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Characterization of Humanized Antibody Produced by Apoptosis-Resistant CHO Cells under Sodium Butyrate-Induced Condition

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Abstract Overexpression of human Bcl-2 protein in recombinant Chinese hamster ovary (rCHO) cells producing humanized antibody (SH2-0.32) considerably suppressed sodium butyrate (NaBu)-induced apoptosis during batch culture by using commercially available serum-free medium, which extended the culture longevity. Due to the extended culture longevity provided by the anti-apoptotic effect of Bcl-2 overexpression, the final antibody concentration of 14C6-bcl-2 culture (Bcl-2 high producer, 23 μg ml^{-1}) was 2 times higher than that of the SH2-0.32-Δbcl-2 culture (cells transfected with *bcl-2*-deficient plasmid, 10.5 μg ml^{-1}) in the presence of NaBu. To determine the effect of NaBu/Bcl-2 overexpression on the molecular integrity of protein products, antibodies purified from 14C6-bcl-2 and SH2-0.32-Δbcl-2 cultures in the presence of NaBu were characterized by using various molecular assay systems. For comparison, antibody purified from the parental rCHO cell culture (SH2-0.32) in the absence of NaBu was also characterized. No significant changes in molecular weight of antibodies could be observed by SDS-PAGE. From GlycoSep-N column analysis, it was found that the core oligosaccharide structure ($GlcNAc_2Man_3GlcNAc_2$) was not affected by NaBu/Bcl-2 overexpression, while the microheterogeneity of N-linked oligosaccharide structure was slightly affected. Compared with the antibody produced in the absence of NaBu, the proportion of neutral oligosaccharides was increased from 10% (14C6-bcl-2) to 16% (SH2-0.32-Δbcl-2) in the presence of NaBu, which was accompanied by the reduced proportion of acidic oligosaccharides, especially of monosialylated and disialylated forms. The changes in microheterogeneous oligoformal structures of antibody in turn affected the mobility of antibody isoforms in isoelectric focusing (IEF), resulting in the occurrence of some more basic antibody isoforms produced in the presence of NaBu. However, the antigen-antibody binding properties were not changed by alteration of glycosylation pattern. The competitive enzyme-linked immunosorbent assay (ELISA) showed that the antibody produced by NaBu/Bcl-2 overexpression maintained its antigen-antibody binding properties with binding affinity of about 2.5×10^9 M^{-1}. Taken together, no significant effects of NaBu/Bcl-2 overexpression on the molecular integrity of antibodies, produced by using serum-free medium, could be observed by the molecular assay systems.

Key words: Apoptosis, Bcl-2, Chinese hamster ovary, humanized antibody, molecular integrity, sodium butyrate

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Sodium butyrate (NaBu), a sodium salt of butyric acid, has been used as a stimulating agent in recombinant Chinese hamster ovary (rCHO) cell cultures to enhance expression of foreign proteins such as erythropoietin (EPO) [5], tissue plasminogen activator [30], nitric oxide synthase [22], and humanized antibody [20]. Since NaBu can also significantly inhibit cell growth, followed by rapid induction of apoptotic cell death [4, 5], its beneficial effect on foreign protein expression should compromise with its cytotoxic effect on cell growth. Recently, we demonstrated that the cytotoxic effect of NaBu could be overcome by overexpression of a human Bcl-2 protein in rCHO cells which produce a humanized antibody [20]. Thereby, the final antibody concentration in a batch culture of rCHO cells could be significantly increased by the enhanced transcription efficiency endowed by NaBu and also by the extended culture longevity provided by the anti-apoptotic effect of Bcl-2 overexpression.

Apart from the expression level, the molecular integrity of recombinant proteins is important for their therapeutic use. A number of intra- and extracellular factors such as polypeptide structure [25], cell type [18], medium [27], and culture duration [13] can affect the molecular integrity of recombinant proteins that are known to be microheterogeneous

in regard to molecular weight, pI value, and glycosylation pattern. Thus, to meet regulatory requirements related with consistency of the product, the molecular integrity of recombinant proteins produced by mammalian cells overexpressing a survival protein needs to be examined, particularly in regard to their carbohydrate residues. The carbohydrate residues on recombinant therapeutic glycoproteins can directly affect the secretion from the host cell, the biological activity, and the circulatory half-life in the plasma [9, 18]. Despite increasing interests in the use of survival protein in CHO cells [14, 37], the molecular integrity of recombinant glycoproteins produced by CHO cells overexpressing a survival protein has not been systematically characterized.

In this study, the molecular integrity of a humanized antibody produced by apoptosis-resistant CHO cells using NaBu/Bcl-2 overexpression was investigated by using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for molecular weight variation, isoelectric focusing (IEF) analysis of isoforms, and competitive enzyme-linked immunosorbent assay (ELISA) for antigen-antibody binding properties. Furthermore, the changes in carbohydrate residues of N-linked oligosaccharide structures were quantitatively analyzed by a high performance liquid chromatography (HPLC) method.

MATERIALS AND METHODS

Cell Lines and Culture Maintenance

Three rCHO cell lines (SH2-0.32, 14C6-bcl-2, and SH2-0.32-Δbcl-2) were used in this study. The 14C6-bcl-2 overexpressing a human Bcl-2 protein was established by transfecting Bcl-2 expression vector (pBcl-2/Zeo) into SH2-0.32 cells producing a humanized antibody IgG_1 directed against the S surface antigen of hepatitis B virus (HBsAg), as described previously [20]. The SH2-0.32-Δbcl-2 was established by transfecting SH2-0.32 cells with the null pcDNA3.1/Zeo(+) without *bcl-2* gene.

The 14C6-bcl-2 and SH2-0.32-Δbcl-2 cell lines were grown in a minimum essential medium (α-MEM, Gibco, Grand Island, NY, U.S.A.) supplemented with 10% dialyzed fetal bovine serum (dFBS, Gibco), 0.32 μM methotrexate (MTX, Sigma, St. Louis, MO, U.S.A.), and 500 μg ml^{-1} of zeocin (Invitrogen, Carlsbad, CA, U.S.A.). The SH2-0.32 cells were grown in the same medium without zeocin. The cells were maintained as monolayer cultures in 25-cm^2 T-flasks (Nunc, Roskilde, Denmark) in a 5% CO_2/air mixture, humidified at 37°C.

