B-cell display-based one-step method to generate chimeric human IgG monoclonal antibodies

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ABSTRACT

The recent development of screening strategies based on the generation and display of large libraries of antibody fragments has allowed considerable advances for the *in vitro* isolation of monoclonal antibodies (mAbs). We previously developed a technology referred to as the 'ADLib (Autonomously Diversifying Library) system', which allows the rapid screening and isolation *in vitro* of antigen-specific monoclonal antibodies (mAbs) from libraries of immunoglobulin M (IgM) displayed by the chicken B-cell line DT40. Here, we report a novel application of the ADLib system to the production of chimeric human mAbs. We have designed gene knock-in constructs to generate DT40 strains that coexpress chimeric human IgG and chicken IgM via B-cell-specific RNA alternative splicing. We demonstrate that the application of the ADLib system to these strains allows the one-step selection of antigen-specific human chimeric IgG. In addition, the production of chimeric IgG can be selectively increased when we modulate RNA processing by overexpressing the polyadenylation factor CstF-64. This method provides a new way to efficiently design mAbs suitable for a wide range of purposes including antibody therapy.

INTRODUCTION

The use of monoclonal antibodies (mAbs) has raised considerable interest in recent years, notably for diagnostic and therapeutic applications. The development of the hybridoma technology (1) has allowed the extensive selection and production of mAbs specifically binding to target antigens of interest, including several mAbs approved for clinical use against human diseases such as cancer (2).

Still, conventional technologies based on animal immunization to isolate new mAbs remain time-consuming, and exclude the generation of mAbs against poorly immunogenic antigens such as auto-antigens and small compounds.

To overcome these issues, various successful *in vitro* screening systems of mAb fragment libraries have emerged from molecular display technologies such as phage display (3,4). Recently, we have developed a new method for the rapid generation of mAbs using the chicken B-cell-derived cell line DT40 (5,6). By enhancing gene conversion (5,7), which is the main diversification process of the immunoglobulin (Ig) variable region in chicken B cells (8,9), we obtained naturally expanding libraries of mAbs displayed at the cell surface as membrane-bound IgM. DT40 clones expressing antigen-specific mAbs can then be isolated using magnetic beads conjugated to any target antigen of interest. This technology, named the ADLib system (Autonomously Diversifying Library system) (5,6), has the advantage of allowing the acquisition of whole IgM molecules in a rapid and convenient manner. Moreover, given the ease of genetic manipulation and culture of DT40 cells (10), the selected clones can be readily expanded and manipulated to accommodate the needs for a scaled-up production or an improved immunoreactivity, including, for instance, the development of *in vitro* affinity maturation systems based on the genetic enhancement of Ig hypermutation (11,12).

The ADLib system has proved to be an effective method for the *de novo* acquisition of antigen-specific mAbs of interest (5,6). However, one limitation was that DT40 cells can produce only chicken IgM, in either its membrane-bound or secreted form. Conversion to other Ig isoforms, notably IgG, is often desirable for practical use, especially for applications involving the recognition of the Fc region. The development of *in vivo* assays and of medical applications also requires the immunogenicity of the mAb itself to be reduced as much as possible, which is

usually achieved by engineering chimeric or humanized versions of the selected mAb. In this report, we present a novel approach for the direct generation of chimeric human IgG in DT40 cell display libraries. By knock-in of the human IgG constant region into the chicken IgM heavy-chain locus, we designed DT40 derivatives that express by alternative splicing both chicken IgM and chimeric IgG sharing the same antigen-binding domain. These strains can generate a library to screen for antigen-specific mAbs by direct application of the ADLib system, thus allowing the simultaneous isolation of specific chimeric IgG of interest. In addition, we show that the production of chimeric IgG can be selectively increased over chicken IgM by modulating the efficiency of RNA processing.

MATERIALS AND METHODS

Cell culture conditions

DT40 cells were cultured as previously described (5,7) in IMDM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, SAFC Biosciences), 1% chicken serum (Invitrogen), 50 U/ml penicillin–50 μg/ml streptomycin (Invitrogen), 55 μM 2-mercaptoethanol (Invitrogen), at $39.5°C$ in 5% CO_2 incubator. Media were changed regularly every 1 or 2 days to maintain the cell density at 2×10^5 to 1.5×10^6 cells/ml. TSA (Wako) was added in the medium at each passage at 2.5 ng/ml when indicated. For purification of chimeric IgG, cultures were transferred the preceding day into a medium containing 10% Ultra-Low IgG FBS (Invitrogen) instead of usual FBS and no chicken serum to reduce contamination with serum IgG.

Plasmid constructs

For the chimeric ch/hu-IgHG1 construct, we first amplified the full-length human IgHG1 constant region (about 3 kb comprising the exons C_H1, H (hinge), C_H2, C_H3 and the downstream polyadenylation signal) and parts of the chicken IgHM constant region from human genomic DNA using the expand long range PCR system (Roche). Primer sequences are provided in Supplementary Data S1. The fragments were cloned into the pCR2.1TOPO vector with the TOPO TA cloning kit (Invitrogen). As the direct amplification of a large region of chicken IgHM proved to be difficult, we first cloned two overlapping fragments, from exon C_H1 to C_H2 then from C_H2 to C_H3, and we ligated them at the PmlI restriction site in exon C_H2 using the DNA Ligation Kit Ver.2.1 (Takara) to reconstitute the entire region from C_H1 to C_H3 (~9 kb). The DNA sequences of the cloned IgHG1 and IgHM regions were analyzed with an ABI3730xl sequencer (Applied Biosystems). We next inserted a Blasticidin S resistance (bsr) marker cassette under control of a β-actin promoter at the EcoRV site downstream of human IgHG1. We then extracted the MluI–XhoI fragment comprising the IgHG1 exons H, C_H2, C_H3 and the bsr marker. This fragment was inserted by blunt ligation at the region digested by BseRI, in the

intronic region between exons C_H1 and C_H2 of chicken IgHM, to make the final ch/hu-IgHG1 plasmid.

