Biotechnology and Genetic Engineering Reviews - Vol. 24, 179-194 (2007)

Modulation of Immunoglobulin Gene Conversion in Chicken DT40 by Enhancing Histone Acetylation, and its Application to Antibody Engineering

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Introduction

Since monoclonal antibodies (MAbs) were developed more than three decades ago, their importance has been increasing in various fields of biological research, clinical diagnosis and therapy. MAbs enable us to supply antibodies of uniform quality that can be produced without limit. In addition, each particular MAb recognizes a single epitope on the antigen of interest. Therefore, MAbs are suitable for medical purposes such as diagnostic reagents and antibody-based medicines, which require rigorous quality controls and a stable and sustainable supply.

Currently, MAbs are actively utilized as antibody medicines against cancer and some chronic diseases (reviewed by Adams and Weiner, 2005). Antibody molecules have a long lifespan in the human body and they cause few side effects, since they

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Abbreviations: MAb: monoclonal antibody; Ig: immunoglobulin; HAT: histone acetyltransferase; IgH: Ig heavy; IgL: Ig light; TSA: Trichostatin A; HDAC: histone deacetylase; ADLib: Autonomously Diversifying Library; ELISA: enzyme linked immunosolvent assay; FACS; fluorescence activated cell sorting; GPCR: GTP-binding protein coupled receptors; FITC: Fluorescein isothiocyanate; DNP; dinitrophenol.

originally function as a "natural molecule-targeted medicine" in the humoral immune systems of vertebrates evolved later than cyclostomes. One of the most successful antibody medicines is Herceptin (Trastuzumab), which targets the extracellular segment of the HER2/erbB2 receptor. Herceptin is used for the therapy of a certain type of metastatic breast cancer for patients whose tumors overexpress this receptor. It has been reported that the combination of Herceptin with chemotherapy greatly improves both survival and response rate of the patients. Owing to the success of these pioneer antibody medicines, many pharmaceutical companies are actively involved in the development of MAbs for antibody medicines.

However, the current standard system of monoclonal antibody production presents several technical bottlenecks that remain unsolved. The monoclonal antibody system of Köhler and Milstein is based on the cell fusion of myeloma and B cells obtained from spleens of immunized mice (Köhler and Milstein, 1975). The procedure includes steps for immunization of animals, selection of hybridoma cells, multiple rounds of limited dilution, screening of positive clones producing appropriate MAbs, and clonal expansion for antibody preparation. Thus, the whole process of MAb preparation using this method is laborious and often very time-consuming (*e.g.,* several months). In addition, the initial immunization process poses the fundamental problem of "immune tolerance" for external antigens. In the thymus, the adaptive immune system excludes lymphocytes that are involved in the immune response to autoantigens, as well as to evolutionally conserved proteins. Hence, raising antibodies against autoantigens and highly conserved antigens needs additional elaboration. Small molecules and saccharide chains are also known to be tough antigens; preparing antibodies that show high affinity and specificity against them is very challenging.

To overcome such problems, and especially to bypass the immune tolerance bottleneck, an *in vitro*-based system for antibody or protein design was developed (reviewed in Rothe *et al.*, 2006). In the middle of 1980, Smith's group established a system called "phage display" (Griffiths and Duncan, 1998; Smith, 1985; Winter *et al.*, 1994), with which one can screen polypeptides interacting with any antigen of interest from a library of bacteriophages displaying foreign proteins fused with the phage coat protein. Alternative *in vitro* systems are called polysome display, ribosome display, or *in vitro* virus system (Mattheakis, *et al.*, 1994; Hanes and Plückthun., 1997; Gersuk *et al.*, 1997; Nemoto *et al*., 1997; Hanes *et al.*, 1998). For example, ribosome display and *in vitro* virus systems are based on the formation of stable antibody-ribosome-mRNA complexes in which the antibody protein is directly linked to its encoding DNA sequence. Binz and coworkers have reported another *in vitro* based system for high-affinity binder selection using ankyrin repeat protein libraries (Binz *et al.,* 2004). Although these *in vitro*-based methods have made great progress, they still require additional preparation time after screening in order to produce full antibodies by the use of recombinant DNA techniques.

Here we describe an *ex vivo* MAb design system using a chicken B-cell derived DT40 cell line which undergoes an enhanced genetic rearrangement at the immunoglobulin (Ig) locus. The system is based on our previous findings on the regulatory role of histone acetylation in yeast homologous recombination during meiosis. In the next section, we briefly describe the background of this system.

