Research Report

Production of Antigen-Specific Human Monoclonal Antibodies: Comparison of Mice Carrying IgH/ κ or IgH/ κ / λ Transloci

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S. Magadán, M. Valladares, E. Suarez¹, I. Sanjuán², A. Molina, C. Ayling³, S.L. Davies⁴, X. Zou³, G.T. Williams⁴, M.S. Neuberger⁴, M. Brüggemann³, F. Gambón², F. Díaz-Espada¹, and A. González-Fernández

Universidad de Vigo, Pontevedra, ¹Clínica Puerta de Hierro, Madrid, ²Hospital Meixoeiro de Vigo, Pontevedra, Spain, ³The Babraham Institute, Babraham, and ⁴Medical Research Council, Cambridge, UK

ABSTRACT

Here we compare human monoclonal antibody (MAb) production from mouse strains that carry disruptions of their endogenous mouse IgH/Ig κ loci and harbor human IgM + Ig κ (BAB κ) or human IgM + Ig κ + Ig λ transloci (BAB κ λ). We found that whereas both strains proved effective for the isolation of antigen-specific IgM antibodies, many of the IgM MAbs elicited from BAB κ comprise human μ chains that are associated with mouse λ chains. In contrast, BAB κ λ mice gave rise to fully functional, polymeric human IgM antibodies comprising both human IgH and human IgL chains. Therefore, the inclusion of a human Ig λ translocus (in addition to the human IgH + Ig κ transloci) not only diminishes problems of endogenous mouse Ig λ expression but also provides a strain of mice that yields fully human MAbs to a wide range of antigens, as witnessed by the isolation of MAbs to human blood cells, tumor cell lines, and an immunoglobulin idiotype.

INTRODUCTION

Since the first description of antigen-specific mouse monoclonal antibodies (MAbs) (22), efforts have been directed at attempting to generate antigen-specific human MAbs. One approach was to engineer the mouse genome so that the mouse produces antibodies encoded by human (as opposed to mouse) gene segments (4,5,9,16). Transloci bearing human Ig genes in germline configuration are introduced into the mouse germline, and their gene segments can undergo productive rearrangement, leading to the generation of B cells expressing human Ig chains. For this approach to work optimally, the endogenous mouse Ig loci are silenced by gene-targeting techniques to allow the expression of only the human Ig genes (17,23,31). This has been achieved with both the mouse IgH and Ig κ loci but not with the mouse Ig λ locus.

Whereas early experiments were performed using small human Ig transloci (human IgH locus of 25 kb or human Ig κ of 85 kb, both with only two Vs genes) (4,8), more recent mouse strains carry much larger transloci (encompassing many gene segments or much of the human germline loci as 1 Mb with 40 Vs in the human IgH locus or 1.3 Mb with 80 Vs in the human Ig κ locus). This has been achieved by the incorporation in the mouse germline of either YACs or human chromosomal fragments (17,26,30).

Here we have used two different mouse strains containing YAC-based human Ig transloci (26). BAB κ mice carry the human IgH YAC and the 1.3-Mb human Ig κ YAC, while BAB κ λ

mice carry an additional human Ig λ YAC transgene (27). Both strains have been crossed with mice in which the endogenous IgH locus and the Ig κ locus were rendered nonfunctional by gene targeting (20,32). In this report, we have compared the efficiency with which human MAbs can be produced from these mouse strains and conclude that mice with human IgH + Ig κ + Ig λ transloci are readily useful to produce antigen-specific fully human MAbs that may prove effective for the treatment of human diseases including cancer.

MATERIALS AND METHODS

Translocus Mouse Strains

The strains BAB κ and BAB κ λ (26) were bred and maintained in a homozygous state. Individual mice from the colonies were regularly tested by ELISA, PCR, and Southern blot analysis. The mice were kept in a pathogen-free environment.