For the CstF-64 overexpression construct, we extracted total RNA from wild-type DT40 cells with Trizol reagent (Invitrogen) and we amplified the CstF-64 cDNA flanked by the ClaI and NheI restriction enzyme sites (primers provided in Supplementary Data S1) by reverse transcription-polymerase chain reaction (RT-PCR) (Superscript III One-step RT-PCR, Invitrogen). After sequence verification and digestion with ClaI and NheI, we inserted the cDNA fragment in pIEn-NT, a vector derived from pIRES-neo (Clontech) in which the CMV expression promoter has been replaced by the CAGGS promoter [containing the CMV enhancer and a chicken β-actin promoter (13)].

Transformation of DT40 cells and constructs verification

Transformation was performed following a standard protocol. For the chimeric ch/hu-IgHG1 construct, 50 μg of the corresponding plasmid were linearized with NcoI to transfect 1×10^7 wild-type DT40 cells by electroporation (Bio-Rad Gene Pulser, 0.4 cm gap, 550 V, 25 μFD). After 24 h of incubation at $37°C$ in 5% CO_2 incubator, single colonies of stable clones were selected in 96-well plates with a medium containing 25 μg/ml Blasticidin S. The positive ch/hu-IgHG1 transformant strain used in this study was named CX13. For the CstF-64 overexpression construct, 50 μg of plasmid were linearized with PvuI to transfect 1×10^7 cells of the CX13 chimera strain and stable clones were selected in medium containing 2 mg/ml Geneticin (Invitrogen).

The expression of ch/hu-IgHG1 messenger RNA (mRNA) was confirmed by RT-PCR (Superscript III One-Step RT-PCR, Invitrogen) after isolation of total RNA using Trizol reagent (Invitrogen). Correct integration of the constructs was confirmed by PCR analysis (primers provided in Supplementary Data S1) using the KOD FX enzyme (Toyobo) after isolation of genomic DNA using the Illustra GenomicPrep Mini Spin Kit (GE Healthcare). We also confirmed by sequence analysis the full sequence of the chimeric ch/hu-IgHG1 cDNA, from the chicken IgHM V-region to the human IgHG1 constant region stop codon (sequence available upon request).

Flow cytometry

1×10^6 cells were harvested and washed in staining buffer [phosphate-buffered saline (PBS), 0.5% bovine serum albumin (BSA, Sigma), 2 mM EDTA], labeled for surface IgM with 4 μg/ml fluorescein isothiocyanate (FITC)-conjugated goat anti-chicken IgM antibody (Bethyl), then with 5 μg/ml propidium iodide (Sigma) to gate out dead cells. The percentage of surface IgM-positive cells (IgM^+) was determined by measuring the FITC fluorescence intensity with the Cytomics FC500 flow cytometer (Beckman Coulter).

Protein immunoblotting

Two to five microliters of WCE or serum supernatant samples were diluted 1:1 in 2x Laemmli loading buffer

[4% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, 20% glycerol, 0.004 % bromophenol blue, 0.125 M Tris–HCl pH 6.8], denatured 5 min at 95°C, separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 10% acrylamide gels in a XCell SureLock Mini-Cell (Invitrogen) and transferred onto nitrocellulose membranes (Hybond-ECL, GE-Healthcare). The blots were blocked in PBST (1x PBS, 0.05% Tween) with 5% skim milk, incubated with the appropriate primary antibody [1:10 000 HFP-conjugated anti-human IgG-Fc (Bethyl), HFP-conjugated anti-chicken IgM (Bethyl) or 1:5000 anti-FLAG antibody (Sigma)] diluted in PBST with 1% skim milk for 1 h at room temperature, then with a secondary antibody if necessary [1:10 000 HFP-conjugated anti-mouse IgG (sigma) for the anti-FLAG immunoblotting]. Signals were detected by exposure on Hyperfilm (GE Healthcare) using the ECL western blotting reagents (GE Healthcare). Preparation of deglycosylated proteins was performed by removal of N-glycans using the enzyme PNGase F (New England Biolabs) according to the protocol of the manufacturer, with 2 h incubation at 37°C for denatured proteins, or overnight to 24 h incubation for native proteins. Whole human IgG1 Kappa (Sigma), diluted at 1 µg/ml in PBS or in the same culture medium as the samples, was used as positive control for the detection of human IgG.

Purification and concentration of chimeric IgG

Chimeric IgG expressing clones were cultured for 24 h in 30 ml medium with 10% Ultra-Low IgG FBS (Gibco-Invitrogen) to a density of 2×10^6 cells/ml. After centrifugation for 10 min at 190 g, the culture supernatant was filtered through a 22-µm filter membrane and purified with a protein G affinity chromatography column (Mab Trap Kit, GE Healthcare) according to the protocol of the manufacturer. Purified immunoglobulins were finally eluted in 3 ml buffer, thus resulting in an ~10-fold concentration.

Generation of diversified cell cultures for the ADLib system

The selected chimeric CX13 cells were passaged every day for 4 weeks in 12 ml medium + 2.5 ng/ml TSA, then 2 weeks in 50 ml medium + 2.5 ng/ml TSA. Analysis of sequence diversification at the IgL and IgH V-regions were essentially performed as described (5,7). Genomic DNA was extracted from 1×10^6 cells using the Illustra GenomicPrep Mini Spin Kit (GE Healthcare) and 1:200 of each sample (corresponding to 5000 cells) was used as template for PCR amplification of the V-regions (primers in Supplementary Data S1). After purification of the PCR products and cloning into the pCR2.1 TOPO vector (Invitrogen), 15 clones for IgL and 24 clones for IgH were sequenced using the M13 universal forward or reverse primers with an ABI 3730xl sequencer (Applied Biosystems). Sequences were aligned to the parental clone before expansion and to published V pseudogene sequences (8,9) to determine the frequency of sequence alterations due to gene conversion or point mutation events.

Selection of antibodies with the ADLib system

The selection of monoclonal antibodies was performed as described (5,6). Briefly, we first conjugated the target antigen (apoferritin, Sigma) to M280 tosyl-activated magnetic beads (Dynal) according to a standard protocol (6). Then 5 µl of antigen-coated beads (about 1.25×10^8 beads) were put into contact with the cells from a 50 ml ADLib culture (about 1×10^8 cells). After several washing steps, the beads – along with the cells bound to the target antigen, if any – were distributed into a 96-well plate with fresh medium and incubated at 39.5°C, 5% CO_2 for 1 week. Culture supernatants from the wells containing growing colonies of antigen-binding cells were analyzed by enzyme-linked immunosorbent assay (ELISA) to screen for antigen-specific antibodies secreted into the medium.