Background 1: histone acetylation and yeast recombination initiation

In eukaryotes, chromosomal DNA is associated with many proteins, including histones, and packaged into a highly condensed structure termed chromatin (Wolffe, 1997). Chromatin structure generally serves as an obstacle to DNA-templated processes like transcription or recombination since its extreme compactness inhibits access of *trans*-acting factors to DNA. Accordingly, chromatin is modified to alleviate the sterical hindrance when DNA is metabolized. Extensive studies on transcriptional activation revealed two systems for such chromatin modifications, ATP-dependent chromatin remodeling and the covalent modifications of histones. The former physically makes "accessible" chromatin DNA by mobilizing, evicting, or exchanging histones (Eberharter and Becker, 2004). The latter activates transcription by modulating interactions between DNA and histones, and/or functioning as landmarks to recruit other effectors, most likely transcription machineries (Kurdistani and Grunstein, 2003). For example, acetylation of lysines in histones H3 and H4, by neutralizing positive charge, is thought to weaken DNA-histone association. In addition, acetylated lysines are recognized and bound by transcriptional activators carrying "bromodomains".

More than a decade ago, we set out to address how recombination occurs in chromatin using yeasts. These basic studies revealed connections between recombination and chromatin regulations. Described in this section is one such study that uncovered the roles of histone acetylation (Yamada *et al*., 2004). The model system is the fission yeast *ade6-M26* locus where homologous recombination frequency elevates during meiosis. *M26* is a G/T transversion that creates a stop codon in the 5' region of the *ade6*$^{+}$ ORF, which offers a binding site for ATF-CREB family proteins Atf1-Pcr1 (Kon *et al*., 1997). The binding of Atf1-Pcr1 to *M26* is indispensable for meiotic activation of recombination at *M26*. We showed that the chromatin structure around *M26* undergoes drastic alteration prior to meiotic recombination (Mizuno *et al*., 1997). We extended our study to explore whether histone acetylation was involved in the recombination activation at *M26* (Yamada *et al*., 2004).

We first examined histone acetylation levels at *ade6-M26* and then compared them to those at the negative control *ade6-M375* locus, an identical nonsense mutation at the adjacent codon of *M26*. Both histones H3 and H4 were more acetylated around *M26* than *M375* and this was dependent on Atf1, suggesting histone hyperacetylation correlates with recombination activation. Then, seeking to find an enzyme responsible for increased acetylation at *M26*, we chose to test the possible involvement of SpGcn5, a homologue of a conserved and well-characterized histone acetyltransferase (HAT) Gcn5. The results clearly indicated that histone acetylation around *M26* required SpGcn5. The observed hyperacetylation appears to be biologically significant, because both Atf1 and SpGcn5, whose impairment severely compromised acetylation levels, played important roles at *ade6-M26* in meiotic chromatin remodeling as well as recombination. Based on these results, we proposed the following model. Prior to meiotic recombination, *M26*-associated Atf1 recruits HAT activity, including that of SpGcn5, to facilitate acetylation of histones H3 and H4 around it. Acetylated histones in turn would promote chromatin remodeling so that recombinases can easily access *M26*, leading to locally activated recombination (*Figure 1*).

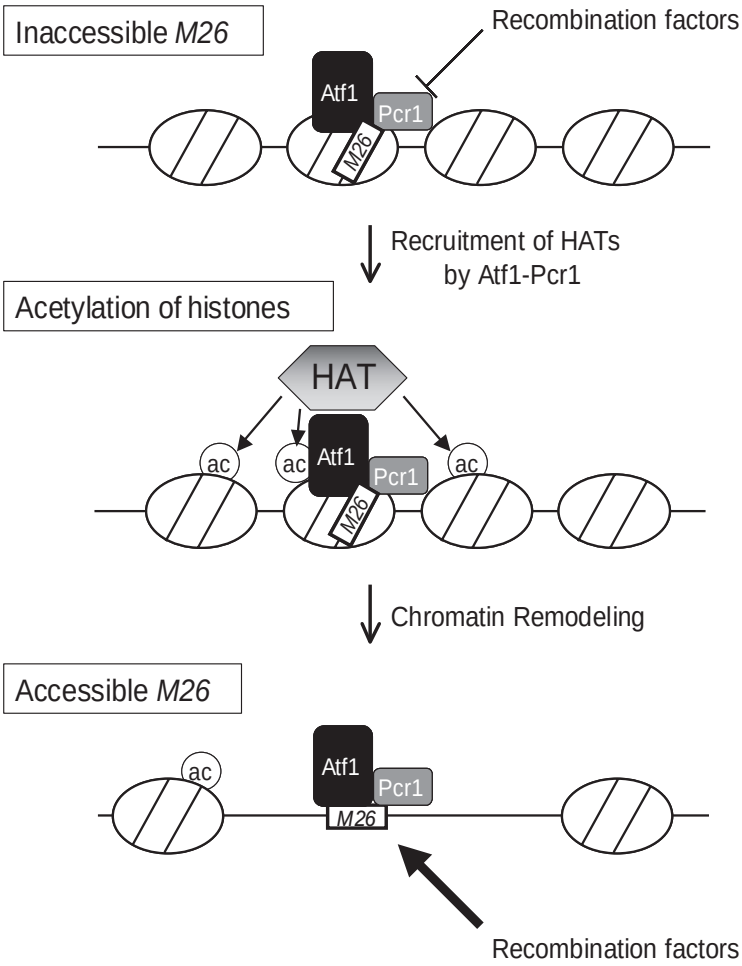

Figure 1. Histone acetylation locally enhances meiotic recombination. Histone acetylation facilitates local activation of meiotic homologous recombination in fission yeast. Before tethering of histone acetyltransferases(HATs), chromatin structure inhibits recombination factors from accessing *ade6-M26*, in spite of stable localization of Atf1-Pcr1 to *M26* (top). Upon recruitment of HATs by Atf1-Pcr1, histones around *M26* are acetylated, rendering chromatin structure open (middle). Altered chromatin is then accessible for recombination factors to be ready for recombination (bottom).

