Improvement of antigen binding ability of human antibodies by light chain shifting

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Received 29 May 1997; accepted 24 July 1997

Key words: affinity improvement, cancer specific antibody, human monoclonal antibody, hybrid antibody, light chain shifting

Abstract

Human HB4C5 hybridoma cells produce a lung cancer-specific IgM human monoclonal antibody (mAb). HB4C5 human mAb cross-reacts with *Candida* cytochrome c (Cyt c) and carboxypeptidase (Cpase). Concanavalin A (ConA)-resistant variants of HB4C5 cells loss the original light chain followed by expression of various new light chains at a high incidence (light chain shifting) (Tachibana *et al.*, 1996). HTD8 cells, one of the ConA-resistant variant subclones of HB4C5 cells, undergo the active light chain shifting and produce various sublines, each of which stably secretes new mAb consisting of a new light chain and a HB4C5 heavy chain. The new mAb exhibits altered antigen binding ability from that of the original antibody. We could expect that HTD8 cells can be used as 'a light chain stem cell line' to improve antigen binding ability and specificity of established human mAbs. A BD9D12 IgG human mAb recognizes lung cancer cells and cross-reacts with cytokeratin 8. Introduction of the heavy chain gene of BD9D12 mAb into HTD8 cells resulted in establishment of various sublines which secreted various kinds of hybrid antibodies consisting of different light chains derived from HTD8 subclones which underwent light chain shifting and a common IgG heavy chain derived from BD9D12. These hybrid antibodies exhibited different or improved reactivities to Cyt, Cpase, cytokeratin 8 and various cancer cells from those of parental mAb, demonstrating that light chain shifting can be applied to improve the affinity and specificity of human mAb.

Abbreviations: Con A – concanavalin A; Cpase – carboxypeptidase; Cyt c – cytochrome c; Ig – immunoglobulin; mAb – monoclonal antibody

Introduction

Many researchers have investigated about the possible application of human monoclonal antibodies (mAbs) in diagnosis and therapy during the past decade. Although various human mAbs specific to human cancer tissues have been generated, human mAbs with higher binding ability and higher specificity to cancer tissues are still requested for many applications. Binding ability of mAb to specific antigen is thought to be improved via somatic hypermutation *in vivo*, but no *in vitro* system corresponding to the *in vivo* affinity maturation is avail-

able (Dhalenborg *et al.*, 1997; Jeffrey and Neuman, 1997). Recently, phage display technology has been utilized to screen affinity-improved mAbs in combination with site-directed mutagenesis techniques (Ohlin *et al.*, 1996; Li *et al.*, 1996).

A human-human hybridoma clone HB4C5 was generated by fusing human lymphoblastoid cells and lymphocytes from a lung cancer patient (Murakami *et al.*, 1985). The HB4C5 cells secreted mAb which specifically recognizes lung cancer cells, especially adenocarcinoma (Yano *et al.*, 1988). Histone 2B was identified as antigen recognized by HB4C5 mAb (Kato

et al., 1991). HB4C5 mAb cross-reacts with *Candida* cytochrome c (Cyt c) (Hashizume *et al.*, 1991a) and carboxypeptidase A (Hashizume *et al.*, 1991b). BD9D12 and AE6F4 hybridoma cell lines were established by an in vitro immunization method (Kawahara *et al.*, 1992). AE6F4 mAb recognizes 14-3-3 protein in lung cancer cells (Shoji *et al.*, 1994a; 1994b; Kawamoto *et al.*, 1995) and cytokeratin 8 (Ichikawa *et al.*, 1997). HB4C5 mAb has been applied to RI imaging of lung cancer tissues (Kusakabe *et al.*, 1994) and AE6F4 to diagnosis of lung cancer cells in sputa (Shoji *et al.*, 1994a). To apply human mAbs to a computer-aided autodiagnosis of lung cancer cells, mAbs which recognize all types of lung cancer cells are desirable. HB4C5 mAb specifically recognizes lung adenocarcinoma. AE6F4 mAb reacts with wide types of lung cancer cells but not with lung small cell carcinoma. Although a combined use of several mAbs which have different specificities is desirable for exact diagnosis of cancer, it is not easy to obtain many cancer-specific human mAbs by the hybridoma method.