ELISA

ELISA experiments were performed as described (6). Briefly, the wells of a U-bottom maxisorp immunoplate (Nunc) were incubated overnight at 4°C with the antigen of interest diluted at 3 µg/ml in PBS. After blocking with a solution of 1% BSA in PBS for 30 min and washing with PBST (1× phosphate saline buffer, 0.05% Tween), 100 µl of culture supernatants were added to each well and incubated for 1 h at room temperature. After washing five times with PBST, 100 µl of secondary antibody [1:10 000 HRP-conjugated anti-chicken antibody or 1:2000 HRP-conjugated anti-human antibody (Bethyl)] was added for 1 h at room temperature. After washing five times with PBST, immunoreactivity was revealed by addition of 100 µl 3,3',5,5'-tetramethylbenzidine (TMB) (Dako Cytomation) for 3 min for anti-chicken antibody and 5 min for anti-human antibody. The reaction was stopped with 100 µl 1 N sulfuric acid and the optical density at 450 nm was measured with a microplate reader (Bio-Rad)

Quantitative ELISA

For sandwich ELISA, the wells of a U-bottom maxisorp immunoplate (Nunc) were incubated for 1 h at room temperature with 100 µl of anti-human IgG-Fc or anti-chicken IgM antibody (Bethyl) diluted at 10 µg/ml in PBS. After blocking with a solution of 1% BSA in PBS for 30 min and washing with PBST, 100 µl of a serial dilutions of culture supernatant were added for 1 h at room temperature, washed, then 100 µl of 1:10 000 HRP-conjugated anti-human IgG-Fc or anti-chicken IgM (Bethyl) were added for 1 h at room temperature. After washing, immunoreactivity was revealed by addition of 100 µl TMB (Dako Cytomation) for 3 min, the reaction stopped with 100 µl 1N sulfuric acid, and the optical density at 450 nm was measured with a microplate reader (Bio-Rad). A standard curve was generated from the O.D. measurements of a control human IgG1 Kappa or (Sigma) chicken IgM (Invitrogen) serially diluted in culture medium from a starting concentration of 1 µg/ml. Then the concentrations of chimeric IgG or of chicken IgM in each sample were estimated from the

O.D. measured at least at two different dilutions in a range allowing linear regression (usually between O.D. = 0.6 and 1.2).

Competition ELISA

Comparison of mAb binding affinities by competition ELISA has been performed as previously described (14), with some modifications. The selected samples of culture supernatants were first serially diluted in fresh culture medium and titrated by anti-apoferritin ELISA as above to determine the dilution required to obtain a maximal signal of O.D. = 0.7. The samples were then diluted accordingly and incubated overnight at room temperature with serial dilutions of apoferritin with concentrations ranging from to 0.05 to 500 nM. The mixed solutions were then analyzed by anti-apoferritin ELISA to determine the concentration of antigen, which inhibits 50% of the maximal binding by free antibodies (IC50) by plotting the percentage of the maximal reactivity signal as function of the concentration of competitor antigen.

RESULTS

Construction and characterization of DT40 clones expressing chicken/human chimeric IgG

The DT40 B-cell line constitutively produces membrane-bound and secreted-form chicken IgM. We engineered DT40 cells by targeted integration, so that they would express chicken/human chimeric IgG, in which the Fc domain of the IgM heavy-chain constant region has been replaced by its human IgG1 counterpart (Figure 1). To make the targeting vector, we first cloned a human genomic region including the last three exons (hinge H, C_H2, C_H3) of the Ig gamma-1 heavy-chain constant region (human IgHG1). As the first exon C_H1 is known to be essential for correct assembly and secretion of chicken light-heavy Ig chains (15), we decided to keep the chicken IgM sequence for C_H1 in this experiment. We inserted the human IgHG1 fragment into the intronic sequences between exons C_H1 and C_H2 of the chicken Ig mu heavy-chain constant region (chicken IgHM). After RNA splicing, this construct is expected to transcribe mRNA with chicken C_H1 adjacent to the human H-C_H2-C_H3 exons.

The chicken IgHM locus contained much longer introns (over 4 kb between C_H1 and C_H2) than human or mouse Ig genes (~0.1–0.3 kb) and composed of large tandem repeats, including regions possibly related to avian-specific CNM and PIR repeats with a 21-bp consensus sequence (16,17). One of these segments could be digested by the restriction enzyme BseRI to be replaced by the human IgHG1 fragment and a flanking selectable marker cassette (Figure 1A). To verify the construct integration (Figure 1B), selected stable DT40 transformants were analyzed by PCR amplification of a 4.5-kb fragment from the beginning of chicken C_H1 to the marker gene (data not shown). We confirmed by RT-PCR and sequence analysis the mRNA expression of the chimeric chicken/human IgG1 heavy-chain (ch/hu-IgHG1) which

contained as predicted the VDJ-C_H1 region of chicken IgHM fused to the H-C_H2-C_H3 region of human IgHG1 (Figure 1B and C). We also detected the simultaneous expression of the full-length chicken IgHM transcripts, presumed to be produced by alternative splicing leading to the removal of the human IgHG1 insert (Figure 1B and C).

We choose one of the positive chimera transformants, referred to as the strain CX13, for further analysis of the expression of chicken IgM and of chimeric IgG (Figure 2). We first confirmed that the chimeric CX13 cells expressed membrane-bound IgM, which were detectable at levels comparable to the parental wild-type (WT) cells by flow-cytometry using a fluorescein-conjugated anti-chicken IgM antibody (Figure 2A). The presence of chicken IgM and of chimeric IgG secreted into the culture medium was also detected by quantitative sandwich ELISA using anti-chicken IgM and anti-human IgG antibodies. The concentration of chimeric IgG in culture supernatants was about 5-fold lower than chicken IgM (Figure 2B). Nonetheless, the concentration of chimeric IgG reached between 0.5 and 1.1 μg/ml after 1 day in cultures containing around 1×10^6 cells (data not shown, estimation based on five random independent cultures), which is comparable to the productivity of recombinant antibodies in stable CHO cells under nonoptimized conditions [0.1–1.1 pg/cell/day (18)].