Acetylation of histones have been reported to be involved in a certain type of site-specific recombination such as V(D)J recombination (see below), but previous observations underscore the importance of histone acetylation in regulating homologous recombination. It is therefore tempting to explore how deeply histone acetylation contributes to the process of homologous recombination. For example, it is worth trying to test the roles of different HATs, since cells may have evolved several redundant pathways to ensure recombination. Indeed, the deletion of the gene coding for SpGcn5 did not completely abolish recombination at *ade6-M26*, suggesting possible involvement of other factors (Yamada *et al*., 2004). Also, and even more

interesting is the opposite: what happens to homologous recombination if histones are aberrantly hyperacetylated? That is in fact a starting point for our followup work explained below.

Background 2: Diversification of chicken Ig genes and histone modification

We next briefly describe the avian system for generating antibody diversity. Avian species (Reynaud *et al*., 1987), as well as rabbits, cattle, swine, and horses (Butler, 1998) utilize gene conversion (see below) to produce Ig gene diversification. In the avian lymphoid organ called bursa of Fabricius, in which B cells develop, B cells undergo rearrangement and diversification of Ig heavy (IgH) and light (IgL) chain genes. In contrast to mouse and human, each of the IgH and IgL genes of chicken has only one pair of V segment and J segment sequences with recombination signal sequences. A cluster of pseudo V segments exists upstream of the functional V segment. These pseudo V segments are non-functional, since they do not have any promoter or recombination signal sequence, and often are truncated. During embryonic development, unidirectional homologous recombination takes place from any of the pseudo V segments to the functional V regions in IgH and IgL genes. Thereafter, upstream pseudo V segments replace V region sequence by gene conversion (*Figure 2*).

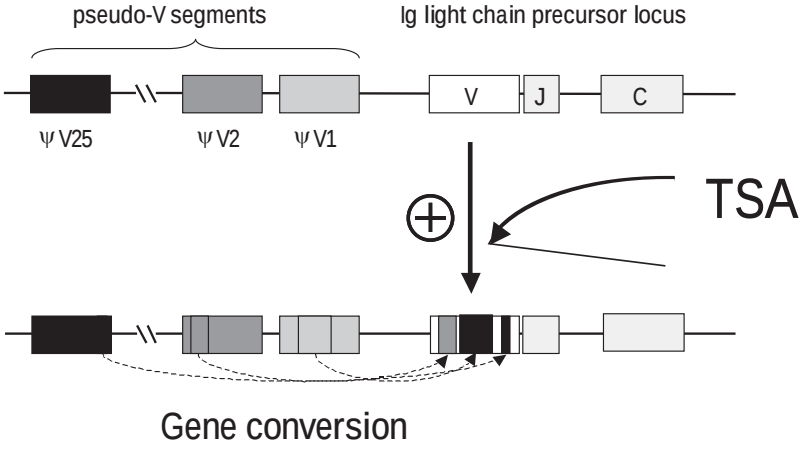

Figure 2. Gene conversion in recombination-active chicken Ig locus. V(D)J recombination occurs once in one of the two chicken Ig allele to generate the transcription- and recombination-active Ig allele. Using the upstream pseudo V segments (25 segments in the case of IgL) as templates, gene conversion, a type of homologous recombination, occurs unidirectionally and continuously at the recombination-active IgL and IgH V regions. Such gene conversion events create diversity of the IgL and IgH V regions in chicken B-cells. We discovered that TSA treatment of the DT40 cell line enhances the Ig gene conversion *in vivo.*

These phenomena have been extensively investigated using the DT40 chicken B lymphoma cell line, which was first utilized by Buerstedde and Takeda (Buerstedde & Takeda, 1991). DT40 has several interesting features as follows: 1) it is an immortalized cell line with relatively fast growth rate (doubling time 7-8 hours); 2)

easy to handle; 3) stable in karyotype; 4) can produce both membrane-bound (surface) and secreted types of IgM type antibody; 5) can undergo a low level of continuous gene conversion at the functional Ig locus; and 6) can be genetically modified by relatively high levels of targeted homologous recombination. For these reasons, DT40 offers a good model system to investigate the roles of histone acetylation in the control of gene conversion (homologous recombination) in higher eukaryotes. In addition, since DT40 displays surface IgMs on the cell membrane, we thought that it could be used for a B-cell based antibody display and production system. However, the gene conversion frequency at Ig locus in DT40 is not high: only a few % of the cells undergo gene conversion after passages of a couple of weeks. Therefore, we need to significantly increase the level of Ig gene conversion in order to apply the use of DT40 for antibody design.