Immunization

Human (either normal or tumoral) cells and a human monoclonal IgM κ protein were used for immunization (Table 1). The human B, T, and myelomonocytic cell lines were HMY (11), JURKAT (14), and U937 (28), respectively. Peripheral blood leukocytes were obtained from blood from healthy volunteers by centrifugation at 1210 $\times$ g over Ficoll-HypaqueTM. Human monocytes were obtained by the incubation of peripheral blood leukocyte cells on plastic Petri dishes. Human IgM was

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

Table 1. Production of Hybridomas from BAB κ and BAB κ , λ Mice Immunized with Human Cells or Human IgM

Antigen	Serum Dilution ^d	Wells Tested	Specific Human IgM Hybridomas
Hu Monocytes ^a	1/50	432	6
Hu monocytes ^a	1/50	324	1
Hu PBL ^a	1/500	2260	25
Hu PBL ^a	1/50	480	10
Hu PBL	1/800	2400	7
U937	1/1000	970	3
U937	1/1000	864	3
Jurkat	1/100	210	0
HMY	1/500	400	14
HuIgM-KLH	1/200	47	5 ^e
HuIgMKLH ^b	1/2000	82	4 ^e
HuIgM-KLH ^c	1/1000	227	30 ^e

The mouse strains immunized were BAB κ ^a or BAB κ , λ (the rest).

The adjuvants used in primary immunizations were complete Freund's Adjuvant (CFA) followed by incomplete Freund's Adjuvant (IFA) in subsequent immunizations, except in mice^b that received alum and cholera toxin^c, respectively.

^dTested by indirect immunofluorescence and expressed as dilution able to detect binding. Immunizations with Hu (human) IgM-KLH were scored at half maximum binding in ELISA.

^eTo discriminate between administered and produced antibody, only human IgM λ MAbs were selected.

obtained from a patient with an IgM, κ non-Hodgkin's lymphoma by fusion of the tumor cells with the K6H6 heteromyeloma (2). The secreted IgM was purified from hybridoma supernatants and coupled to KLH (Calbiochem-Novabiochem, San Diego, CA, USA) as previously described (2).

Groups of 3–8 mice, 2–4 months old, were immunized intraperitoneally with $1\text{--}2 \times 10^6$ human cells or with 100 μg human IgM coupled to KLH. Complete and incomplete Freund's Adjuvant, cholera toxin, or alum were used as adjuvants. The animals were immunized again three weeks later and bled. A last intravenous immunization was carried out 10–20 days later. Three days later, the animals were sacrificed, and the spleen was used for fusion.

Hybridomas

The fusion was carried out as previously described (12). SP2/0 or NSO myeloma cells were mixed with spleen cells at a ratio of $2\text{--}3 \times 10^7$ myeloma cells/ 10^8 spleen cells in the presence of 50% PEG 1500 (Sigma, St. Louis, MO,

USA). After fusion, cells were resuspended in HAT medium and plated on either 24- or 96-well plates (Corning Costar, Acton, MA, USA). Human antibodies produced by the hybridomas were tested by ELISA or flow cytometry, as described below.

Analysis of Human Ig in Mouse Serum or from Hybridoma Supernatants by ELISA

The 96-well plates (Falcon®; BD Biosciences Clontech, Palo Alto, CA, USA) were coated with 10 $\mu\text{g}/\text{mL}$ either rabbit anti-human κ , λ , or μ H chain (Dako, Glostrup, Denmark), blocked with PBS/1% BSA, and incubated with different mouse serum dilutions or hybridoma supernatants. Bound Igs were detected using biotinylated rabbit anti-human μ H chain (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or biotinylated goat anti-mouse λ (Caltag Laboratories, Burlingame, CA, USA). Avidin-HRP (Dako) was added to the wells, the reaction was visualized with 2,2' azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)

(Sigma), and measured on a Multiskan® EX ELISA plate reader (Thermo Lab-systems, Helsinki, Finland) at 405 nm. Human serum IgM of known concentration was used as a standard and established the sensitivity of the ELISA as approximately 10 ng/mL.

Antigen-specific serum or hybridoma-derived antibodies were tested by coating plates with 10 $\mu\text{g}/\text{mL}$ either with human monoclonal IgM, κ protein or KLH carrier protein. An unrelated monoclonal IgM, κ protein was used as a control. The binding of specific MAbs was detected with HRP-labeled goat anti-human λ antibodies. The reaction was visualized with *O*-Phenylenediamine (Sigma) and measured at 492 nm.

Flow Cytometric Analysis

Fluorescein isothiocyanate (FITC)-conjugated rabbit antibodies anti-human μ , κ (both from Dako), and λ (Immunotech, Marseille, France), R-phycoerythrin (PE)-conjugated rabbit anti-human μ H chain (Pharmingen, San Diego, CA, USA), and biotinylated goat antibodies anti-mouse λ (Caltag), followed by streptavidin-PE (Dako), were used to analyze surface Ig expression in mouse spleen cells by direct staining.