The expression of chimeric ch/hu-IgHG1 in the CX13 culture supernatant was confirmed by western blotting (Figure 2C, lane 3), but the corresponding band appeared at a higher position than expected compared to the control human IgHG1 (lane 1). This difference is most likely due to the higher glycosylation levels of ch/hu-IgHG1, as ch/hu-IgHG1 includes the C_H1 exon of chicken IgHM, which contains an asparagine N-glycosylation consensus site (Asn-Asn-Ser) evolutionary conserved in IgHM, but absent from human IgHG1. After purification of chimeric IgG with a protein G affinity column followed by enzymatic removal of N-glycans by PNGase F, the band of ch/hu-IgHG1 migrated at the correct predicted size of about 50 kDa (lane 6).

These results demonstrated that DT40 cells stably transformed with the ch/hu-IgHG1 construct generate at the same time whole secreted-form chimeric IgG, secreted-form chicken IgM and membrane-bound chicken IgM. In a same cell, all three Ig variants are presumed to share the same antigen-binding domain and thus potentially recognize the same antigen. We therefore used the chimera transformant CX13 to generate a library of chimeric IgG that could be adapted to the screening of specific antibodies with the ADLib system. The overall strategy used in this method is illustrated in Figure 1D.

Application to the ADLib system for the selection of chimeric IgG antibodies

To prepare a diversified library of cells producing chimeric IgG, we cultured CX13 cells for 6 weeks in the presence of 2.5 ng/ml TSA. We confirmed by sequence analysis that the prolonged treatment with TSA stimulated sequence
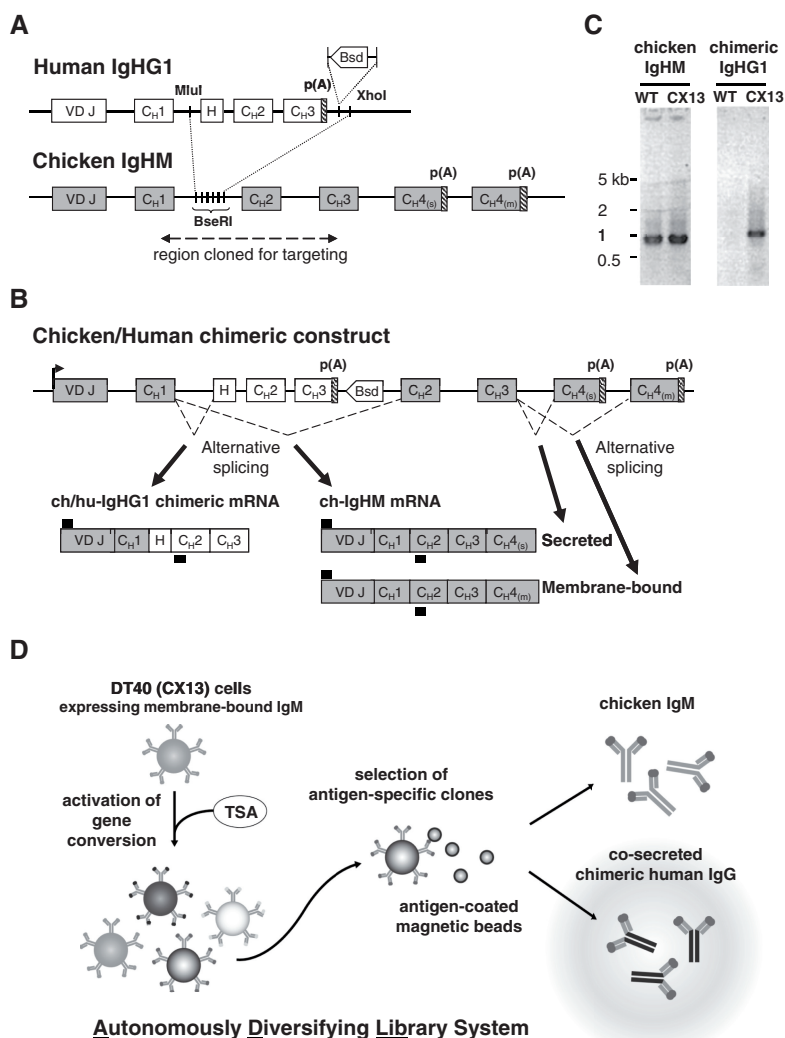
Figure 1. The chicken/human chimeric IgG expression construct. (**A**) Strategy for the knock-in of the human IgHG1 constant region at the chicken IgHM locus. The chicken IgHM region from the exon C_H1 to C_H3 was cloned to make the targeting vector. The intronic region digested by BseRI was replaced by the human IgHG1 region from H to C_H3 and a selectable marker. Bsd = blasticidin S deaminase. p(A) = polyadenylation sites. Genomic size and distances are not to scale (distance of chicken C_H1 to C_H2 = 4.5 kb; human H-C_H2-C_H3 insert = 1 kb). (**B**) Final genomic structure after integration of the knock-in plasmid and expected mRNA variants produced by alternative splicing. Thick horizontal bars indicate the approximate positions of the primers for PCR and RT–PCR used to confirm genomic integration and expression of IgHM and IgHG1 transcripts. (**C**) Expression of IgHM and of chimeric IgHG1 detected by RT–PCR in the wild-type parental strain (WT) and a positive chimeric transformant (CX13). (**D**) Schematic outline of the ADLib system applied to the selection of chimeric human IgG. (From left to right) The enhancement of sequence diversification at the Ig variable locus in TSA-treated DT40 cultures allows the generation of an autonomously diversifying library of cells expressing various surface IgM; the clones specific for the target antigen are isolated using antigen-coated magnetic beads; CX13 cells co-express secreted-form chicken IgM and chimeric IgG with the same antigen-binding domain; antigen-specific chimeric IgG can therefore be isolated directly from the culture supernatant for further use.

diversification at the Ig variable region. Compared to the parental sequence of CX13 before expansion, 100% of the sequence patterns analyzed at the IgL locus and 54% at the IgH locus showed alterations, the majority of which could be attributed to gene conversion events (Figure 2D). The frequency of sequence alterations was comparable to the frequency usually observed in TSA-treated wild-type DT40 (5,7). Using primers specific to ch/hu-IgHG1, we also confirmed that the transcripts corresponding to the

chimeric IgG also contained diversified sequence patterns (data not shown).