Diversification of Ig locus in the presence of TSA

Using chromatin immunoprecipitation assays with anti-acetylated histones, we discovered that the VJ rearranged, recombination- and transcription-active IgL locus of DT40 exhibits higher histone H4 acetylation levels compared to the VJ unrearranged, recombination-inactive IgL locus (*Figure 3*). This suggested that histone acetylation controls gene conversion at the Ig locus in DT40. To test this notion, we treated DT40 cells with Trichostatin A (TSA), which is an antifungal hydroxamate produced by *Streptomyces* and the first natural molecule discovered to induce an accumulation of acetylated histones *in vivo* (Yoshida *et al.*, 1990). TSA is a recognized reference as one of the most potent reversible inhibitors of histone deacetylase (HDACs) catalytic activity, efficient at nanomolar concentrations. Since histone acetylation levels are regulated by the balance of histone acetylation by HATs and deacetylation by HDACs, histone acetylation is enhanced when treating the cells with HDAC inhibitors including TSA. Importantly, it was reported that TSA treatment of human B lymphocytes produces an elevation in V(D)J recombination frequency. Histone acetylation presumably facilitates the recruitment of recombination activating gene (RAG) proteins to the recombination signal sequence (Kwon et al., 2000; McBlane & Boyes, 2000; McMurry and Krangel, 2000; Gellert 2002). Thus, we speculated that TSA treatment of DT40 might enhance Ig gene conversion.

As predicted, we discovered that the gene conversion frequency and histone H4 acetylation at the recombination-active Ig locus is markedly increased upon treatment of DT40 cells with TSA (*Figure 3*). Indeed, using a surface IgM-negative DT40 strain containing a frameshift mutation in the Ig light-chain V region, we observed that prolonged treatment by adding TSA at different concentrations (0.625 to 2.5 ng/ml) in the culture medium leads to an increased reversion rate to surface IgM-positive cells (up to >90% cell population compared to <10 % without TSA treatment). This stimulation of repair by gene conversion was dependent on the TSA treatment time and its concentration, and concomitant with a local increase of H4 histone acetylation level as shown by chromatin immunoprecipitation experiments.

Furthermore, sequence analysis of the IgL V region of subclones randomly taken from a TSA-treated culture (*Figure 4*) revealed that their sequences were actually diversified (27 subclones out of 42 showing alterations from the original sequence

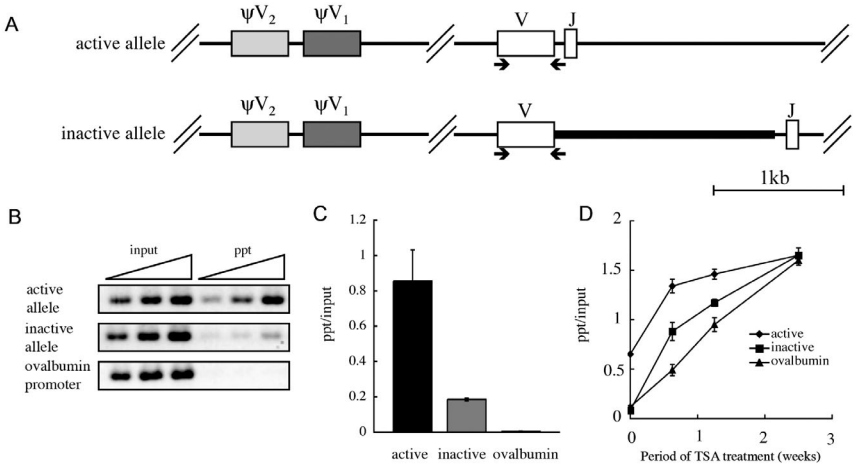

Figure 3. Higher histone acetylation at recombination-active V region. **A**, Schematic diagrams of recombination-active and -inactive Ig alleles. **B**, Chromatin immunoprecipitation assay using anti-acetylated histones reveals that the recombination-active V region exhibits higher histone H4 levels as compared to the recombination inactive Ig and ovalbumin alleles. **C**, quantification of results in **B**. The vertical axis represents the relative efficiency of chromatin immunoprecipitation (precipitated DNA *vs.* total input DNA) with anti acetylated histone H4. **D**, TSA dose dependency of histone acetylation. TSA induces histone acetylation of both Ig alleles, although transcription and gene conversion occurs only at the active allele possibly because it lacks a silencer for transcription that is located in the inactive allele (within a region shown in the thick line in **A**). Error bars (s.d.) in C and D are based on two independent experiments. Modified from the previous report (Seo *et al.*, 2005).

after a 3-week treatment), in contrast to subclones from a mock-treated culture that did not show any change in the absence of TSA. Comparison with the sequences of the 25 pseudo- IgL V regions then allowed us to recognize that the majority of genetic alteration at the IgL V region can be attributed to one or several independent conversion events (10 out of 16 different patterns), although we also observed a few mutation events suggesting that somatic hypermutation is slightly stimulated in the presence of TSA, as reported in the case of human BL2 cell line (Woo *et al.*, 2003). In addition, we confirmed that the IgH V region of DT40 is also diversified in the presence of TSA. These studies demonstrated that the diversification of the Ig locus could be accelerated by treatment of the cells with TSA. In other words, TSA treatment of DT40 can autonomously evolve an Ig diversified B-cell library, in which each cell presents surface IgMs with distinct specificity. We referred to this library as ADLib (Autonomously Diversifying Library), and we considered the use of this B-cell based library to develop an *ex vivo* antibody production system.