It has been reported that concanavalin A (Con A) treatment of HB4C5 cells led to obtain sublines which underwent light chain shifting, where new light chain was secreted alternative to original one (Tachibana *et al.*, 1994). It was also shown that mAbs secreted from these sublines were consisted of various λ chains and a common heavy chain, and had altered antigen binding specificity (Tachibana *et al.*, 1996). The human hybridoma cell line B4TN produce lung cancer reactive mAbs. It was shown that Con A-resistant variant clones derived from B4TN cells also produced various light chains which demonstrated a range of reactivity spanning from completely lost to significantly improved (Siripen *et al.*, 1997). Recently, we found that one of the ConA-resistant variants of HB4C5 cells, HTD8, undergoes active light chain shifting and produce various sublines in a high incidence, each of which secretes a new IgM mAb consisting of a new light chain and an original heavy chain. To improve antigen binding ability and specificity, we could expect that HTD8 cells can be used as a 'light chain stem cell line.' In this report, we attempted to introduce the gene of the heavy chain with known specificity into the HTD8 cells, and analyzed the binding ability and specificity of hybrid mAbs secreted from sublines of HTD8 transfected with heavy chain gene.

Materials and methods

Cell culture

HB4C5 is a human hybridoma cell line generated by fusing human lymphoblastoid NAT-30 cells with lymphnode lymphocytes from a patient with lung large cell carcinoma (Murakami *et al.*, 1985). ConA-resistant clones derived from this hybridoma cell line were isolated as previously described (Tachibana *et al.*, 1994). HTD8 cell is one of the ConA-resistant variant clones of HB4C5 cell. Other hybridoma cell lines BD9D12 and AE6F4 were established by in vitro immunization with a lung cancer cell line A549 (Kawahara *et al.*, 1992). The BD9D12 cells secrete IgG type human mAb reactive with various cancer cells and AE6F4 cells IgM type human mAb. Six lung cancer cell lines (QG-56, PC-10, QG-90, PC-13, PC-8, A549), a breast cancer cell line (MCF-7), an epidermoid cancer cell line (A431) and a normal lung fibroblast cell line (Flow 2000) were used to examine the specificities of the antibodies. These cells were maintained in ERDF medium (Kyokuto Pharmaceutical, Tokyo, Japan) supplemented with 5% fetal bovine serum (FBS; Hyclone laboratory, Logan, UT, USA) at 37 °C in a 95% air/5% CO₂ atmosphere.

Extraction of cytokeratin 8 from MCF-7 cells

Cytokeratin 8 was extracted as described (Achtstaetter *et al.*, 1986). In brief, the MCF-7 cells (1×10^8 cells) were incubated with a detergent buffer for 1–2 min. The supernatant is gently poured off, and the remained cells were gently immersed in high salt buffer (10 mM Tris-HCl, pH 7.6, 140 mM NaCl, 1.5 M KCl, 5 mM EDTA, 0.5% Triton X-100) for 30 min at room temperature. The supernatant was then decanted and the remained insoluble cytoskeletal residues are scraped off with a rubber scraper, resuspended in the same buffer, and briefly homogenized. The high salt buffer-resistant cytoskeletal proteins were then separated by two-dimensional polyacrylamide gel electrophoresis (PAGE) (Model 491 Prep Cell, Bio-Rad, Hercules, CA, USA).