Having obtained a display library of cells with diversified antibodies on their surface, we applied the ADLib system to select antigen-specific clones (5,6), using apoferritin as target antigen. We screened a repertoire of about 10^8 cells from the above-diversified culture with apoferritin-conjugated magnetic beads, and we succeeded in isolating several clones producing
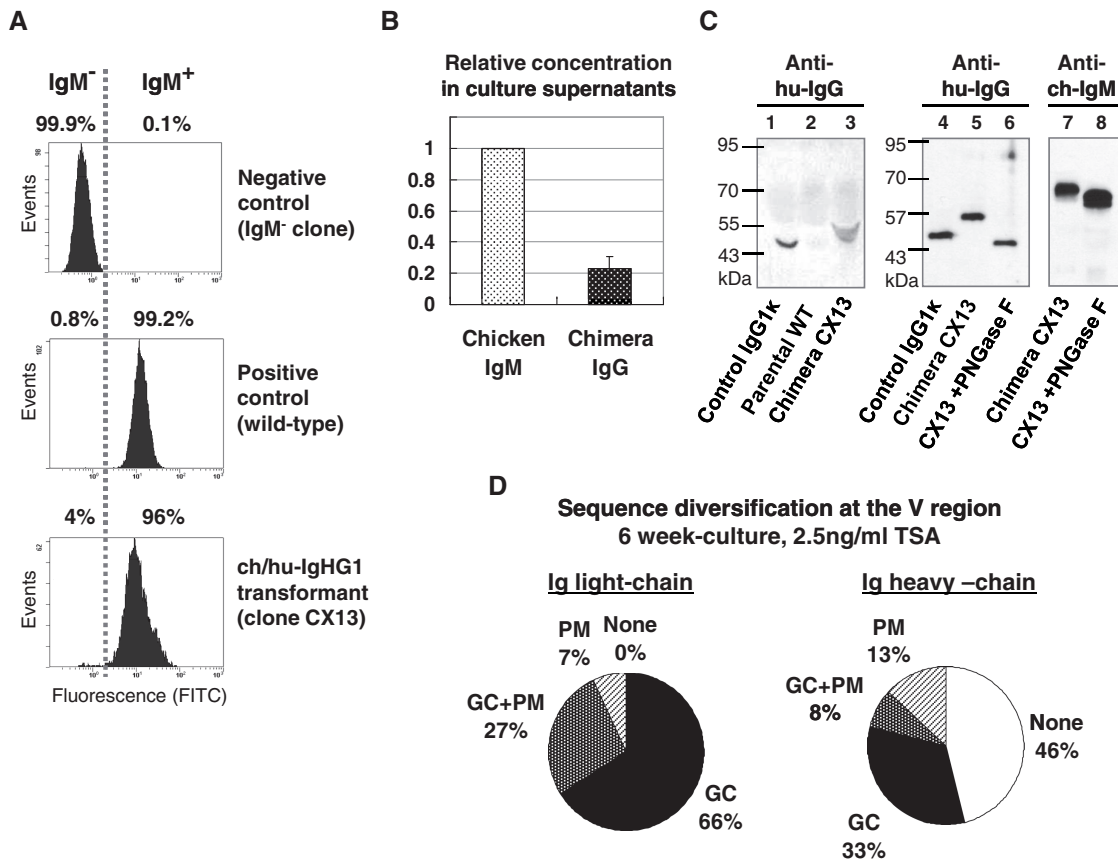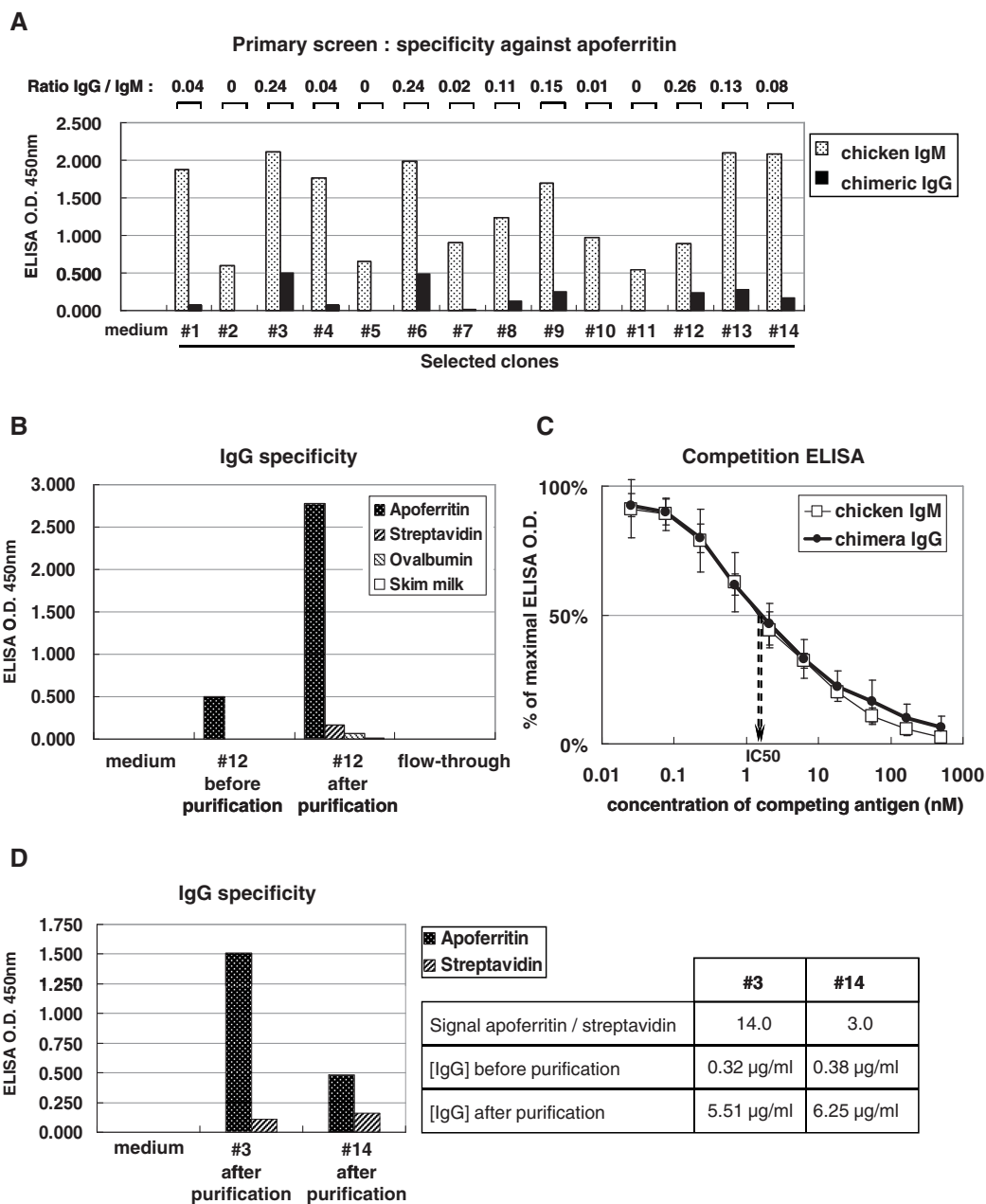
Figure 2. Expression and diversification of chicken IgM and of chimeric IgG in transformed DT40 cells. (**A**) Flow-cytomeric analysis of surface displayed chicken IgM. Expression of membrane-bound IgM was detected by immunostaining of DT40 cells with FITC-conjugated anti-chicken IgM antibody. The measure of fluorescence intensity allows the definition of two distinct populations of cells expressing membrane-bound IgM (IgM+) or not (IgM–) as shown respectively by the WT control (middle graph) and a mutant negative control (upper graph). As shown in the bottom graph, the great majority (96%) of the chimera CX13 cells are contained in the IgM+ fraction. (**B**) Relative concentration of chicken IgM and of chimera IgG secreted in the culture medium. The concentrations estimated by quantitative ELISA ranged from 0.5 to 1.2 µg/ml for chimera IgG and from 1.8 to 6.7 µg/ml for chicken IgM depending on the culture conditions. The graphs indicate the average and standard deviation of the chimera IgG concentration relative to the chicken IgM concentration (arbitrarily set to 1) calculated from five independent cultures. (**C**) Western blot with anti-chicken IgM or anti-human IgG antibodies. The loading of non-diluted culture supernatant (first blot) leads to deformed band because of the over-loading of serum proteins. Further analyses (second and third blots) were performed after purification of IgG by affinity chromatography and dilution in PBS. Lanes 1 and 4: human IgG1 positive control. Lane 2: parental WT showing no expression of chimera IgG. Lanes 3, 5 and 7: CX13 coexpressing chimeric IgG and chicken IgM. Lanes 6 and 8: CX13 after deglycosylation with PNGase F. (**D**) Sequence diversification induced in the Ig variable region after culture expansion for 6 weeks in presence of TSA. The proportion of cells containing sequence alterations compared to the parental chimera CX13 clone was determined by sequence analysis: GC = gene conversion tracts, PM = point mutations, none = no alteration.