Cell Culture with NaBu Addition

The 14C6-bcl-2 cells were cultivated as monolayer cultures in 6-well tissue culture plates (Nunc) containing 3 ml of α-MEM supplemented with 5% dFBS and 0.32 μM MTX. The SH2-0.32-Δbcl-2 cells were also cultivated for comparison. Exponentially grown cells were seeded at a concentration of 6.7×10^4 cells ml^{-1}. After a 3-day cultivation process, the spent medium was replaced with 3 ml of an antibody production medium, a commercially available serum-free medium (CHO-S-SFMII, Gibco) supplemented with 5 mM NaBu (Sigma).

Culture plates were periodically examined for cell concentration and viability. Culture supernatants were aliquoted and kept frozen at 70°C for the antibody assay.

Cell Concentration, Viability, and Antibody Assays

Cell concentration was estimated by using a hemacytometer. Viable cells were distinguished from dead cells by using the trypan blue dye exclusion method. Secreted antibody concentration was measured by sandwich ELISA as described previously [19].

Production and Purification of Antibody

The 14C6-bcl-2 and SH2-0.32-Δbcl-2 cells were cultivated as monolayer cultures in 75-cm^2 T-flasks (Nunc) containing 15 ml of α-MEM supplemented with 5% dFBS and 0.32 mM MTX. Exponentially growing cells were seeded at a concentration of 1×10^5 cells ml^{-1}. After a 3-day cultivation process, the spent medium was replaced with an antibody production medium. Thereafter, the spent medium was replaced with the fresh antibody production medium every 2 days. The spent medium containing secreted antibodies was stored at 4°C for purification. For preparation of the control antibody, parental rCHO cells (SH2-0.32) were also cultivated identically, except that NaBu was not included in the antibody production medium. The antibody production was performed for 6 days.

For antibody purification, the spent medium collected for 6 days of antibody production period was first pooled, clarified by filtering with 0.2 μm membrane, and then loaded into Protein G Sepharose 4 Fast Flow Column (Amersham Pharmacia Biotech, Uppsala, Sweden), previously equilibrated with binding buffer (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.02% NaN_3). After sample application and washing, the bound antibodies were eluted with 0.1 M glycine-HCl buffer (pH 2.7) and fractions were immediately neutralized with 1 M Tris-HCl (pH 8.0). The eluted antibodies were concentrated with Centricon®100 (Amicon, Beverly, MA, U.S.A.). For the quantification of the purified antibody solution, OD of 1.38 at 280 nm was taken as 1 mg ml^{-1} protein concentration [2].

SDS-PAGE and IEF

For SDS-PAGE analysis, antibody samples (3 μg) obtained under the different conditions as well as prestained protein molecular weight markers (Bio-Rad, Hercules, CA, U.S.A.) were run on 15% and 8% acrylamide resolving gels with (reducing condition) and without (nonreducing condition)

2-mercaptoethanol, respectively, as described by Bollag and Edelstein [2].

For IEF analysis, antibody samples (10 μg) obtained under the different conditions as well as IEF standards (pI 4.45 to 9.6, Bio-Rad) were run on 5% native IEF ready gel, pH 3-10 (Bio-Rad), and focused at increasing voltages (100 V for 1 h, 250 V for 1 h, and 500 V for 30 min). The catholyte was 20 mM lysine/20 mM arginine (Bio-Rad) and the anolyte was 7 mM phosphoric acid (Bio-Rad). Gel fixation was performed by soaking the gel in 10% trichloroacetic acid (TCA) for 10 min and 1% TCA overnight [2]. The focused bands were stained with Coomassie Blue R-250 (Sigma).

Competitive ELISA

For competition binding assay, the control antibody purified from SH2-0.32 cell culture in the absence of NaBu was conjugated with biotin by using ECL™ protein biotinylation module (Amersham Pharmacia Biotech) according to the protocol provided by the manufacturer. The biotinylated control antibody (25 ng) and varying amounts of competing sample antibodies (0-3 μg) in 200 μl of BSA/PBS (2% bovine serum albumin in phosphate buffered saline, pH 7.4) solution were loaded to each well of immunoplate (Nunc) precoated with HBsAg. After 2 h incubation at 37°C, the biotinylated control antibodies bound to precoated HBsAg were quantified by detecing the streptavidin-horseradish peroxidase (HRP) complex.

To determine the binding affinity of sample antibodies, BSA/PBS solution (200 μl) containing 3 ng of sample antibodies and various concentrations of HBsAg (1.0×10^{-12}-1.0×10^{-7} M) was first incubated for 2 h at 37°C, and then loaded to each well of immunoplate precoated with HBsAg. After 2 h incubation at 37°C, the antibodies bound to precoated HBsAg were quantified with HRP-conjugated goat anti-human IgG (Fc specific, Sigma, U.S.A.). An apparent affinity was determined as the reciprocal of the HBsAg concentration required to inhibit 50% of the maximal binding in the competitive ELISA, which is a close approximation to the affinity determined by Friguet *et al.* [10].

Glycoform Analyses

Selective Staining of Glycoprotein. For selective identification of glycosylated moieties of antibody, periodic acid Schiff (PAS) staining was performed as described previously [11]. After SDS-PAGE of sample antibodies (3 μg) on 15% resolving gel under the reducing condition, the gel was first incubated for 1 h in the fixation solution (acetic acid:methanol:H_2O=10:35:55) and then for 1 h in the periodate solution (0.7% periodic acid in 5% acetic acid solution). After rinsing the gel briefly with H_2O, the gel was immersed in the meta-bisulfite solution (0.2% sodium meta-bisulfite in 5% acetic acid). After 10 min, the gel was then incubated in Schiffs reagent (Sigma) until red bands of glycosylated protein appeared.

