transgenic milk samples with the mouse anti-human kappa L-chain antibody on a blot of a nonreduced gel revealed a strong band with a MW of around 150 kD, migrating at the same position as the control human IgG1/κ antibody (Figure 1B). This indicates the presence of correctly assembled chimeric IgG1/κ antibodies (H_2L_2). Also some additional weak bands of lower MW were observed, probably representing incompletely assembled IgG1 molecules such as H_2L, HL_2 and L_2 (Figure 1B and data not shown). The amount of L-chain protein that is produced in excess over the H-chain protein in the epithelial cells, is presumably secreted as L-chain dimers (L_2) and as a combination of one H-chain with two L-chain molecules (HL_2). Some single H-chain and L-chain bands were also seen on blots of higher percentage nonreduced SDS/PAGE gels (data not shown), demonstrating the existence of non-covalently bound single chains. The concentration of chimeric antibodies in the milk was determined densitometrically on the 150 kD band (Figure 1B), using the band obtained with 50 ng of the control human IgG1/κ antibody as a reference. By this method, the concentration of chimeric antibodies in the milk was estimated to be approximately 0.3 mg/ml (mouse 21428) and 0.5 mg/ml (mouse 25069).

Recently, it has been reported that the bovine *BLG* gene including 2.8 kb of 5′ and 1.9 kb of 3′ flanking region was expressed mammary gland-specifically in transgenic mice, with expression levels in milk exceeding 1 mg/ml (Hyttinen et al., 1998). When the ovine *BLG* promoter was used to drive the expression of the human α_1-antitrypsin gene, levels up to 7 mg/ml were produced in the transgenic mouse milk (Archibald et al., 1990). However, in the same study, the promoter was shown to function in a position-dependent manner. Using the bovine *BLG* promoter, we also found the transgene expression to be integration site dependent. Therefore, more transgenic lines have to be made to see whether or not higher expression levels can be achieved.

To test whether the chimeric antibodies are functional, their CD19-binding ability was determined by fluorocytometric analysis on cells of the human B cell line JY, that had been transfected previously with a vector carrying the cDNA encoding the human CD19 antigen (Hooijberg et al., 1995). The surface expression of the CD19 antigen on these JY*hCD19 transfectants is about one log higher than on JY-wild type cells and is comparable to the CD20 antigen

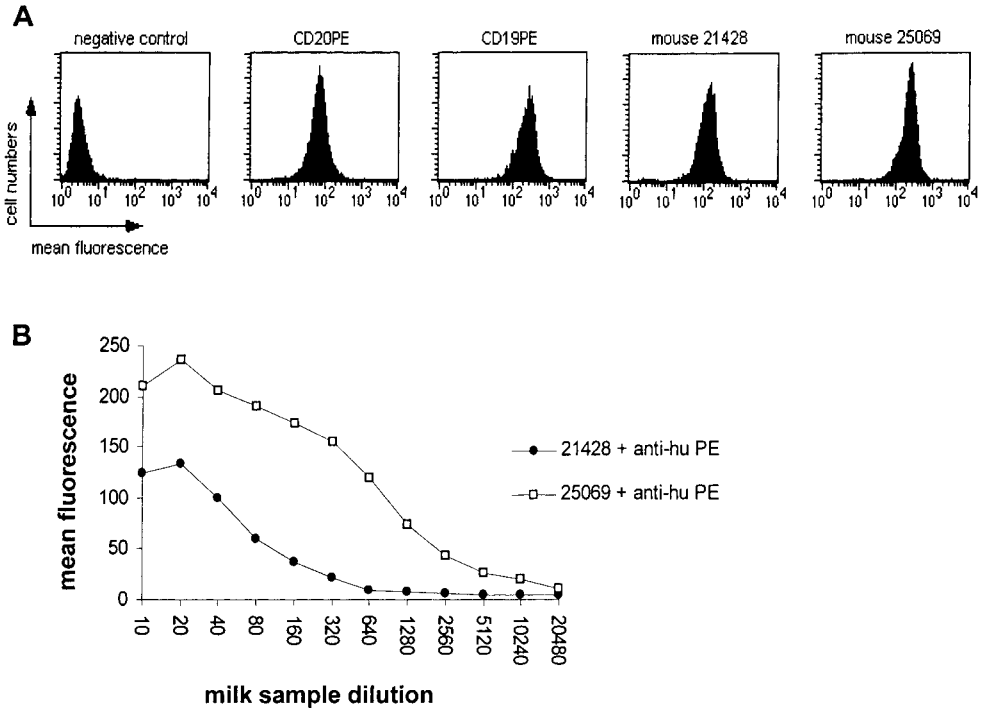

Figure 2. Fluorocytometric analysis of milk from two transgenic females of line 2. (A) FACS patterns obtained after incubation of human JY*hCD19 cells with 10 µl of diluted (1:20) milk samples from two transgenic females of line 2 (mouse 21428, 10 dL; mouse 25069, 12 dL), or 10 µl of a diluted (1:10) milk sample from a female non-transgenic littermate (negative control, 12 dL), followed by PE-labeled $F(ab)_2$ fragments of a mouse anti-human IgG(Fc). Expression of the CD20 and CD19 surface antigens on the cells was determined by direct staining with PE-conjugated primary antibodies (CD20PE and CD19PE). (B) In order to determine relative quantities, JY*hCD19 cells were incubated with 10 µl of serial dilutions of the same two milk samples of line 2 as in panel A, and bound chimeric immunoglobulin was detected with PE-labeled $F(ab)_2$ fragments of a mouse anti-human IgG(Fc) (anti-hu PE). The mean fluorescence intensity is plotted against the milk sample dilution factor.

surface expression on the same cells (Figure 2A; Hooijberg et al., 1995). Incubation of these cells with diluted milk samples of the two mice of line 2, followed by detection of bound immunoglobulin with PE-conjugated $F(ab)_2$ fragments of goat anti-human IgG(Fc), resulted in a high mean fluorescence intensity (Figure 2A) illustrating binding of the chimeric antibodies to the CD19 antigen. In order to determine relative quantities of the antibodies, cells were incubated with serial dilutions of the milk samples. The dose-response curve (Figure 2B) shows that the chimeric antibody level in the milk of mouse 25069 is higher than the level in the milk of mouse 21428, as could also be seen on the western blot (Figure 1B).

To confirm the specificity of the chimeric antibodies as being truly anti-CD19, a blocking experiment was performed. JY*hCD19 cells were incubated with serial dilutions of the transgenic milk samples, followed by incubation with PE-conjugated anti-human CD19 or anti-human CD20 antibodies and measurement of the fluorescence intensity. The chimeric antibodies present in the milk samples at a 1 : 10 dilution completely blocked binding of the anti-CD19-PE antibodies (data not shown). Further dilution of the milk samples resulted in a decrease of blocking and an increase in binding of CD19-PE. A milk sample of a non-transgenic mouse did not inhibit binding of CD19-PE. Pre-incubation with the transgenic milk samples had no effect on the binding of anti-CD20 antibodies (data not shown).

Expression of mouse-human chimeric anti-CD6 mAbs in the milk of transgenic mice has been reported (Limonta et al., 1995), as well as expression of mouse-human and porcine-mouse chimeric anti-viral mAbs (Castilla et al., 1998; Sola et al., 1998). In case of the anti-CD6 mAbs the therapeutical application is not clear, while the anti-viral mAbs have been designed to provide newborn piglets with resistance to an infection of the enteric tract. In contrast, we report on the

expression of a mouse-human chimeric mAb that is aimed at treatment of human B cell lymphomas, as is the case for an anti-CD19 specificity.

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