The ADLib system for selection of antibody producing DT40 clones

For the selection of DT40 clones producing an antigen specific antibody, we prepared magnetic beads conjugated with the antigen of interest. Conjugation of antigens to

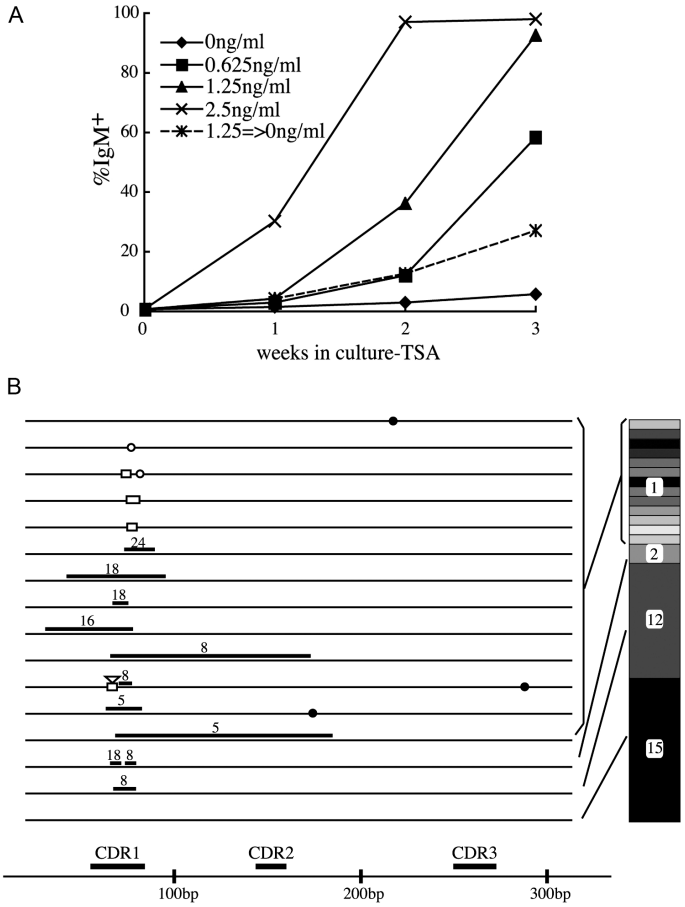

Figure 4. Treatment of DT40 with TSA enhances the Ig gene conversion. Prolonged TSA treatment causes autonomous diversification of both IgH and IgL V regions. The figure illustrates diversification of IgL V regions. Data are from Seo *et al*., 2005. **A**, Time course experiments of gene conversion monitored by FACS. The DT40 CL18 clone has a frameshift mutation in the complementarity-determining region-1 (CDR1), therefore no surface IgM is produced. When some gene conversion events rescue this frameshift mutation, CL18 can produce surface IgM, which can be detected by FACS with fluorescein-conjugated anti-IgM antibodies. Note that most of the cells are converted to surface IgM positive in the presence of at least 1.25 ng/ml TSA, but little conversion occurs without TSA. If TSA treatment is terminated (1.25 ng/ml to 0 ng/ml), we detect a marked slowdown of the rate at which the population of surface IgM positive cells increases. This suggests that the action of TSA may be reversible. **B**, DNA sequence analysis of the diversified IgL V region. In the left panel, thick horizontal bars, open boxes, filled and open circles, and an open triangle represent DNA sequence alterations attributed as gene conversion, deletion, single mutation and deletion, and insertion events, respectively. The numbers above the bars represent the possible donor pseudo gene number. The right bar graph indicates the proportion of each sequence alteration with indication of numbers for each pattern.

magnetic beads is mediated by covalent crosslinking, by metal chelating of a histidine tag fused with antigen proteins, or avidin-biotin interaction for biotinylated antigens. The antigen-conjugated magnetic beads are then placed into contact with the diversified DT40 libraries *in vitro*. After extensive wash, selected clones are further

diluted, and then placed into each well of 96-well microtiter plates. Since DT40 grows fast, we can obtain culture supernatants containing secreted IgMs that are ready for enzyme linked immunosolvent assay (ELISA) in a week. The specificities of antibody produced by the selected clones can be directly examined either by ELISA with culture supernatant, or alternatively, fluorescence activated cell sorting (FACS) of the selected clones. As described below, we have succeeded in producing antibodies specific to various antigens using the "ADLib system" (*Figure 5*).