Preparation of N-Linked Oligosaccharides from Antibody. Antibodies (100 μg) obtained under the different conditions were completely freeze-dried in microtubes and denatured by boiling for 10 min in 50 μl of the reaction buffer (50 mM of sodium phosphate, pH 7.0, 12.5 mM of EDTA, 0.1% SDS). The reaction mixture was treated with 2 μl of Triton X-100 and 2 units of N-glycosidase F (Boehringer Mannheim, Mannheim, Germany), and incubated at 37°C for 2 h. Oligosaccharides were recovered by using Sep-Pak C_{18} cartridge (Waters, Milford, MA, U.S.A.). Eluted oligosaccharides were freeze-dried and fluorescently labeled with 2-aminobenzamide (2AB) for the HPLC analysis, as described previously [1].

HPLC Analysis of 2AB-Labeled Oligosaccharides. The 2AB-labeled oligosaccharides were separated into neutral and acidic fractions by a strong anion-exchange column according to the number of sialic acid substituents MonoQ HR 5/5 (Amersham Pharmacia Biotech). A neutral fraction was eluted with water and then acidic fractions were eluted with a gradient formed by 0-1 M ammonium acetate, pH 4.0, at a flow rate of 1 ml/min at room temperature. The pools of oligosaccharides obtained from MonoQ were further resolved into individual oligosaccharide components by an amide column, GlycoSep-N (Oxford GlycoScience, Oxford, U.K.) according to glucose unit (gu) values. A glucose-homopolymer standard was injected primarily to form a dextran ladder for the calculation of gu value. Acetonitrile (solvent A) and 250 mM ammonium acetate (solvent B, pH 4.0) were used for the gradient conditions. Initial condition was 80% solvent A at a flow rate of 1 ml/min at 30°C, followed by a gradient of 20-53% solvent B over 160 min. Structures of oligosaccharides were assigned from the relationship between gu value and oligosaccharide structure, as described previously [17].

RESULTS

Batch Culture with NaBu

To determine whether the delay of NaBu-induced apoptotic cell death by overexpression of Bcl-2 protein extends culture longevity in a serum-free medium (CHO-S-SFMII), thereby increasing antibody production, batch cultures of 14C6-bcl-2 and SH2-0.32-Δbcl-2 cells were carried out as monolayer cultures containing 3 ml of α-MEM supplemented with 5% dFBS and 0.32 μM MTX. After a 3-day cultivation, the spent medium was replaced with an antibody production medium (CHO-S-SFMII supplemented with 5 mM NaBu).

Figure 1 shows cell growth and antibody production of SH2-0.32-Δbcl-2 and 14C6-bcl-2 cells. When SH2-0.32-Δbcl-2 cells were subjected to NaBu, viable cell concentration began to decrease rapidly. Cell viability decreased from

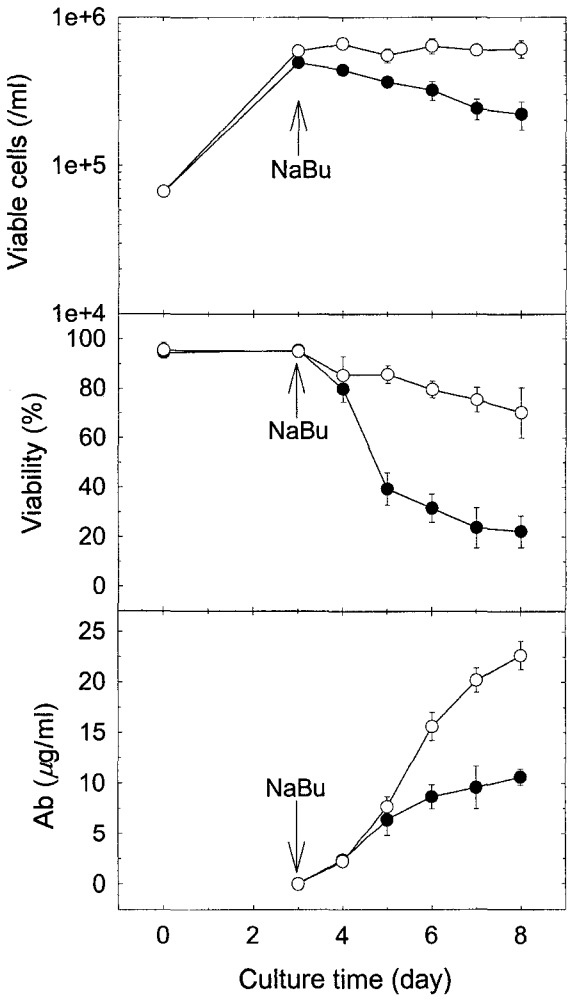

Fig. 1. Comparison of batch cultures of SH2-0.32-Δbcl-2 (●) and 14C6-bcl-2 cells (○) in the presence of 5 mM NaBu.
When cells were in mid-exponential growth phase (3 days after inoculation), the spent medium was replaced with CHO-S-SFMII containing 5 mM NaBu as indicated by arrows. Error bars represent the standard deviations calculated from data obtained in duplicate experiments.

95% to 39% during 48 h after the NaBu addition. Our previous study showed that this significant loss of viability resulted from the NaBu-induced apoptosis mediated by the activation of apoptotic protease, caspase 3 [20]. The q_{Ab} of SH2-0.32-Δbcl-2, calculated from the data obtained during 48 h after the NaBu addition, was 8.0 μg 10^6 cells^{-1} day^{-1}. The final antibody concentration obtained in SH2-0.32-Δbcl-2 culture was approximately 11 μg ml^{-1}.