Cell transformation

The heavy chain cDNA of BD9D12 human mAb was inserted into pEFneoFc76 vector (Liu *et al.*, 1993; Ohashi *et al.*, 1994), which was a kind gift from Dr. H. Ohashi, to construct a pEF-BD9D12H plasmid which

contains the Fd and IgG1 Fc region. The plasmid was introduced into HTD8 cells (1×10^6 cells) by electroporation. Cells were suspended in the phosphate buffered saline (PBS) solution containing 10 mg of the plasmid DNA. An electric pulse (0.45 kV cm^{-1}) was discharged across the cell suspension using an electroporation apparatus (BTX electrocell manipulator 600, BTX electroporation system, CA, USA). Then the cells were cultivated in 5% FBS-ERDF for 48 hr at 37°C in humidified 7% O_2 /5% CO_2 . The cells expressing the heavy chain gene were selected in 5% FBS-ERDF medium containing G418 (2 mg ml^{-1}). The resistant cells were cloned by a limiting dilution method and screened for transformant cell clones secreting new light chains.

Antigen binding assay

Antibodies were tested by enzyme-linked immunosorbent assay (ELISA) for binding to Cyt c (Sigma, St. Louis, USA), Cpase (Sigma) and cytokeratin 8. After Cyt c, Cpase and cytokeratin 8 were absorbed onto microtiter plates (Nunc, Kamstrup, Denmark) for 1 hr at 37°C , and washed three times with PBS containing 0.05% Tween20 (TPBS), cells were treated with 1% bovine serum albumin (BSA) in PBS for 1 hr to minimize nonspecific adsorption of antibodies, then culture supernatants from the transfected hybridomas were added. After washing as described above, the plates were treated with horseradish peroxidase (HRP)-conjugated goat anti-human antibody (Biosource, CA, USA). After incubation at 37°C for 1 hr, the plate was washed with TPBS, followed by the addition of the substrate solution containing 0.003% H_2O_2 , 0.1 M citrate buffer (pH 4.0), and ABTS (2, 2'-azino-bis (3-ethylbenzothiazolin-6-sulfonic acid) di-ammonium salts), and continued incubation for 15 min at room temperature. The reaction was stopped by the addition of 100 μl of 1.5% Oxalic acid and absorbance at 405 nm was determined using a microplate reader (Sanko Junyaku, Japan).

Reactivity of mAb to various cell lines (cell-ELISA)

Human cancer cells and normal fibroblast cells were grown in 96 well microtiter plates until confluency in ERDF medium supplemented with 5% FBS. After removing the medium, cells were fixed with 0.05% glutaraldehyde (Sigma) for 15 min as described (Aihara *et al.*, 1988). After washing the cells three times with a TPBS, cells were treated with 0.5% BSA contain-

ing 0.2% gelatin in PBS for 1 hr. Then supernatant from hybridomas were added and incubated at 37°C for 1 hr. After washing, a HRP-conjugated goat anti human antibody was further added and incubated for 1 hr at 37°C . The following process was same as ELISA method as described above.

Western Blot analysis

Culture supernatants of transformed hybridomas were electrophoresed in a gel containing 10% SDS and 10% polyacrylamide under reducing conditions (Laemmli, 1970) and electroblotted onto a nitrocellulose membrane (Schleicher and Schuell, Germany) as described (Johnson *et al.*, 1988). The membrane was blocked with Block Ace (Dainippon Pharmacy, Tokyo, Japan) for 1 hr at room temperature, washed three times with TPBS, then incubated with a HRP-conjugated goat anti-human λ light chain for 1 hr. The membranes was washed as above and reacted with 1.6 mM 4-chloro-1-naphthol and 0.01% H_2O_2 in PBS containing 20% methanol.

Results

HTD8 cells as light chain stem cells

HTD8 cells, one of the HB4C5 ConA-resistant clones, were found to undergo active light chain shifting in which active loss of production of the original HB4C5 light chain and alternative production of the new light chain occur. In addition to the original 32 kDa λ light chain, some clones derived from HTD 8 cells also secreted the 28 and 26 kDa light chain species (data not shown). The original light chain has a carbohydrate chain in the variable region (Tachibana *et al.*, 1993), while new light chain are thought to have no carbohydrate chain (Tachibana *et al.*, 1994). Then we could easily confirm the occurrence of light chain shifting by the difference in molecular weight of light chain in Western blotting analysis. After 4-weeks' culture without any stimulation, about 70% of clones derived from HTD8 cells continues to express the original light chain, while about 30% of them lost the production of the original light chain and started to express the new light chains (Figure 1).