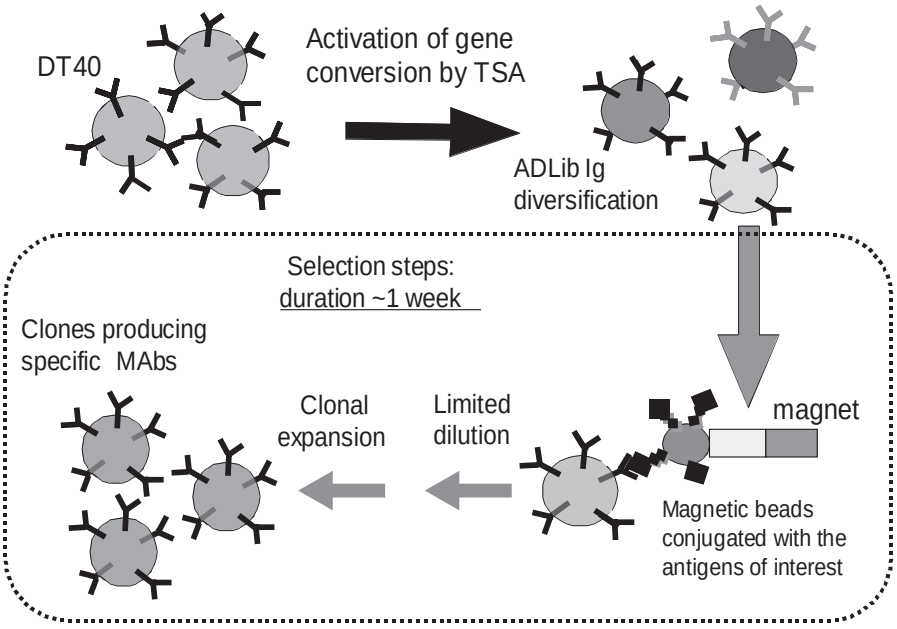

Figure 5. Schematic of the ADLib system. Once an ADLib has been prepared, screening steps require about one week, since the doubling time of DT40 is very short (7-8 days).

Advantages and current limitations of the ADLib system

Speed and flexibility are major advantages of the ADLib system. Regarding speed, one can obtain DT40 clones producing specific antibody by the ADLib system within one week in the quickest case. This is at least ten fold faster than conventional MAb preparation methods using hybridomas. In addition, it is even faster than the phage display system, since the ADLib system does not need any additional recombinant DNA steps for converting phage antibody to full antibody. This advantage can be very practical. In many cases, MAb production problems arise from the lack of purity, improper folding, or instability of the antigen used for immunization. After one round of the ADLib system, such critical antigen obstacles in antibody preparation can be readily recognized and then eliminated. By contrast, recognition of antigen problems typically takes several months with the conventional MAb preparation methods.

As for the flexibility, the ADLib System enables wider application to various antigens. In addition, the ADLib System is readily applicable to Mab customizations

such as artificial class conversion to IgG and additional affinity maturation, once appropriate DT40 clones have been isolated. It should be noted that the ADLib system is a complete *ex vivo* system; hence it can be applied to various so-called "tough" antigens, such as evolutionally conserved proteins, autoantigens, small compounds, and toxins. Against these antigens, high-affinity specific antibodies are difficult to prepare, because of their extremely low or too high antigenicity due to immune tolerance or toxicity *in vivo*.

As already mentioned, homologous gene targeting of DT40 functions very efficiently. Therefore, once the positive DT40 clones are selected, it is relatively easy to conduct relevant knock-in or knock-out of a gene of interest. For example, we have succeeded in terminating the residual Ig gene conversion in the isolated clones by knocking out the gene encoding the activation induced cytidine deaminase (AID), which has been shown to be critical in both gene conversion and somatic hypermutation (Arakawa *et al.*, 2002).

On the other hand, there are still some challenges to be solved in the present version of the ADLib system. For instance, DT40 produces only the IgM class of antibody. Many researchers prefer to use the IgG class instead of IgM, since IgM has lower stability and larger mass (IgG is dimeric, while IgM is pentameric). In addition, purification and affinity pull down of IgG are easier by using *Staphylococcus aureus* Protein A, which specifically binds IgG. We are currently developing several methods (including the production of chicken/mouse or chicken/human chimeric antibodies) to convert chicken IgM of DT40 to IgG of mouse, human, and rabbit.

Another important technical issue is affinity maturation. Many MAbs prepared by the ADLib system have monovalent dissociation constants ranging from 10^{-7}-10^{-8} M. This reflects a moderate level of affinity, but this is sufficient for many immunoassays such as ELISA and Western blotting. However, for antibody medicines and high sensitivity immunoassays, it is essential to prepare MAbs with much higher affinity (dissociation constants, 10^{-10}-10^{-12} M). In order to improve the affinity of the MAbs raised by diversified libraries, deletion of Rad51 family proteins XRCC2 and XRCC3 in the isolated clones could be useful, since their deletions have been shown to activate somatic hypermutation of the V regions in DT40 (Sale *et al.*, 2001). Considering the *in vivo* mechanism of antibody diversification and maturation, performing the recombination-based ADLib system followed by the mutation-based affinity maturation process might be a reasonable procedure.

