Deletion of the κ genes from mouse hybridomas established with NS-1 myelomas

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Abstract

Monoclonal antibodies (mAbs) of the IgG class produced by mouse hybridomas raised with NS-1 myelomas have been shown to contain two types of immunoglobulin light (κ) chains derived from the myelomas and antigen-stimulated spleen lymphocytes, and the hybridomas produce three mAb species with light chain heterogeneity (Abe and Inouye, 1993). In the present study, 9 hybridoma lines secreting homogeneous mAbs have been isolated from 63 lines cloned from an established hybridoma line producing three mAbs. They secrete homogeneous mAbs containing light chains derived from either myeloma or spleen cells. They contain either κ gene derived from the respective cells, and the other gene was deleted during the cultivation. The deletion frequency of the κ gene of myelomas is 3 times higher than that of spleen cells, although 80–85% of hybridomas reach the stable state containing both κ genes.

Abbreviations: mAb – monoclonal antibody; PBS – phosphate-buffered saline; SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC – high-performance liquid chromatography; L chain – light chain; H chain – heavy chain; SSC – saline supplemented with sodium citrate.

Introduction

Various myeloma lines are used as fusion partners in the preparation of hybridomas producing monoclonal antibodies (mAbs). Myeloma cell lines such as SP2/0 and X63.6.5.3 can not synthesize the heavy (H) and light (L) chains of immunoglobulins, and thus hybridomas established with these myeloma lines secrete mAbs bearing H and L chains derived only from spleen lymphocytes. In contrast, myelomas such as NS-1 and P3U1 synthesize L (κ) chains, but do not secrete free L chains. Intracellular κ chains are only secreted from somatic hybrid cells by attaching with H chains (Köhler et al., 1976; Köhler and Milstein, 1976). If random association of two types of L chains (Ls and Lm, being derived from spleen lymphocytes and myelomas, respectively) with one heavy chain (Hs) from lymphocytes occurs, three species of hybrid mAb (Hs₂Ls₂, Hs₂LsLm, and Hs₂Lm₂) will be formed in the

culture fluids.

Hybridoma lines were raised by using NS-1 myeloma as a fusion partner (Abe and Inouye, 1993). mAbs of the IgG1 class produced by the hybridomas were purified by the conventional method using anion-exchange HPLC (Campbell, 1984; Deschamps et al., 1985), and were judged to be apparently homogeneous by SDS-PAGE and analytical gel filtration HPLC. The purification and judgement for purity have been widely accepted. However, we have confirmed that the mAb was separated into three species by hydrophobic interaction HPLC, and purified them separately (Abe and Inouye, 1993; Inouye and Abe, 1994). Although this result seems to be reasonable from the theoretical consideration mentioned above, we have noticed that the ratio of the three mAbs varies depending on the cultivation conditions of the hybridoma. In the present study, we demonstrate that hybridoma lines secreting homogeneous mAbs were isolated from the population

of hybridoma cells raised with NS-1 myeloma, and that they contained L chains derived from only myelomas or spleen lymphocytes.

Materials and methods

Hybridoma and monoclonal antibody (mAb)

A mouse mAb (FK021.5) of the IgG1 class was used, and the specific antigen is human ferritin (Abe and Inouye, 1993). The hybridoma secreting the mAb was established by fusing spleen cells from an antigen-immunized BALB/c mouse with NS-1 myeloma according to Köhler and Milstein (1975). The hybridoma cells were injected into pristane-primed BALB/c mice and grown in ascites. Purification of the mAb from the ascites was performed at 4 °C. The collected ascites were centrifuged at $3,000 \times g$ for 20 min to remove cells, and the supernatants were passed through Millipore filters (pore size: 0.8 μm ; AA type). Ammonium sulfate (solid) was added to the filtrate to give 50% saturation, followed by centrifugation at $10,000 \times g$ for 20 min after standing 2 h. The precipitates were dissolved in phosphate-buffered saline (PBS, pH 7.4) and salted out with 50% saturated ammonium sulfate under the same conditions as described above. The precipitates were dissolved in PBS, giving partially purified mAbs and then applied to anion-exchange HPLC using TSKgel DEAE-5PW (Tosoh, Tokyo). The mAb preparation collected from the major peak was apparently homogeneous by SDS-PAGE and analytical gel filtration HPLC with TSKgel G3000SW_{XL} (Abe and Inouye, 1993).