anti-apoferritin Ig. ELISA analysis showed that the culture supernatants of these clones contained secreted-form chicken IgM, which were highly specific to apoferritin (Figure 3A). We also observed that the same samples contained apoferritin-specific chimeric IgG, although the ELISA signal intensity was lower than for chicken IgM (Figure 3A). The lower reactivity might be due to the 5-fold lower concentration of chimeric human IgG as compared to chicken IgM (Figure 2B). To test this idea, we selected a clone with a high IgG to IgM signal ratio (clone #12 of Figure 3A) and we purified the IgG antibodies from a saturated culture supernatant using protein G columns. The concentration of chimeric IgG before purification as determined by quantitative ELISA was 0.54 µg/ml. We eluted the sample after purification in a buffer volume that allowed an over 10-fold enrichment of chimeric IgG (final concentration 5.61 µg/ml).

The ELISA reactivity to apoferritin increased accordingly as expected (Figure 3B). These results indicate that the purified chimeric IgG is highly specific to the target antigen but exhibits less reactivity in the initial culture supernatants due to interference by the excess of coexisting chicken IgM.

We also compared the affinity of chicken IgM and of chimeric IgG to apoferritin by the method of Rath *et al.* (14), which provides a measurement of the relative antibody affinity by competitive ELISA. By plotting the percentage of binding as a function of competitor antigen concentration (Figure 3C), we determined the concentration of competitor required to inhibit 50% of the binding of the free antibody in solution (IC50). The IC50 is correlated to the dissociation constant of the antibody. The average IC50 values for two independent clones (clone #3 and #12 of Figure 3A) were very similar

A

Primary screen : specificity against apoferritin

Ratio IgG / IgM : 0.04 0 0.24 0.04 0 0.24 0.02 0.11 0.15 0.01 0 0.26 0.13 0.08

B

IgG specificity

C

Competition ELISA

D

IgG specificity

	#3	#14
Signal apoferritin / streptavidin	14.0	3.0
[IgG] before purification	0.32 µg/ml	0.38 µg/ml
[IgG] after purification	5.51 µg/ml	6.25 µg/ml