Application of the ADLib technology

In the following section, we present some examples of antibody design by the ADLib system.

Standard ADLib protocols are currently in place in our laboratory. The success rates of antibody production of unexceptional protein antigens have reached relatively high levels (70-90%, depending on the protein species, purity and quality). If positive clones are included in the ADLib diversified library (normally consisting of about 10^8 clones) used in the screening, we can obtain a few to several dozens (maximum over one hundred in some microtiter plates) of positive clones in a single round of screening within one week. Occasionally, we obtain some clones expressing antibody

reactive with multiple unrelated antigens (multireactive MAbs). These multireactive MAbs do not have good antigen specificities. We can easily eliminate such clones by selecting only those clones scoring high ratios of ELISA signals for target *vs.* reference antigens (for example, chicken ovalbumin) in simultaneous ELISA experiments. *Figure 6* indicates the ELISA results of production of anti-human and rabbit IgG (Seo *et al.*, 2005). In either case, antibodies are specifically reactive with the antigens used for the selection, but not with unrelated antigens. Under normal culture conditions, the yield of MAbs (IgM) in the culture supernatants is in the range of 0.5 to dozens of micrograms IgM/mL. The yield can be improved to hundreds of micrograms of IgM/mL when a high-density culture system is employed. Some of these MAbs can be applicable to Western blotting.

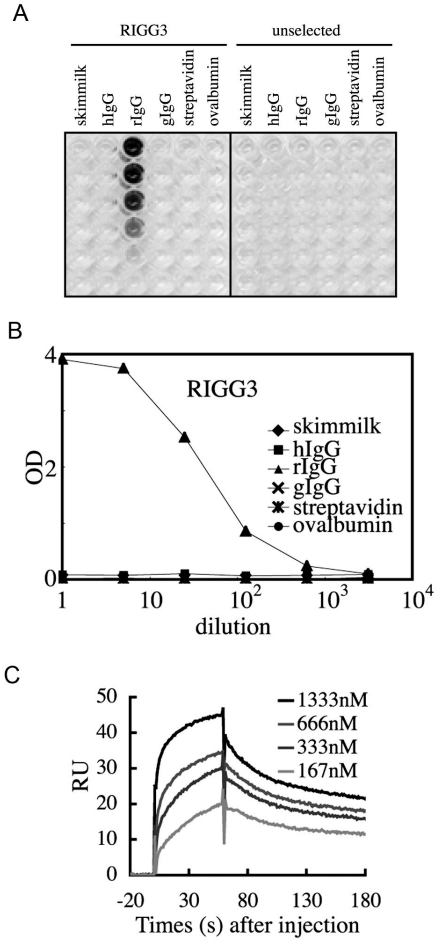

Figure 6. Preparation of anti-rabbit IgG MAbs by the ADLib system. DT40 clones in an ADLib are contact with rabbit IgG-magnetic beads. **A** and **B**, data of ELISA for the specificity check of anti-rabbit IgG monoclonal antibody (RIGG3). The RIGG3 MAb is only reactive with rabbit IgG (rIgG), but not other unrelated antigens such as skim milk, human IgG (hIgG), goat IgG (gIgG), streptavidin, and ovalbumin. **C**, Affinity measurements using surface plasmon resonance. Chicken IgMs are adsorbed to sensor chips to measure affinity (dissociation constant) of the monovalent antigen binding site. The monovalent dissociation constant of RIGG3 is estimated at 47nM. Modified from the previous report (Seo *et al*., 2005).

Since the ADLib system is a pure *ex vivo* antibody design system, it is applicable to autoantigens and highly conserved protein antigens, beyond the problem of immune tolerance. For example, we can produce chicken MAbs against chicken lysozymes (Seo *et al*., 2006), namely autoantibodies that are difficult to raise *in vivo*. This means that the ADLib system is also suitable for the generation of MAbs against highly conserved proteins (e.g., ubiquitin) and toxins. We do not have any example of anti-toxin MAb preparation as yet, but we are currently planning to obtain MAbs with toxin-neutralizing activity.

In the ADLib system, DT40 cells are kept in contact with antigen-conjugated magnetic beads under moderate conditions for a relatively short period (at 4°C for 30 min). Therefore, the ADLib system is assumed to be suitable for the production of MAbs that recognize steric or conformational differences of antigens. Using the ADLib system, we have obtained MAbs that distinguish the secreted form of human IgA from the plasma type IgA. This feature of the ADLib system may be advantageous in raising antibodies against some membrane proteins (*e.g.,* GTP-binding protein coupled receptors, GPCRs), which are hard to be maintained in their native and functional conformations in solution. In this case, membrane protein antigens could be presented in detergent micelles or cell surfaces to preserve functional conformation. Resulting MAbs that recognize such membrane proteins could function as powerful tools for stabilizing membrane proteins during crystallization processes for X-ray structural analysis. However, raising those kinds of MAbs is considered to be so difficult that the development of a high throughput version the ADLib system is probably necessary.