Specific antibodies were tested by indirect immunofluorescence using the immunizing human cells. Cells (4×10^5) were incubated with dilutions of mouse serum or with hybridoma supernatants at 4°C for 30 min, washed twice, and stained with FITC-conjugated rabbit anti-human μ (Dako). Peripheral blood leukocyte cells and granulocytes from blood were gated by conventional scatter parameters to allow the analysis of individual populations in a flow cytometer (Coulter Electronics, Hialeah, FL, USA).

Chromatographic and Electrophoretic Analysis of Human IgM

The M_r of the human monoclonal IgM antibodies was analyzed by size-exclusion chromatography. The cell culture supernatant was concentrated approximately 10-fold, and 200 μL were applied to a Superose® 12 HR10/30 column (Amersham Biosciences, Uppsala, Sweden). Fractions (0.5-mL) were collected and analyzed by ELISA and flow

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

cytometry. The column was calibrated with normal human serum, and the eluted fractions corresponding to the positions for IgM, IgG, and albumin were determined by double immunodiffusion.

To investigate the presence of free L chain, 5 μ L culture supernatant were subjected to electrophoresis on a 1% SPE agarose gel (Beckman Instruments, Fullerton, CA, USA) and allowed to transfer by pressure blotting on a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA) (1). The membrane was blocked with 3% fat-milk and revealed with HRP-labeled goat anti-human κ , λ , or μ chain-specific antibodies (Sigma), followed by detection using chemiluminescence (Lumi-Light^{Plus}; Roche Applied Science, Indianapolis, IN, USA).

Complement-Mediated Cell Lysis Using Human Mabs

DAUDI cells (21) were incubated with supernatant from hybridoma clones, followed by the addition of rabbit (Dade Boehringer, Newark, NJ, USA) or human complement (fresh human serum). Cell viability was assessed in an EPICSTM cytometer after treatment with 10 μ g/mL propidium iodide or in an optical microscope after a conventional microlymphocytotoxicity assay (29) on Terasaki plates (Robbins Scientific, Sunnyvale, CA, USA).

RESULTS AND DISCUSSION

Human Igs on B Cells and in Serum of Transgenic Mice

The level of human Igs in the serum of the translocus mouse strains BAB κ and BAB κ λ was titrated by ELISA. Human IgM was present at approximately 50 μ g/mL before immunization in both strains, which could rise up to approximately 200 μ g/mL after immunization. No significant differences were found, despite the additional human λ locus in the BAB κ λ mice. We also found that the mouse IgH locus was reactivated upon human H chain expression, which allowed the production of mouse Igs (data not shown and Reference 25).

The spleens of the BAB κ and BAB κ λ mice had a reduced number of

cells compared to the normal mice. The range was $2\text{--}5 \times 10^7$ spleen cells in BAB λ and $0.7\text{--}1 \times 10^8$ in BAB κ λ , a near-normal level that was $1\text{--}2 \times 10^8$ in unmanipulated conventional mice with a similar genetic background. Similarly, the analysis of lymph nodes and Peyer's patches revealed a significant reduction in cell numbers in the translocus mice (data not shown).

Flow cytometric analysis was conducted to identify the levels of human IgM⁺ cells in the spleen and the ratio of L chain expression in both strains of mice (Figure 1). In BAB κ mice, approximately 16% of the lymphocytes express human IgM, κ and approximately 17% human IgM express mouse λ . In the BAB κ λ mice, the level of human IgM, κ ⁺ lymphocytes (14%) was very similar, while the introduced human Ig λ locus allowed the additional generation of 32% human IgM⁺, λ ⁺ cells. This is in sharp contrast to the BAB κ mice, which produce about half of their Abs with mouse λ L chain. Furthermore, BAB κ produces 6% of double-positive human κ ⁺ and mouse λ ⁺ cells, which may imply incomplete allelic exclusion in this translocus mouse strain. Cells expressing different L chains are much more rare in BAB κ λ mice, and a reason for this may be the increase in the lymphocyte population caused by human λ expression (Figure 1, bottom panel, and Reference 26). Increased numbers of splenic lymphocytes expressing mouse surface IgG were not identified in any of the strains (data not shown).

Production of Antigen-Specific Antibodies to Human Antigens

For the immunization of the translocus mouse strains, we used several types of human cells (either peripheral blood leukocytes, monocytes, or tumoral cells lines) or a human monoclonal IgM protein conjugated to KLH (Table 1). Mice immunized with purified peripheral blood monocytes and, separately, the U937 cell line, revealed a 1:50 detection titer in BAB κ and an exceptionally high titer detectable in a 1:1000 dilution in BAB κ λ . A high titer by ELISA was also found in mice immunized with human IgM coupled to KLH. Immunization without adjuvants did not yield a serum response (data not shown), and although the use of Freund's Adjuvants allowed efficient priming, this appeared to be equal to other adjuvants.