Cloning and culture of hybridoma cells

Cloning of hybridoma cells was performed by limiting dilution. They were plated at one cell per well in a microtiter plate (MaxiSorp, 96-wells; Nunc-Intermed, Roskilde, Denmark), cultivated for 2 weeks in a RDF medium (Kyokuto pharmaceutical, Tokyo) supplemented with 10% fetal calf serum (Cell Culture Laboratories, Cleveland, OH) and incubated at 37 °C in 5% CO₂ plus 95% air. The cells were cultured for 3 days after thawing, the culture medium changed once and then cloned by limiting dilution.

After confirming colonies of a single hybridoma, the cells were expanded to 10-cm diameter dishes (Falcon). When they were confluent, the cultured medium was collected and centrifuged at $1,500 \times g$ for 5 min,

and the supernatant was used as the cultured medium.

Purification of mAbs from the cultured medium

mAbs were purified from the cultured medium by using a protein A-Topopearl column (Tosoh). The medium was applied to a TSKgel AF Protein A-Toyopearl 650M column (5 mm (inner diameter) $\times$ 5 cm; Tosoh) equilibrated with PBS. The column was thoroughly washed with PBS, and then proteins were eluted with 0.1M sodium citrate, pH 4.0 at a flow-rate of 1 ml min⁻¹. Approximately 200 μg IgG1 was recovered from 10 ml cultured medium and the purity was about 80% by gel filtration HPLC with TSKgel G3000SW_{XL}. The concentration of mAbs was determined spectrophotometrically with an absorption coefficient at 280 nm, A_{280} (1 mg ml⁻¹), of 1.4 (Inouye and Gohzu, 1991).

High-performance liquid chromatography (HPLC)

The HPLC apparatus consisted of a solvent-delivery system CCPM, a UV monitoring system UV-8010, a fraction collector FC-8000 and a computer-control system CS-8010 was purchased from Tosoh (Tokyo). Throughout this study, the elution was monitored by absorbance at 280 nm, and fractions (1 ml) were collected.

Hydrophobic interaction HPLC was performed on a TSKgel Phenyl-5PW column (7.5 mm (inner diameter) $\times$ 75 mm) (Tosoh) (Abe and Inouye, 1993; Morimoto and Inouye, 1992). Fifty μg of the purified IgG1 dissolved in PBS containing 1 M ammonium sulfate was injected into the column equilibrated with the same buffer. A linear gradient of ammonia sulfate from 1 to 0 M in the equilibrating buffer was generated over 35 min from 5 min after the sample injection. Gel filtration HPLC was done using a TSKgel G3000SW_{XL} (7.8 mm (inner diameter) $\times$ 30 cm) with PBS (Inouye and Gohzu, 1991).

Isolation of cellular DNA

DNA was extracted from hybridoma cells with SDS and proteinase K (Gross-Bellard et al., 1973; Köhler et al., 1982). Two $\times 10^7$ cells were washed twice in PBS and resuspend in 10 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and 150 mM NaCl. The cells were lysed by adding 0.2% SDS, and treated with 100 μg ml⁻¹ proteinase K (Sigma, St. Louis, MO) at 37 °C, for 12 h. The cell digests were treated with

phenol-chloroform and ethanol, followed by dialysis, and treated by ribonuclease A (50 g ml^{-1}) at 37°C for 2–3 h.