Unlike SH2-0.32-Δbcl-2 cells, viable cell concentration of 14C6-bcl-2 cells overexpressing Bcl-2 protein was maintained at approximately 6.1×10^5 cells ml^{-1} after the NaBu addition, and then decreased gradually from 95% to 70% at the end of culture. Based on the data obtained during 48 h after the NaBu addition, the qAb of 14C6-bcl-2 was 11.0 μg 10^6 cells^{-1} day^{-1}. The final antibody concentration obtained in 14C6-bcl-2 culture was 23 μg ml^{-1}, which is approximately 2 times higher than that obtained in SH2-0.32-Δbcl-2 culture. This enhanced final antibody titer of 14C6-bcl-2 was mainly due to the extended culture longevity by the anti-apoptotic effect of Bcl-2 overexpression. These results were consistent with our previous results obtained from the batch culture by

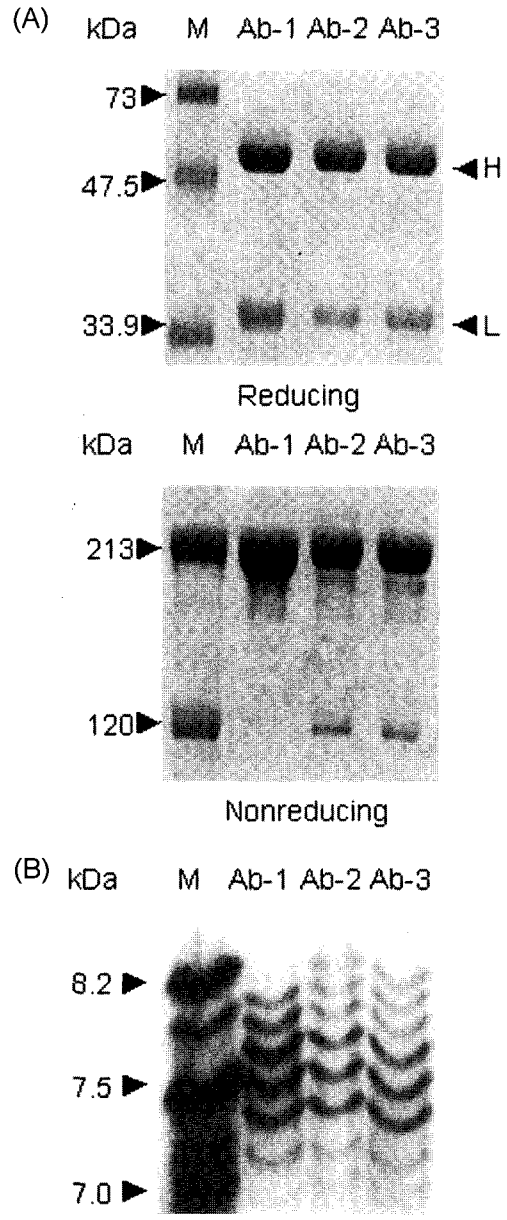

Fig. 2. Humanized antibody analyses by SDS-PAGE (A) and IEF (B).
For SDS-PAGE, 3 μg of each antibody sample obtained in the absence (Ab-1: SH2-0.32) and the presence (Ab-2: SH2-0.32-Δbcl-2; Ab-3: 14C6-bcl-2) of NaBu was loaded, and separated on 15% (reducing) and 8% (nonreducing) acrylamide resolving gels. M is a molecular size marker. H: heavy chain; L: light chain of antibody. For IEF analysis, 10 μg of each antibody sample was run on 5% native IEF ready gel, pH 3–10, and focused at increasing voltage. M is an IEF standard (pI 4.45 to 9.6).

using a serum-supplemented medium [20]. Thus, regardless of the media used, overexpression of Bcl-2 protein can extend culture longevity by suppressing NaBu-induced apoptotic cell death, thereby increasing antibody production.

SDS-PAGE and IEF

To determine the effect of NaBu/Bcl-2 overexpression on the biochemical characteristics of secreted antibody, the antibodies from SH2-0.32-Δbcl-2 and 14C6-bcl-2 cell cultures in the presence of NaBu were purified as described in Materials and Methods, and characterized by SDS-PAGE and IEF analyses. For comparison, control antibody produced from SH2-0.32 cells in the absence of NaBu was also characterized.

Figure 2a shows the SDS-PAGE analysis of control antibody (Ab-1; antibody produced from SH2-0.32 in the absence of NaBu) and sample antibodies (Ab-2 and Ab-3; antibodies produced from SH2-0.32-Δbcl-2 and 14C6-bcl-2 in the presence of NaBu, respectively) under the reducing and nonreducing conditions. Under the reducing condition, the heavy (H) and light (L) chains of all three antibodies were detected as a single band, indicating that there was no observable heterogeneity in molecular weight of each chain. The molecular weight of heavy chain was 55 kDa as expected. However, that of the light chain was 35 kDa rather than 25 kDa. The higher molecular weight of the light chain in this study was caused by introducing a new N-glycosylation site during the humanization process of V_L framework and, therefore, N-glycosylation of the light chain, which will be discussed in the following glycoform analysis section.

Under the nonreducing condition, although the major bands of all three antibodies showed an equivalent electrophoretic mobility, two antibodies (Ab-2 and Ab-3) produced in the presence of NaBu showed minor bands at approximately 110 kDa. These minor bands are thought to be immature antibodies assembled with only two heavy chains during the post-translational modification. Thus, compared with Ab-2, no significant changes in structural properties of Ab-3, which were produced using the NaBu/Bcl-2 overexpression system, could be observed by SDS-PAGE analysis.

Figure 2b shows the IEF analysis of three antibodies. All three antibodies were characterized by several distinct isoforms with pI range of 7.0–8.2, and most antibody isoforms were focused at around pI 7.5. No significant differences in band patterns were observed between Ab-2 and Ab-3 produced in the presence of NaBu. However, compared with control antibody (Ab-1) produced in the absence of NaBu, one more basic band was found in Ab-2 and Ab-3. This IEF result was in contrast with that reported by other groups. Chotigeat *et al.* [7] reported that the isoforms of human follicle stimulating hormone (hFSH) were shifted to the lower pI fractions when the molar concentration of NaBu was raised from 0 to 1.5 mM in CHO cell culture, while Cherlet and Marc [6] reported no significant change in pI in the monoclonal antibody produced from hybridoma cells when NaBu was added to the culture medium at a concentration of 1 mM. These conflicting results may be attributed to a number of factors such as the nature of secreted proteins, cell lines, and culture conditions.