Figure 1. Active light chain shifting in HTD8 cells. During 4 week's culturing, HTD8 cells produces about 70% of subclones which secrete the original HTD8 antibody and the rest subclones which undergo active loss of production of original light chain and alternative production of new light chains.

Introduction of human heavy chain gene into HTD8 cell

The variable regions of both the heavy and light chains generally contribute to antigen binding ability and specificity. We attempted to improve antigen binding ability and specificity by introducing a heavy chain gene of another human mAb into 'a light chain stem cell line', HTD8, leading to generation of novel mAbs with various light chain and a common heavy chain with known specificity. The strategy was shown in Figure 2. For this purpose, we used the γ heavy chain gene derived from another anti-lung cancer BD9D12 mAb as a source of heavy chain. The subclones of these transformed HTD8 cells could produce various kind of hybrid antibody molecules. After selecting recombinant HTD8 cells secreting hybrid antibody which was composed of HTD8-derived light chain and BD9D12-derived γ heavy chain by ELISA using anti- γ heavy chain antibody, the sizes of light chains were analyzed by Western blotting analysis. Of 84 clones, about 70% of antibody secreted from recombinant HTD8 cells had

γ heavy chain. Since HTD8 cells shifts its light chain during culturing, some of antibodies having a common IgG-type heavy chain are thought to have different light chains, leading to the altered antigen binding ability and specificity. Then we selected some clones for further experiments.

Alteration of antigen binding ability of human mAb

We selected four recombinant HTD8 clones secreting antibodies consisting of HTD8-derived light chain and BD9D12-derived heavy chain. We termed these recombinant HTD8 clones clone A, clone B, clone C and clone D, which secreted various size of light chains, 26 kDa, 28 kDa, 28 kDa and 32 kDa, respectively. Then we tested the reactivity of these hybrid antibodies to Cyt c, Cpase and cytokeratin 8 (Table 1). Clone A secreting 26 kDa new light chain had higher binding abilities to Cyt c, Cpase and cytokeratin 8 than other clones. Clone B and C showed slightly enhanced reactivity to cytokeratin 8 as compared to HTD8 cells and that to Cpase and Cyt c was comparable to HTD8 cells.

Figure 2. The strategy to improve reactivity of human monoclonal antibody by using light chain shifting. HTD8 cells were used as a 'light chain stem cell line' and transfected with a certain human antibody heavy chain gene (BD9D12H) by electroporation method. The transfected cells were selected with G418. The subclones producing various kinds of hybrid antibody molecules which have only the heavy chain protein in common, were screened and selected for a hybrid cell line which expresses improved antigen binding monoclonal antibodies.

Although parental BD9D12 and HTD8 mAbs did not react with Cpase and Cyt c, mAbs derived from clone A strongly reacted with these antigens. In contrast, clone D which secreted 32 kDa light chain showed no reactivity to the present antigens. The antibody produc-

tion rate were demonstrated to be comparable between these clones and HTD8 cells.

Figure 3. Reactivity of hybrid antibodies derived from transformed HTD8 cells against various cancer cell lines. The hybrid antibodies (clone A, B, C and D), AE6F4 and HTD8 antibodies were reacted at various concentrations with an human epidermoid cancer cell line (A-431), a lung cancer cell line (A-549) and a human breast cancer cell line (MCF-7) by cell-ELISA method. Clone A, ●; clone B, ■; clone C, ▲; clone D, ▴; AE6F4, △; HTD8, ×.

Table 1. Characterization and production of antibodies from transformed-HTD8 subclones compared with original BD9D12 and HTD8 antibodies by using ELISA assay

Cell	λ (kDa)	Anti Cpase	Anti Cyt c	Anti cytokeratin 8	Productivity (ng/ml/3 days)
Clone A	26	+++ ^a	+++	+++	480
Clone B	28	+	+	++	359
Clone C	28	+	+	+	304
Clone D	32	–	–	–	412
AE6F4	ND ^b	–	–	+++	450
BD9D12	ND ^b	–	–	++	ND ^b
HTD 8	32	+	+	–	360

Antibodies (500 ng/ml) were examined for the reactivity to Cpase, Cyt c and cytokeratin 8 by ELISA.