Figure 3. Selection of antigen-specific chimeric IgG. (**A**) Primary screen of anti-apoferritin mAbs selected with the ADLib system from the chimera CX13 derived library. Isolated clones (1–15) were grown for 1 week after selection. Antigen-specific chicken IgM and chimeric IgG were detected by ELISA using apoferritin-coated immunoplates incubated with the culture supernatant samples and with respectively anti-chicken IgM and anti-human IgG secondary antibodies. O.D. values were corrected with the medium background value. The ratio of the O.D. for human IgG relative to chicken IgM is indicated above the graphs for each selected clone. The clones with the highest ratio (#12, #3) were kept for further analysis. (**B**) Reactivity of the chimeric IgG of apoferritin-specific clone #12 to different antigens before and after purification with a protein G affinity chromatography column. (**C**) Determination of the relative binding affinities of purified apoferritin-specific chimera IgG and chicken IgM. Culture supernatants were incubated with serial dilutions of apoferritin and then analyzed by competitive ELISA to compare the reactivity of chicken IgM and of chimeric IgG in the presence of increasing concentrations of competing antigen. By plotting the ELISA O.D. (represented as a percentage of the maximal O.D. measured in the absence of any competitor antigen) in function of the concentration of antigen, we determined the concentration needed to inhibit the binding of free antibody by 50% (IC50), which is representative of the antibody affinity. The graphs show the average and standard deviation values from four independent experiments using two different apoferritin-specific clones (#3 and #12). Calculated IC50 for chimera IgG = 1.7 nM; IC50 for chicken IgM = 1.8 nM. (**D**) Comparison of two apoferritin-specific clones (#3 and #14) showing different antigen-binding reactivity after purification of chimeric IgG.

between chicken IgM (1.8 nM) and affinity-purified chimera IgG (1.7 nM). These results suggest that the selection of antigen-specific chicken IgM from the CX13 chimera-derived library allowed the simultaneous selection of chimeric IgG with the same specificity and affinity for the target antigen.

We also noticed that, in some particular cases, the selected candidates appeared to produce chimeric IgG

with lower specificity, despite the high specificity shown by the corresponding IgM. For instance, the clones #3 and # 14 of Figure 3A showed a similar specificity of IgM to apoferritin, whereas the specificity of chimeric IgG appeared significantly reduced for #14 compared to #3 as shown by the difference in their IgG to IgM specificity signal ratio (respectively 0.08 and 0.24). Even after purification with protein G columns, the specificity of chimeric IgG from clone #14 stayed at a much lower level than the IgG from clone #3, although the concentrations of IgG were similar (Figure 3D). This suggests that some clones such as #14 might not be suited to the conversion of IgM into chimeric IgG, possibly because structural alterations affect the conformation of their antigen-binding site. Such problems are often experienced during the process of genetically engineering conventional monoclonal antibodies to convert them into human IgG. The present system can readily eliminate such 'class-switch sensitive' clones at the stage of primary screenings by simply considering the IgG to IgM signal ratio.

Increase of chimeric IgG secretion by enhancement of RNA cleavage-polyadenylation

We next wondered if we could shift the IgG to IgM expression ratio in favor of a higher concentration of IgG in the culture supernatant, in order to increase the production of chimeric IgG. In B cells, the simultaneous expression of membrane-bound IgM and of secreted-form IgM is controlled by the balance between competing RNA cleavage–polyadenylation and RNA splicing reactions (19): the use of a promoter–proximal poly(A) site leads to the production of secreted-form IgM, while exon-skipping by alternative splicing and the use of a distal poly(A) site lead to the production of membrane-bound IgM. When B cells differentiate into mature plasma cells, the use of the promoter–proximal poly(A) site is enhanced by the overexpression of polyadenylation and elongation factors such as CstF-64 and ELL2 (20,21), leading to an increased production of secreted-form IgM over membrane-bound IgM.

We investigated whether the production of chimeric IgG could be increased in a similar manner by overexpression of CstF-64, as our construct includes a poly(A) termination site after the human IgHG1 insert. We transfected the CX13 chimera cells by random integration of an expression plasmid with the N-terminal FLAG-tagged CstF-64 under the CAGGS promoter, and verified CstF-64 protein expression by western blotting using anti-FLAG antibody (Figure 4A).

RT–PCR analysis showed that the transcript level of ch/hu-IgHG1 was not affected in CstF-64 overexpressing cells (CstF-64$^+$ cells) compared to the parental CX13 cells (Figure 4B). In striking contrast, the ch-IgHM transcripts were drastically reduced in CstF-64$^+$ cells (over 27-fold reduction). This indicates that chicken IgM expression was severely repressed in CstF-64$^+$ cells, consistent with the loss of membrane-bound IgM in FACS analysis (Figure 4C).

We also analyzed the amounts of chimeric IgG secreted in the culture supernatants by quantitative ELISA (Figure 4D). To ensure that any differences in the concentrations of IgG were not due to variations in cell growth, we started cultures using two independent clones with different initial cell densities (from 0.5×10^6 to 2×10^6 cells/ml) and we took supernatants after 16 h (shorter than the doubling time in medium without chicken serum). The concentrations of chimeric IgG in the CstF-64$^+$ supernatants were 2–3-fold higher than those from the parental CX13 cells (Figure 4D). By contrast, as expected from the transcription analysis, the concentration of IgM in CstF-64$^+$ supernatants was strongly reduced by at least 10-fold (Figure 4E).

Taken together, these experiments demonstrated that the overexpression of the polyadenylation factor CstF-64 in CX13 cells induced the shutdown of chicken IgM expression and the relative increase of IgG synthesis, which resulted in a shift of the ratio of the concentration of IgG to IgM from 1:5 to about 4:1 and thus in a substantial improvement of the chimeric IgG production.

DISCUSSION

We described in this report new DT40 strains designed to express chimeric IgG antibodies containing chicken antigen-binding regions and human constant regions of the IgG isoform. The production of chimeric human IgG would allow easier purification and wider application than IgM, notably for therapeutic uses dependent on Fc region-mediated functions. By exploiting the alternative splicing processes specifically enabled in B cells, our constructs allow the coexpression of secreted chimeric IgG and of membrane-bound chicken IgM in the same cell. These cells can therefore be used to generate an autonomously diversifying library of antibodies displayed at the cell surface, from which antigen-specific clones can be isolated by the direct application of the ADLib system. This method presents the great advantage of allowing the selection and production of whole chimeric IgG mAbs in a single step, without the need of further manipulation such as making recombinant proteins from phage DNA. Other successful systems of antibody generation by display on mammalian cells have been reported, but still require a conversion to secreted-form IgG before re-expression in productive cells (22,23). Moreover, we showed that the co-selection of secreted IgG with the native IgM allows the immediate discrimination of the mAbs that do not retain their antigenic specificity after conversion to IgG. The direct acquisition of IgG would therefore facilitate the implementation of validation assays and may bring considerable gain in the time and effort required for the screening and lead optimization of new mAb candidates.