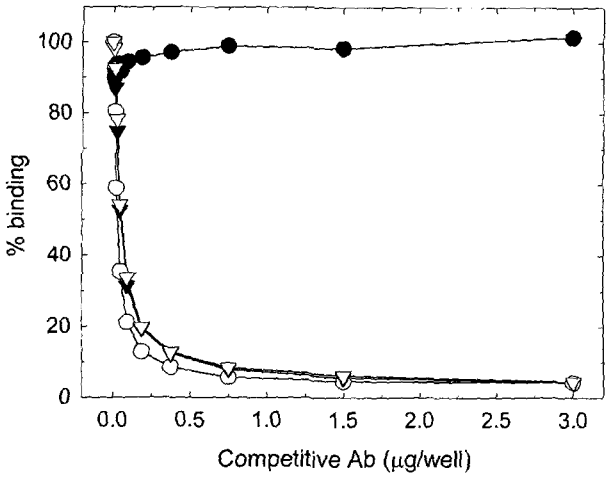

Fig. 3. Comparison of antibody samples obtained in the absence (Ab-1) and the presence (Ab-2, Ab-3) of NaBu by competition binding assay.
Various concentrations of sample antibodies were used to compete the binding of biotinylated Ab-1 to precoated HBsAg. (●) Unrelated chimeric antibody against preS2 antigen of HBV; (○) Ab-1; (▼) Ab-2; (▽) Ab-3.

Competitive ELISA

To investigate whether antibodies produced under the different culture conditions maintain the antigen-antibody binding properties, competitive ELISA was performed by using various concentrations of control and sample antibodies as competitors. Figure 3 shows that the competition profiles of sample antibodies (Ab-2 and Ab-3) were similar to that of the control antibody (Ab-1). An unrelated antibody used as a negative control did not compete the binding of biotin-conjugated control antibody to antigen. Thus, it was found that the antigen-antibody binding specificity of sample antibodies was not affected by the NaBu/Bcl-2 overexpression.

Figure 4 shows the antigen binding affinity ($K_{affinity}$) of control (Ab-1) and sample antibodies (Ab-2 and Ab-3), determined by using various concentrations of antigen. All three antibodies showed almost equivalent binding affinity to antigen. The $K_{affinity}$ was $2.5\times10^9\ M^{-1}$ for Ab-2 and Ab-3, and $2.0\times10^9\ M^{-1}$ for Ab-1. According to competitive ELISA results, no significant changes in antigen-antibody binding properties of antibodies, which were produced using the NaBu/Bcl-2 overexpression system, could be observed.

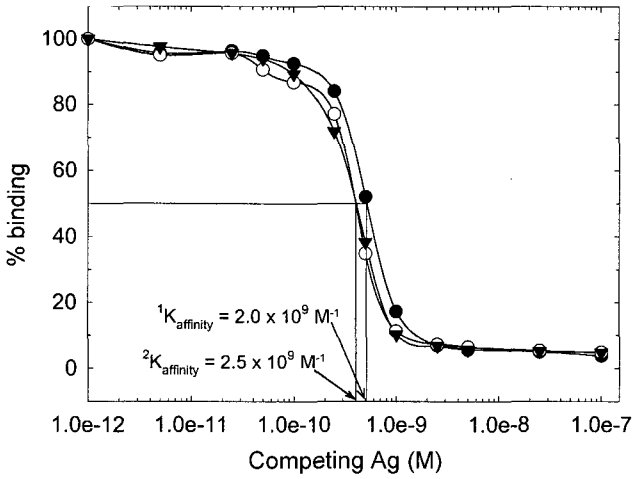

Fig. 4. Affinity profiles of antibody samples obtained in the absence (Ab-1) and the presence (Ab-2, Ab-3) of NaBu.
Various concentrations of HBsAg were previously incubated with antibodies and used as competitors for precoated HBsAg. (●) Ab-1; (○) Ab-2; (▼) Ab-3.

Glycoform Analyses

PAS Staining of Glycoprotein. In order to confirm the glycosylation of secreted antibodies (Ab-1, Ab-2, and Ab-3), carbohydrate-specific PAS staining was carried out. Figure 5 shows the Coomassie and PAS staining of Ab-1 after SDS-PAGE under the reducing condition. Similar results were obtained for Ab-2 and Ab-3 (data not shown). The PAS staining shows that post-translational glycosylation was processed in L chain as well as H chain. This was due to a new N-glycosylation site (Asn-X-Ser/Thr) that was introduced during the humanization process of V_L framework

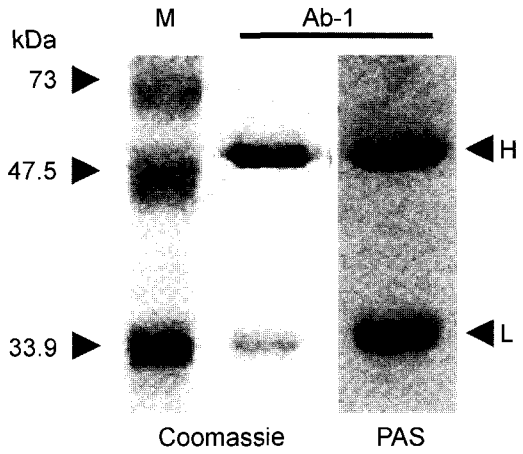

Fig. 5. Selective identification of the glycosylated moiety of humanized antibody produced from SH-0.32 in the absence of NaBu (Ab-1) using PAS staining.
Ab-1 (3 μg) was separated on 15% resolving gel under the reducing condition and stained with PAS and Coomassie Blue R-250 for comparison. M is a molecular size marker. H: heavy chain; L: light chain of antibody.

residues onto which murine complementarity determining regions can be grafted (data not shown). This additional N-glycosylation of light chain might be the cause of band shift toward a higher molecular weight of the light chain (35 kDa) when compared to the conventional light chain (25 kDa) in SDS-PAGE analysis (Fig. 2a). Since equal molecular contents of H and L chains were loaded in each lane, the similar degree of PAS staining to H chain and L chain suggests that the glycosylation of IgG chains is not biased to one chain.

MonoQ Column Analysis. For quantitative analysis of the variation in N-linked oligosaccharide structures of secreted antibodies, the mixture of N-glycans liberated from antibodies by digestion with N-glycosidase F was fluorescently labeled with 2AB and then resolved into neural and acidic oligosaccharides (monosialylated and disialylated) according to the number of sialic acid substituents, using an anion-exchange column (MonoQ).