^a Binding ability of antibodies to these antigens was assessed as follows; the absorbance at 405 nm in the ELISA was below 0.1, –; 0.1~0.5, +; 0.5~1.0, ++ and over 1.0, +++.

^b ND, not determined.

Reactivity of transformed-HTD8 antibodies to various cancer cell lines

To clarify if the variant antibodies were capable of binding to various cancer cell lines, we examined the reactivity of the recombinant HTD8-derived antibodies to an epidermoid cancer cell line (A-431), lung cancer cell line (A-549) and a human breast cancer cell line (MCF-7) by cell-ELISA. Figure 3 showed the reactivities of clone A to clone D antibodies against these cancer cell lines. Although AE6F4 antibody reacted strongly to A-431 cells, and weakly to A-549 cells and MCF-7 cells, HTD8 antibody could not react to these three cancer cell lines. Clone A antibody most strongly reacted to these three cancer cell lines, demonstrating that Clone A antibody generated by hybridization of HTD8 variant light chain and BD9D12 heavy chain showed stronger reactivity than anti-lung cancer mAb AE6F4. Reactivity of clone B antibody to A431 cells was similar to that of AE6F4 antibody but much stronger than that of parental HTD8. Clone D antibody, on the other hand, showed no reactivity to these cancer cells.

Reactivities of these antibodies to various cancer cell lines and a normal cell line were examined. Table 2 shows the reactivities of antibodies secreted by recombinant-HTD8 cells to lung adenocarcinoma (PC-8), lung squamous carcinoma (QG-56, PC-10), lung small cell anaplastic carcinoma (QG-90), lung large cell anaplastic carcinoma (PC-13), MCF-7, A-431 and a normal human lung fibroblast cell line (Flow 2000) compared with original HB4C5, HTD8, AE6F4 and BD9D12 antibodies. Among these antibodies, clone A

reacted most widely with a variety of lung cancer cell lines and other cancer cell lines derived from breast cancer and epidermoid cancer, while did not react to normal fibroblast, indicating that clone A specifically reacts to cancer cell lines. Clone C antibody had narrower specificity against cancer cell line. Clone C antibody reacted only to MCF-7 cells. Clone B antibody showed similar reactivity to these cancer cell lines as BD9D12 antibody, although reactivities to PC-8 which were observed for BD9D12 antibody were diminished. Clone D antibody also showed no reactivity to any cancer cell lines examined.

Discussion

We have developed lung cancer-specific human mAbs such as HB4C5 and AE6F4 and applied them to diagnosis of human lung cancer. Various cancer-specific human mAbs are demanded for exact diagnosis of cancer. Since it is laborious and time-consuming work to develop and characterize new cancer-specific human mAbs, we tried to improve antigen binding ability and specificity of established cancer specific human mAbs by the use of light chain shifting phenomenon. Recombination activating genes *RAG-1* and *RAG-2* were expressed in HB4C5 cells (Tachibana *et al.*, 1996). Stimulation by lectines such as Con A and wheat germ agglutinin can induce light chain replacement through secondary rearrangement in HB4C5 cells (Krungrasem *et al.*, 1997). We found that a subline of ConA treated HB4C5 cells, HTD8 actively produce sublines which produce new light chains different from

Table 2. Reactivities of antibodies to various human cancer and normal cell lines by cell-ELISA assaya

Cell line	HB4C5	HTD8	AE6F4	BD9D12	Clone A	Clone B	Clone C	Clone D
Lung squamous carcinoma								
QG-56	— ^a	—	+	+	+	+	—	—
PC-10	—	—	ND ^b	+	+	+	—	—
Lung adenocarcinoma								
PC-8	++	—	+	+	+	—	—	—
Lung small cell anaplastic carcinoma								
QG-90	—	—	—	—	+	—	—	—
Lung large cell anaplastic carcinoma								
PC-13	—	—	++	+	+	+	—	—
Breast cancer cell line								
MCF7	—	—	+	+	++	+	+	—
Epidermoid cancer cell line								
A431	—	—	+	+	++	+	—	—
Lung normal fibroblast								
Flow 2000	—	—	—	—	—	—	—	—

^a Binding ability of antibodies to these cell lines was assessed in the same manner as Table 1.