It is known that generation of robust, high quality MAbs against small compounds, peptides or saccharide chains is difficult. Since the ADLib system does not involve the use of an *in vivo* immunization step, preparation of MAbs against those small molecules seems relatively straightforward. For example, we successfully raised MAbs against fluorescein isocyanate (FITC), dinitrophenol (DNP), carbohydrates such as the blood and cancer-related antigen Lewis X (CD15, Gal*f*ß1–4(Fuc*f*α1–3)GlcNAc-R) (*Figure 7*), peptides (15 amino acids in length), and a glycolipid derivative. Raising MAbs against peptides with chemical modifications (*e.g.*, phosphorylation, acetylation, and methylation) by the ADLib diversified library would be of considerable interest in future projects, since MAbs recognizing posttranslational modifications are known to be powerful tools for research on epigenetics and the cell cycle. In the near future, a modified screening protocol of the ADLib system will provide a powerful solution to preparation of MAbs that distinguish protein antigens with subtle changes (posttranslational modifications and even point mutations).

Conclusion

The ADLib System is an *ex vivo* MAbs production system that is based on the diversification of Ig V regions mediated by enhanced Ig gene conversion of DT40 cells. Since the ADLib system does not involve any *in vivo* immunization steps, it is applicable to various so-called "tough antigens" that, up until now, have been very difficult to raise MAbs against with high affinity and specificity. In addition, one can easily customize the MAbs produced by the ADLib system by using the efficient

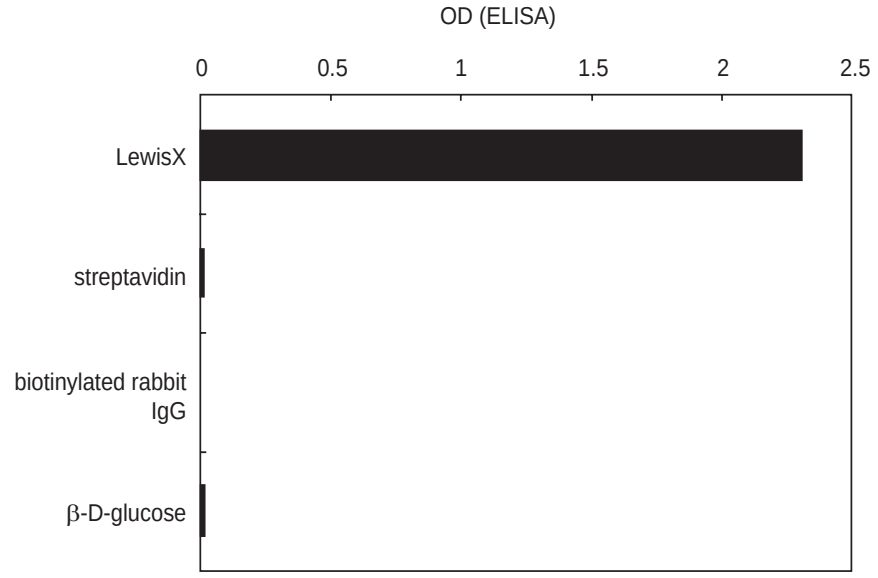

Figure 7. Design of anti-Lewis X monoclonal antibody by the ADLib system. Lewis X-conjugated biotinylated-polyacrylamide polymers are bound to streptavidin-conjugated magnetic beads, and then the resulting Lewis X-conjugated beads are submitted to the ADLib system. The obtained antibodies specifically reacted with the Lewis X-conjugated antigen, but not with other unrelated antigens (streptavidin, biotinylated rabbit IgG, *f*β-D-glucose-conjugated biotinylated-polyacrylamide polymers). Thus, the antibodies are expected to have a high specificity to Lewis X.

gene targeting ability of DT40. Widespread use of the ADLib system for MAb production would greatly improve the efficiency of research in various biological, analytical, and medical sciences. In addition, the ADLib system would be a powerful tool for the immediate preparation of diagnostic regents or antibody medicines to prevent pandemic or newly emerging infective diseases such as avian flu, as well as for the accelerated development of new antibody medicines and diagnostic tools that would be suitable for personalized medicine.

Acknowledgement

We thank Drs. S. Takeda, M Takata, K. Hirota, T. Fukuda, and K. Okuhara for helpful advice and discussion; Mr. H. Sasanuma for helpful advice on antibody selection; Ms K. Arai, A. Nakamura, M. Masuoka, M. Asakawa, and Mr. N. Harigai for their experimental supports; and all members of Chiome Bioscience Inc. for their help. This work was supported by grants from REDS (Rational Evolutionary Design of Advanced Biomolecule, Saitama) project, basic research from the Bio-oriented Technology Research Advancement Institution (to T. Shibata and K. Ohta), New Energy and Industrial Technology Development Organization (to M. Fujiwara), and grants-in-aid for scientific research on priority areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to K. Ohta).

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