Figure 6 shows that neutral oligosaccharides were eluted with water at the beginning. As the gradient was formed by 1 M ammonium acetate, monosialylated and disialylated oligosaccharides were eluted according to their negative charges at about 15 and 22 min of retention time, respectively. The relative amounts of the neutral, monosialylated, and disialylated oligosaccharides were calculated from each peak area, and summarized in Table 1. The antibodies produced in the presence of NaBu (Ab-2 and Ab-3) had a larger proportion of neutral oligosaccharides (67.5 and 60.9%, respectively) than that produced in the absence of NaBu (Ab-1, 51.2%). This increase in the proportion of neutral oligosaccharides in Ab-2 and Ab-3 was accompanied by the reduction in the proportion of monosialylated and disialylated acidic oligosaccharides.

GlycoSep-N Column Analysis. In order to further characterize the oligosaccharides, the neutral, monosialylated,

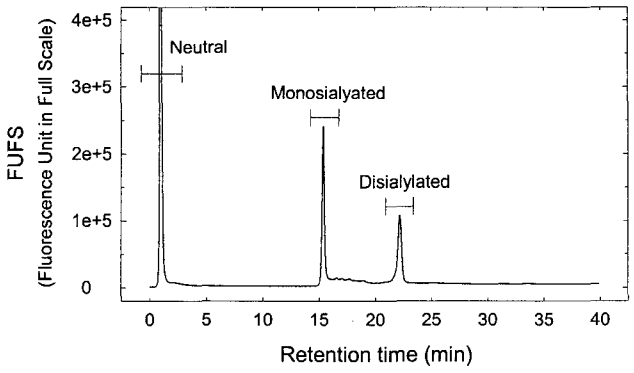

Fig. 6. MonoQ chromatogram of N-linked oligosaccharides purified from humanized antibody.
2AB-labeled oligosaccharides were separated into neutral and acidic pools according to the number of sialic acid substituents. Each peak was collected for further GlycoSep-N chromatography.

Table 1. The structures of identified oligosaccharides and their relative amounts in glycosylated humanized antibodies produced from rCHO in the presence and the absence of NaBu.

	Ab-1	Ab-2	Ab-3
Oligoforms*			
Neutral	51.2±4.6	67.5±0.8	60.9±3.4
Monosialylated	32.6±3.3	23.0±0.9	28.4±2.4
Disialylated	16.2±1.2	9.5±0.1	10.7±1.0
Sum	100.0	100.0	100.0
Neutral			
A2G0F**	43.3	34.3	38.8
A2G0FB	5.3	2.9	4.9
A2G1(1-3)B	6.7	8.9	4.3
A2G1(1-6)FB	20.7	16.8	21.7
A2G2F	7.8	7.8	7.8
Not identified	16.2	29.3	22.5
Sum	100.0	100.0	100.0
Monosialylated			
A2G2S1(1-6)	17.0	12.7	12.7
A2G2S1(1-3)	0.0	6.5	5.5
A2G2FS1(1-6)	58.7	80.8	73.2
Not identified	24.3	0.0	8.6
Sum	100.0	100.0	100.0
Disialylated			
A2G2FS2	68.2	100.0	100.0
Not identified	31.8	0.0	0.0
Sum	100.0	100.0	100.0

*The relative amounts of neutral, monosialylated, and disialylated oligosaccharides were calculated from MonoQ data obtained in duplicate experiments.

**Nomenclature for the description of oligosaccharide structures is as follows: A(1,2), the number of antennae linked to the trimannosyl (Man) core; G(0,1,2), the number of galactose (Gal); F, core fucose (Fuc); B, bisecting N-acetylglucosamine (GlcNAc); S(1,2), the number of N-acetylneuraminic acid (NeuNAc, commonly sialic acid).

and disialylated peaks shown in Fig. 6 were separated into individual oligosaccharide components by an amide column (GlycoSep-N) according to gu value which is related to the hydrophilicity of the glycans. Figure 7 shows the representative elution profiles of glucose-homopolymers (A), neutral (B), monosialylated acidic (C), and disialylated acidic oligosaccharides (D). Glucose-homopolymers were injected primarily to form a dextran ladder for the calculation of gu value. From the corresponding relationship between gu value and retention time of the glucose-homopolymer standard chromatogram, a multi-parameter function (cubic equation) could be deduced, and this function was then used for further calculation of gu value of the sample peak. Oligosaccharide structures were assigned on the basis of gu values and various standard oligosaccharide structures, as reported by Guile *et al.* [17]. The structures of sample peaks shown in fraction F (gu values <4) were not determined because they were thought to be contaminants. The gu values of complex oligosaccharides found in IgG were reported to be >4 [17].

The schematic drawings of identified oligosaccharide structures are shown in Fig. 8. The A2G1(1-3)B can be coeluted with A2G1(1-6)F, and the A2G1(1-6)FB can be coeluted with A2G1(1-3)F. However, the structures of A2G1(1-3)B and A2G1(1-6)FB could be identified based on the degree of decrease in gu values of their peaks after the N-acetyl-β,D-glucosaminidase treatment. After the enzyme treatment, the gu values of A2G1(1-3)B and A2G1(1-6)FB were decreased by 0.42 and 0.40, respectively. If the peaks were A2G1(1-6)F and A2G1(1-3)F, their gu values would be decreased by 0.20 after the enzyme treatment [17]. The relative amounts of oligoforms were calculated from each peak area and summarized in Table 1.