^b ND, not determined.

original light chain and secrete antibodies with altered affinity and specificity.

Since both heavy and light chains are responsible for affinity and specificity of antibody, HTD8 cells were considered to be useful as a 'light chain stem cells' to improve or modify the functions of antibody. The transformed HTD8 cell clones with the heavy chain gene of BD9D12 mAb produced various subclones which expressed various new light chain and common heavy chain protein. They were easily detectable from mAb of the original HTD8 cells because the introduced heavy chain was IgG. Of four transformed HTD8 antibodies, clone A antibody showed the highest affinity to cancer-related antigens such as Cyt c, Cpase and cytokeratin 8 (Table 1). Weber *et al.* (1984) showed that tissue polypeptide antigen (TPA), which is related to cytokeratin 8, 18 and 19 is also found in the sera of patients with some types of cancer. Cytokeratin 8 protein may act a cell surface-associated antigen represented on the adenocarcinoma cells (Hembrough *et al.*, 1995). Therefore, human mAbs against cytokeratin 8 may be potentially useful for detection of adenocarcinoma cells (Ichikawa *et al.*, 1997). When these four clones were examined for their immunological reactivities to lung cancer cell lines of four major histologies, i.e. adenocarcinoma, squamous cell carcinoma, large cell carcinoma and small cell carcinoma. Clone A antibody was found to be highly reactive to all types of lung cancer cell lines examined, although AE6F4 and BD9D12 mAbs had no reactivity to small cell car-

cinoma (Table 2). Clone A antibody also reacted with various types of cancer cells but not with normal cells. In contrast, clone D antibody did not react with cancer cell lines examined. Although original BD9D12 mAb exhibited a wide range of reactivity to these cancer cell lines, its affinity and specificity was improved in transformed-HTD8, clone A antibody for diagnosis of cancer cells. These results show that replacing the original λ light chain with the variant new light chains leads to alteration of both binding ability and affinity of antibody, suggesting that HTD8 cells will be useful for analysis of the roles of heavy chain and light chain in antigen-antibody binding reaction. Although the somatic hypermutation of rearranged Ig variable region gene is thought to plays a major role in generating antibody diversity, the mechanism can not be direct; it was found that the patterns of germline and somatic diversity are complementary and the number of differences between the gene segments is larger than the number of changes created by somatic hypermutation (Tomlinson *et al.*, 1996). The process of variable region hypermutation is thought to commence in germinal centers in B cells expression surface Ig and is believed to turn off as the B cell differentiates into a plasma cell. In human studies, some cases of acute lymphoblastic leukemia Ig continue rearrangement but lack of somatic hypermutation in Ig heavy chain genes in human B cell precursor leukemia (Bird *et al.*, 1988). Moreover, human B cell tumors might lack the mechanism for somatic hypermutation and represent a stage of nor-

mal B lymphocyte differentiation in which the somatic hypermutation mechanism is not active (Meeker *et al.*, 1988). In this study, we reported the improvement of the antigen binding affinity and specificity of the transformed HTD8 cells via light chain shifting. HTD8 cells undergo secondary rearrangement (Tachibana *et al.*, 1996), and continued producing new light chains, although they were thought to be plasma stage cells. Therefore, we believed that the improvement of the antigen binding ability in the HTD8 antibodies could occur by the germ line rearrangement and was not by somatic hypermutation. Although the mechanism of the light chain shifting to alter antigen binding ability in human plasma cells have not been known fully, we demonstrated that light chain shifting is a new way to easily produce human antibodies with high affinity and specificity by plasma B cell lines.

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