We demonstrated that the modulation of splicing/cleavage–polyadenylation efficiency by CstF-64 overexpression induced the cells to switch from the expression of IgM to an enhanced production of chimeric IgG. Interestingly, when the overexpression of CstF-64 was first reported by Takagaki *et al.* (21) to induce a switch of membrane-bound IgM to secreted-form IgM, the observed increase of membrane-bound to secreted-form
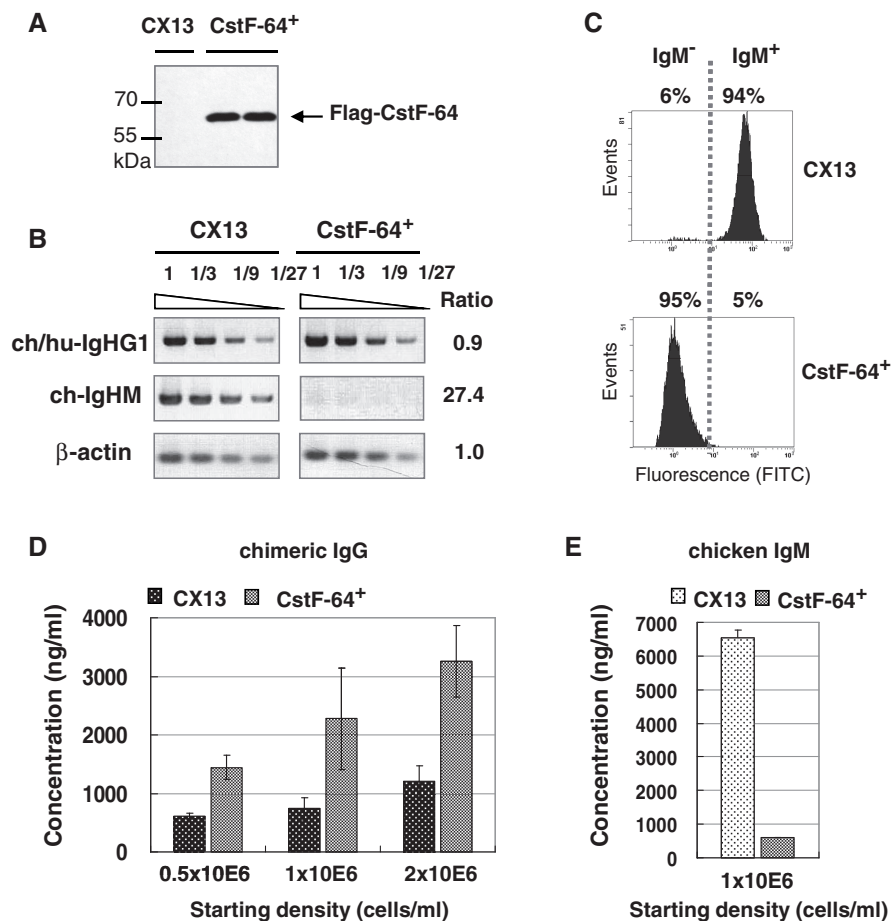
Figure 4. Effects of CstF-64 overexpression on chimera IgG and surface IgM expression. (**A**) Overexpression of exogenous FLAG-tagged CstF-64 shown by anti-FLAG western blot analysis of 5 μl WCE from the parental chimera CX13 and two transfected clones (CstF-64+). (**B**) Semiquantitative RT–PCR analysis of the expression of chicken IgM and chimeric IgG. The same primers as in Figure 1 were used to amplify transcript products from serial dilutions of total RNA starting from a concentration of 50 ng/ml. Amplifications of β-actin transcripts were used as normalization controls. The average ratios of the transcript levels in CX13 relative to the levels in CstF-64+ were estimated by gel band quantification. (**C**) Flow-cytomeric analysis showing the loss of membrane-bound chicken IgM in CstF-64+ cells. Surface IgM-positive and -negative populations were determined in each culture by the same method as in Figure 1a. While the parental CX13 cells are mostly IgM+ as shown previously (upper graph), 95% of the CstF-64+ cells appear IgM– (bottom graph). (**D**) Concentration of chimeric IgG in culture supernatants determined by quantitative ELISA. Cultures of CX13 and of CstF-64+ cells were started with different initial cell densities as indicated and supernatant samples were taken after 16 h. Each bar shows the average and standard deviation values from two independent experiments performed each with two different clones cultured in the same conditions. (**E**) Concentration of chicken IgM in culture supernatants determined by quantitative ELISA. Cultures of CX13 and of CstF-64+ cells were started with a density of 1×10^6 cells/ml as indicated and supernatant samples were taken after 16 h. Each bar shows the average and standard deviation values from two different clones cultured in the same conditions.

mRNA ratio was limited to about 8-fold the initial ratio, whereas in our experiments the IgM expression was much more reduced, resulting in an increase of the IgG to IgM ratio by at least 20-fold. This might be explained by the fact that the cleavage–polyadenylation reaction at the end of the inserted chimeric IgG would be more efficient than at the end of IgM, possibly because the IgG cleavage–polyadenylation site is much closer to the promoter (see Figure 1b), or because there might be some unknown differences between the recognition of splicing and/or cleavage–polyadenylation signal sites of different Ig isotypes or different animal species. Thus, the overexpression of CstF-64, possibly combined with a conditional expression system to prevent the loss of membrane-bound IgM during the ADLib selection phase, would ensure a high productivity of chimeric

human IgG while minimizing the expression of chicken IgM after the selection. This would provide a great practical advantage to facilitate further characterization of the selected IgG and their use in various biological assays.

Finally, it should be noted that the same strategy is presumably applicable to produce chimeras of other isotypes and other animal species at will. These results open promising prospects for systems capable to generate any type of antibodies, including fully humanized IgG for therapeutic purposes or even mimetic molecules with various protein scaffolds (24), by inserting the right expression construct at the appropriate genomic location. The versatility of the ADLib system applicable to a broad range of antigens (5,6), in combination with the recent development of DT40 affinity maturation systems (11,12) and with the direct generation of chimeric mAbs

that we presented in this report, can be expected to provide a fully integrated system to raise high-quality mAbs against any target of choice in a single step.

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