No significant differences in glycosylation patterns between Ab-2 and Ab-3 were observed, indicating that the overexpression of Bcl-2 protein did not affect the glycosylation patterns of antibodies in the presence of NaBu. In contrast, the glycosylation patterns of antibodies

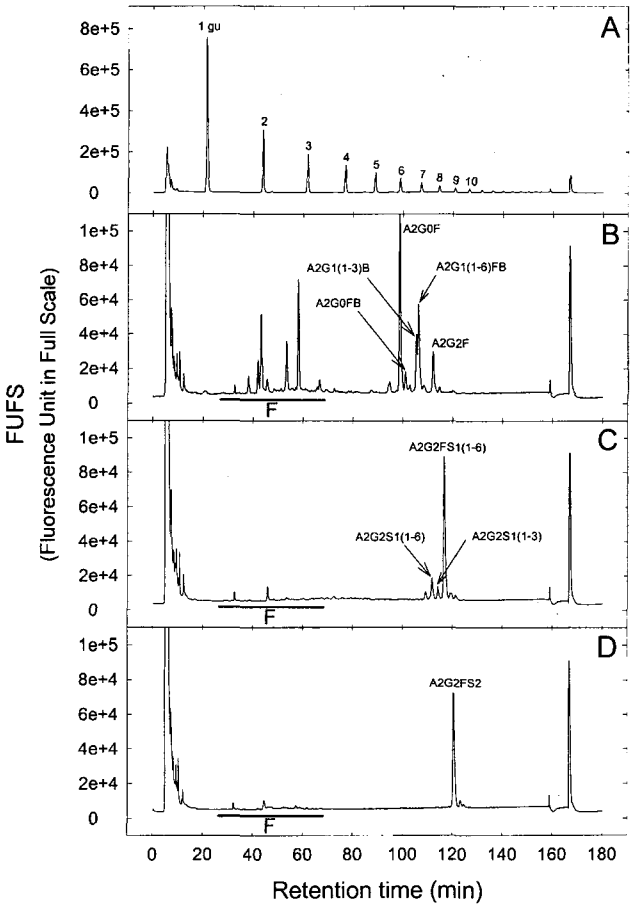

Fig. 7. GlycoSep-N chromatogram of neutral (B), monosialylated (C), and disialylated (D) oligosaccharides collected from MonoQ profile.

For determination of gu value of each peak, glucose-homopolymer standard was also injected to form a dextran ladder (A). Identified oligosaccharide structures were indicated above each peak. F: peaks of contaminants not to be analyzed.

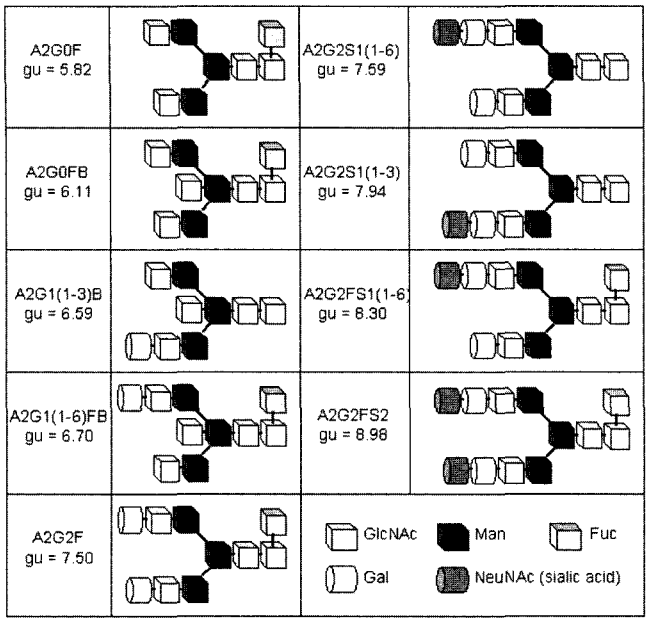

Fig. 8. Schematic drawings of oligosaccharide structures identified by the GlycoSep-N chromatography.

in the presence of NaBu (Ab-2 and Ab-3) were different from those of the control antibody (Ab-1) produced in the absence of NaBu. Thus, NaBu might affect the activities of enzymes involved in intracellular glycosylation pathways of rCHO cells, resulting in the changes in oligoformal microheterogeneity of antibodies. Compared with Ab-1, the major glycoforms of neutral, monosialylated, and disialylated oligosaccharides of Ab-2 and Ab-3 were relatively decreased (A2G0F) or increased [A2G2FS1(1-6) and A2G2FS2].

DISCUSSION

NaBu has been widely used for making a high-level expression of foreign protein in the animal cell culture. The modulation of gene expression by NaBu is known to be related to the open chromatin structure as a result of hyperacetylation of histone H4 by inactivation of histone deacetylase. Therefore, the increased accessibility of transcription factors [24] can be seen. Hyperacetylation of H4, however, can give rise to cellular apoptosis, because the enhanced gene expression may facilitate the translation of putative cell death proteins [28], and/or the appearance of more open chromatin structure may increase the possibility of DNA cleavage by apoptotic endonuclease [35].

Previously, we demonstrated that NaBu-induced apoptotic cell death in rCHO cell cultures by using a serum-containing medium could be inhibited by overexpression of Bcl-2 survival protein, resulting in substantial increase in antibody production [20]. In the present study, it was demonstrated that the enhanced transcription efficiency endowed by NaBu and the extended culture longevity provided by Bcl-2 overexpression also resulted in substantial increase in antibody production in rCHO cell culture by using a serum-free medium that is preferred in the industry.

In order to adopt a new process including expression system and medium for therapeutic protein production, the molecular integrity of proteins produced by a new process should be examined. Accordingly, antibodies were purified from parental (SH2-0.32), Bcl-2 deficient (SH2-0.32-Δbcl-2), and Bcl-2 overexpressing (14C6-bcl-2) rCHO cell cultures in the presence and absence of NaBu to determine the effect of NaBu/Bcl-2 overexpression on their molecular integrity. To the best of our knowledge, there is no information available regarding the characterization of glycosylation patterns of recombinant antibody produced from apoptosis-resistant CHO cells, by using the NaBu/Bcl-2 overexpression system.

SDS-PAGE analysis under the reducing condition indicated that the molecular weights of L and H chains of antibodies were consistent, suggesting that NaBu/Bcl-2 overexpression did not induce significant variations in proteolytic processing and post-translational glycosylation. Under the nonreducing condition, immature antibodies that might have been released from membrane-ruptured cells in the process of NaBu-induced apoptotic cell death were detected in the presence of NaBu. However, these immature antibodies accounted for only small fraction of the total antibodies produced from cells (Fig. 2). Immature antibodies are thought to be composed of two heavy chains on the basis of molecular weight. In addition, they were purified by Protein G column which has a specific affinity to the Fc region of H chains.