Analytical studies

SDS-PAGE was performed in 12% slab gels under reducing conditions (Laemmli, 1970). Two μg of purified IgG1 was reduced by treatment with 1% 2-mercaptoethanol at 100°C for 10 min. Proteins were stained by Coomassie brilliant blue R-250. The molecular-mass marker kit containing rabbit muscle phosphorylase b (94 kDa), bovine serum albumin (67 kDa), chicken ovalbumin (43 kDa), bovine erythrocyte carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20 kDa) was a product of Pharmacia (Uppsala, Sweden).

Western blotting analysis was performed according to the method of Towbin et al. (1979). Proteins were applied to SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was treated by rabbit anti-mouse L (κ) chain serum (Organon Teknica-Cappel, Durham, NC) for 1 h at 25°C , followed by treatment with mouse anti-rabbit IgG antibody conjugated with horseradish peroxidase (Tago, Burlingame, CA) for 30 min at 25°C . The L (κ) chain was detected by immunostain kit HRP (Konica, Tokyo).

Southern blot analysis (Southern, 1975) was performed with 10 μg DNA sample digested by Hind-III. Hybridization after transfer to nitrocellulose membrane (Advantec, Tokyo) was made with a J κ DNA probe in $2 \times \text{SSC}$ buffer, $10 \times$ Denhardt's solution, 0.1% SDS, and 50 $\mu\text{g/ml}$ salmon sperm DNA, at 65°C for 18 h, followed by washing with $2 \times \text{SSC}$ in Denhardt's solution and then with $0.2 \times \text{SSC}$ at the same temperature. A mouse DNA fragment containing the J κ gene (J κ probe) isolated from clone Ig146 (Sakano et al., 1979) was a kind gift of Dr. Y. Kurosawa (Fujita Health University).

Results and discussion

Hydrophobic interaction HPLC

Fractions of mAb FK021.5 purified into a single peak by anion-exchange HPLC with TSKgel DEAE-5PW were separated into three peaks (peaks 1–3) at 25.2, 29.7 and 32.4 min in hydrophobic interaction HPLC with TSKgel Phenyl-5PW (Fig. 1). The ratio of the respective peak areas was 2.0:1.3:1.0. By assuming

Figure 1. Separation of three hybrid mAb species from mAb FK021.5 by means of hydrophobic interaction HPLC. The purified fraction of mAb FK021.5 by anion-exchange HPLC was applied to the HPLC. The chromatographic conditions were described in the text.

the same absorption coefficient for mAbs in the three peaks, the ratio indicates the amounts of mAbs eluted out in the respective peaks. The mAb fractions collected from peaks 1, 2 and 3 were applied to SDS-PAGE under reducing conditions (Fig. 2A). The H chain appeared at 50 kDa, and two L chains at 28 and 29 kDa. Peak 1 contained only the 28-kDa chain, peak 3 the 29-kDa chain while peak 2 contained both chains. Immunoblotting analysis showed that NS-1 lysates contained only the 29-kDa chain, and mAb FK021.5 purified by the anion-exchange HPLC contained two chains of 28 and 29 kDa (Fig. 2B). Therefore, the 28-kDa chain must be derived from the spleen lymphocytes and therefore the 28 and 29-kDa chains are Ls and Lm, respectively. It is suggested that mAbs eluted in peaks 1, 2 and 3 have the composition of H and L chains as Hs_2Ls_2 , Hs_2LsLm and Hs_2Lm_2 , respectively. By considering the ratio of their amounts eluted from the HPLC (Fig. 1), the ratio of Ls and Lm secreted as a component of mAb FK021.5 was estimated to be 3:2. If efficiencies in biosynthesis of both chains are the same, this ratio may reflect that of numbers of chromosomes encoding Ls and Lm in the hybridoma.