The efficacy of generating specific hybridomas secreting human anti-human Mabs was similarly variable in both strains (Table 1), but could be as high as 2% (in BAB κ immunized with human peripheral blood leukocytes) and up to 13% (in BAB κ λ immunized with human IgM-KLH). However, the number of hybrids secreting specific Mabs was relatively low compared to those routinely obtained from normal mice (13), which could be caused by the reduced number of cells in their lymphoid organs.

Supernatants from the hybridoma clones were analyzed by flow cytometry or ELISA for antigen binding (Table

Figure 1. Flow cytometric analysis of B-lymphocyte populations from spleens of BAB κ and BAB κ λ translocus mice. Representative samples show the percentages of lymphocytes expressing combinations of human (Hu) IgM/mouse (Mo) λ ; human IgM/human κ ; human IgM/human λ ; and human κ /mouse λ chain.

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

1 and Figure 2) and from four fusions each. This identified 42 MABs from BAB κ and 27 MABs from BAB κ,λ that recognized human cells. Although the production of specific hybridomas appears to be successful in BAB κ , unfortunately a large number of the clones expressed a mouse λ L chain (33 of

42). The expression of the mouse λ L chain was not found in hybridomas from BAB κ,λ , which allows this strain to produce a larger number of specific, fully human MABs against human cells (25 IgM, κ and 2 IgM, λ) than to BAB κ (9 IgM, κ). The immunization of BAB

κ,λ mice with human IgM-KLH resulted in 39 antigen-specific clones from three fusions, of which only two had the desired anti-idiotypic specificity. In none of the strains, MABs with two different L chains were found, which suggests that cells expressing two L

chains are rare.

Specific hybridomas were expanded, and Figure 2A shows detailed flow cytometric analysis of three human IgM, κ MABs obtained after immunization with human peripheral blood leukocytes (50.2), with U937 (54.3), and with HMY (B11) cells. Clone 50.2 has a broad spectrum of recognition for lymphocytes, monocytes, and granulocytes and, as expected, binds to HMY and JURKAT cells. The binding of MAB 54.3 is directed against human monocytes and T-cells; here JURKAT cells are shown as a representative example of T cells, while MAB B11 is exclusively directed against human B-cells with binding to the HMY cells shown in Figure 2A. The results establish that BAB κ

Figure 2. Human MABs recognize human cells and a human monoclonal IgM, κ lymphoma protein. (A) Peripheral blood leukocytes, granulocytes, HMY, and JURKAT cells were stained with human MABs 50.2, 54.3, and B11, followed by FITC-conjugated anti-human IgM (open tracing) or with secondary antibody (filled tracing, control cells). (B) Specific human IgM, λ MABs against a monoclonal IgM, κ coupled to KLH were selected. The binding of two representative clones, G3 and C10, was compared on ELISA plates coated with either the monoclonal IgM, κ (i-IgM, κ), an unrelated monoclonal IgM, κ (u-IgM, κ) or KLH. Binding in wells coated with the IgM, κ proteins and with KLH was developed with peroxidase-labeled anti-human λ L chain and anti-human μ chain, respectively. Each well was tested in triplicate.

Figure 3. H and L chain association and features of the human MABs. (A) The M_r of MAB 32.4 (human IgM, κ against U937 cells as a representative example) was determined by the size fractionation of the culture supernatant. The eluted fractions (x-axis), numbers 11–30, which excluded the void volume, were tested for IgM by ELISA, indicated in adsorption (A_{492}). The expected elution positions of pentameric IgM (IgMp), monomeric IgM (IgMm), IgG and albumin (Alb) are also indicated on the x-axis. (B) Antigen specificity to the U937 cells was tested by flow cytometry, and only four fractions (P1–P4) are shown. The fractions were concentrated, and binding to U937 cells was detected with FITC-labeled anti-human IgM (open tracing). Cells incubated with anti-human IgM-FITC were used as controls (filled tracing). (C) The identification of H and L chain association in antibodies secreted by 13 hybridomas. Secreted proteins were separated by agarose gel electrophoresis and transferred to PVDF membranes. The Ig chains were identified separately with HRP-labeled anti-human λ , anti-human κ , and anti-human μ antibodies, followed by chemiluminescence. Two clones, 10a and 10b, were from the same hybridoma.