N-glycosylation at the conserved Asn-X-Ser/Thr glycosylation site in the Fc moiety of the IgG C_H2-domain has been known to play an important role in Fc-receptor-mediated functions, and antigen clearance functions such as complement activation, protein assembly, secretion rate, sensitivity to proteases, and even serum half-life in the case of IgG_3 [8, 39]. In general, antibody glycosylation can be influenced by cell type from which antibody is produced, conformation of antibody itself, and cell culture conditions [39].

MonoQ column analysis showed that the relative amounts of acidic (monosialylated and disialylated) oligosaccharides were lower in antibodies produced in the presence of NaBu (Ab-2 and Ab-3) than those produced in the absence of NaBu (Ab-1). The changes in microheterogeneous oligoformal structures of antibody are likely to affect the mobility of antibody isoforms, resulting in the occurrence of some more basic isoforms of Ab-2 and Ab-3 in IEF study (Fig. 2). This result is consistent with previous reports, showing a close relationship between the isoform pI and the sialic acid contents of hFSH [38] along with EPO [40].

The reduced sialylation of antibodies produced in the presence of NaBu may occur due to a number of

intracellular and extracellular factors. If the intracellular sialyltransferase activity is not high enough to meet the elevated secretion rate of antibodies, cells may improperly release sialylated antibodies into the culture medium. As suggested earlier [34], NaBu may enhance reutilization of existing glycoproteins in the culture, generating sialic acid for biosynthesis through lysosomal degradation. Increased extracellular sialidase activity released from dead cells may also result in reduced sialylation of antibodies produced in the presence of cytotoxic NaBu. The observation that the relative amounts of acidic oligosaccharides in the presence of NaBu were marginally increased by overexpression of Bcl-2 supports this hypothesis. Gu *et al.* [15] also reported that a competitive inhibitor of extracellular sialidase could prevent the loss of sialic acid of interferon-γ after an onset of cell death, suggesting that the sialidase released from dead cells can remove the terminal sialic acid from N-glycoform of glycoprotein.

GlycoSep-N column analysis revealed that the core oligosaccharide structure ($GlcNAc_2Man_3GlcNAc_2$) was not affected by NaBu/Bcl-2 overexpression. Regardless of the presence of NaBu, agalactosylated (G0) or monogalactosylated (G1) forms were major structures in neutral oligoforms and they accounted for about 40% of the total antibodies produced from rCHO cells. Routier *et al.* [33] reported a similar result in which 57% of humanized antibodies produced from CHO DUKX cell culture containing 5% serum had agalactosylated and monogalactosylated oligosaccharides. However, they could not observe nonfucosylated core oligosaccharide and bisecting GlcNAc residue which were observed as a small proportion of antibodies in this study. Furthermore, they could not observe any sialylated oligoforms isolated from *N*-glycans. They suggested that the lack of sialylated oligoforms of humanized antibodies was due to CHO cells that normally express β-galactoside (α2,3) sialyltransferase but not β-galactoside (α2,6) sialyltransferase, and the sialylation of IgG was suggested to be (α2,6) only. In contrast, Gawlitzek *et al.* [12] and Mimura *et al.* [29] reported different results where sialylated oligoforms of *N*-glycans were detected from TNFR-IgG fusion protein, human chimeric IgG in CHO DUKX and CHO-KI cell cultures, respectively. Therefore, the heterogeneity in oligosaccharide structures of antibodies may result from a number of factors such as different culture conditions, and clone-specific and polypeptide-specific variations in carbohydrate processing.

For *in vivo* functions of IgG, it was reported that the presence of $GlcNAc_2Man_3GlcNAc_2$ core oligosaccharide in the C_H2 domain is a minimal requirement for IgG binding to Fcγ-receptors, but galactosylated branches are not required for the efficient recognition [8]. The partial presence of nonfucosylated oligosaccharide structure could be observed in neutral and monosialylated oligoforms. In a case of monosialylated oligoforms, the nonfucosylated oligoforms account for about 20%. The partial presence of nonfucosylated glycans, observed in humanized antibodies described here, is thought not to affect significantly *in vivo* in a life time, because such variations in the degree of fucosylation of IgG are also observed in human serum IgG [8].

Although the presence of NaBu in CHO culture can slightly affect the glycosylation patterns, especially terminal sialylation of *N*-glycans, the antigen-antibody binding properties were not changed by making an alteration in the glycosylation pattern (Figs. 3 and 4). Mimura *et al.* [29] demonstrated that the IgG antibody produced from CHO cells in the presence of NaBu showed a similar *in vivo* function such as IgG-Fc effector function. Thus, overexpression of Bcl-2 protein which can reduce marginally the desialylation of antibody glycoforms in the presence of NaBu (Table 1) will be beneficial to the molecular integrity of antibody produced from rCHO cells. However, efforts to maximize the sialylation of glycoprotein, especially of protein for therapeutic use, in the NaBu/Bcl-2 overexpression system are still needed, considering the influence of terminal sialylation of glycoprotein on the solubility, thermal stability, resistance to protease, specific activity, and circulatory lifetime.

Sialylation of recombinant proteins in CHO cells has been increased successfully by feeding a direct intracellular precursor for sialic acid synthesis [16], transfecting a sialyltransferase [21], expressing a sialidase antisense RNA [9], and regulating two glycosyltransferase activities in the O-glycosylation pathway by using simultaneously tetracyclin-regulatable expression of antisense RNA and sense gene [31].

In conclusion, it has been demonstrated that NaBu-induced apoptosis of rCHO cells producing a humanized antibody could be significantly suppressed by overexpression of Bcl-2 protein during a batch culture by using a commercially available serum-free medium. As a consequence, the increased viable cell culture longevity brought final antibody concentration up about 2-fold. Although the NaBu treatment of rCHO culture could slightly affect the glycosylation pattern of antibody, especially the degree of sialylation, there was no significant variation in the molecular weight and antigen-antibody binding properties. Taken together, when a serum-free medium was used, no significant effects of NaBu/Bcl-2 overexpression on the molecular integrity of antibodies were observed by the molecular assay systems, thus demonstrating its potential usefulness for commercial process.

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