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

and BAB κ , λ mice can produce different human MAbs for cell surface markers of a variety of human cells, some of them highly specific for particular subsets of the lymphocyte lineage.

The immunizations with human proteins allowed analysis by ELISA. Figure 2B illustrates the binding of two human IgM, λ MAbs (G3 and C10) obtained from BAB κ , λ after immunization with a human monoclonal IgM, κ protein. G3 recognizes the human IgM used for the immunization but not an unrelated IgM, κ protein, probably because of idiotypic differences, while C10 recognizes the KLH carrier protein.

Polymeric Assembly of the Human IgM MAbs and Presence of Free Light Chains

An important issue to consider is the production in the translocus mice of near-authentic human MAbs endowed

with structural and biological features that permit their use in human therapeutic applications. M_r analysis by gel filtration made it impossible to differentiate between normal human IgM and human IgM produced by the translocus mice (Figure 3). Secreted human IgM is largely pentameric, and size-exclusion chromatography released a main peak, approximately 900 kDa, at fractions 16–18, which was also seen for MAbs produced by the translocus mice (see Figure 3A, MAb 32.4). Other MAbs showed the same size-exclusion chromatography pattern (data not shown). A minor fraction, most likely of H2L2 configuration, was also found for all human IgMs tested (fractions 21–23). Figure 3B shows a flow cytometry analysis of four fractions of the same MAb 32.4 against the immunizing U937 cells. As expected, only the fractions corresponding to polymeric and monomeric IgM (P1 and P2, respectively) gave a positive staining on the cells. The polymeric structure of the secreted human antibody may indicate that association with the mouse J chain provides the necessary support structure, although fully functional hexameric polypeptides can be produced in the absence of the J chain (7,19).

Mouse hybridomas frequently produce an excess of L chain, but complete H chain produced on its own or in excess, has not been found (24). To determine if this is also the case for the human hybridomas, we analyzed Ig from culture supernatant (Figure 3C). From the 12 different hybridomas analyzed, seven produced human λ and five produced human κ , of which two (nos. 1 and 13) and three clones (nos. 8, 10, and 12), respectively, also secreted free L chains.

Human MAbs from Transgenic Mice Trigger Complement-Mediated Cell Lysis

Multivalent IgM is very potent in effector functions, such as complement-dependent cell lysis, opsonization, and phagocytosis (3,6). We wanted to test whether our human MAbs initiate complement-dependent cell lysis. Figure 4A shows that a large proportion of DAUDI cells are lysed in the presence of B11 and rabbit complement, but a non-binder BA5 MAb did not trigger cell lysis. Other cells such as peripheral blood leukocytes, JURKAT, and U937 were also lysed by specific antibodies in the presence of the rabbit complement (data not shown). An important additional evaluation to analyze the known resistance of human cells to a human complement-mediated attack (15,18) was established using human serum as the complement source either by flow cytometry (Figure 4A) or using Terasaki plates (Figure 4B). The incubation of DAUDI cells with B11 and human serum resulted in almost complete cell lysis, while antibody or human serum alone had no effect. Other cells, such as leukemic cells obtained from patients, also seemed to be lysed by some of our human MAbs in the presence of human complement (data not shown).

In summary, the results show that a diverse range of specific polymeric human MAbs can be obtained after immunization in transgenic mice. The generated MAbs are of the IgM isotype, which has been proven beneficial in cell depletion (10), and their target immunogen can be exploited to elicit the removal or possible eradication of tumor cells.

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Figure 4. Complement-mediated cell lysis. (A) DAUDI cells were incubated with MAb B11 raised against B cells or with MAb BA5 raised against monocytes in the presence of rabbit or human complement (human serum). Dead cells were identified by propidium iodide staining in a flow cytometer. Medium without MAb served as the control (indicated in the rectangular boxed region in each graph). (B) Microlymphocytotoxicity assay. DAUDI cells were incubated with MAb B11 and rabbit complement (B11 + rabbit C') or human serum (B11 + human C'). Heat-inactivated human serum (B11 + inactivated C') or complement without relevant MAb (C') were used as controls. Eosin staining visualized the dead cells, which appeared as black dots.

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

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Address correspondence to:
Dra. África González-Fernández
Área de Inmunología, Facultad de Ciencias
Universidad de Vigo
Lagoas Marcosende s/n 36200
Vigo, Pontevedra, Spain
e-mail: africa@uvigo.es