Cloning hybridoma cells producing mAb FK021.5

By limiting dilution of hybridoma cells producing FK021.5, 63 new clones were newly separated. mAbs produced by these clones were purified by protein A chromatography and analysed by SDS-PAGE under reducing conditions. From the migration pattern of the L chains, the clones were categorized into 3 groups:

Figure 2. Analysis of L (κ) chains in mAb species of mAb FK021.5 by SDS-PAGE and immunoblotting. **A.** SDS-PAGE. Lanes 1, molecular-mass marker proteins; 2–4, samples collected from peaks 1–3 of Fig. 1, respectively; and 5, a sample of mAb FK021.5 collected in anion-exchange HPLC. The IgG1 H chain appeared as a band of 50 kDa, and L chains were those of 28 and 29 kDa. **B.** Immunoblotting. L (κ) chain was detected by anti-mouse L (κ) chain rabbit serum and anti-rabbit IgG mouse antibody conjugated with horseradish peroxidase (see Materials and methods). Lanes 1, lysates of NS-1 myeloma; and 2, mAb FK021.5.

54 clones (#1–54) produced mAbs containing both 28- and 29-kDa L chains, however, 6 clones (#55–60) produced mAbs containing only 28-kDa L chain, and 3 clones (#61–63) produced mAbs containing only 29-kDa L chain. The mAb fractions isolated from the cultured medium of clone #1, which showed two L chains in SDS-PAGE, were separated into 3 peaks in the Phenyl-5PW HPLC (Fig. 3A). The elution pattern, peak positions and ratio of the peak areas are the same as those observed with mAb FK021.5 (Fig. 1). On the other hand, the mAb fractions isolated from the cultured media of clones #55 and 61, which contain 28 and 29-kDa L chains, respectively, eluted at 25.2 and 32.4 min as a single peak (Figs. 3B and C), suggesting that these clones produce mAbs of Hs₂LS₂ and Hs₂Lm₂, respectively. The mAb fractions produced by other clones in the respective groups showed the same HPLC behaviors as those of the representative clones as shown in Fig. 3. Clones #1–54 produce three hybrid

mAbs, and clones #55–60 and #61–63 produce Hs₂LS₂ and Hs₂Lm₂, respectively.

Southern blot analysis

Genomic DNA of the separated clones from the parent hybridoma producing mAb FK021.5 was analyzed (Fig. 4). All clones as well as the parent one showed a common band of 6.6 kb. The parent hybridoma (lane 1) and clone #1 (lane 4), both of which produce 3 hybrid mAbs, generated 2 bands of 4.5 and 5.0 kb in addition to that of 6.6 kb. On the other hand, clones #55, 57, and 59 producing only mAb Hs₂LS₂ (lane 2, 3 and 6, respectively) produced a single band of 4.5 kb, and the 5.0-kb band was not observed. Clone #61 producing only mAb Hs₂Lm₂ showed the 5.0-kb band but not the 4.5-kb band (lane 5).

The 2.8-kb band is the embryonic κ gene derived from NS-1 myelomas. It is noteworthy that the band is

Figure 3. Hydrophobic interaction HPLC with TSKgel Phenyl-5PW of mAb samples purified from hybridomas separated from the parent hybridoma producing mAb FK021.5. The chromatographic conditions are described in the text. Hybridoma clones: A, the parent clone (the mAb FK021.5.1 producer); B, # 55; and C, # 61.

Figure 4. Southern blotting analysis of genomic DNAs of hybridoma clones separated from the parent hybridoma producing mAb FK021.5. The blotting conditions were described in the text. Lanes 1, the parent clone (the mAb FK021.5 producer); 2, #55; 3, #57; 4, #1; 5, #61; and 6, #59.

not apparent in lane 4, indicating that the embryonic κ gene derived from NS-1 myelomas is deleted.

The 6.6-kb band is the κ gene which has failed to rearrange and from which the 5.0- and 4.5-kb κ genes are differentiated (Sakano et al., 1979). As the 5.0-kb but not 4.5-kb band is observed in the NS-1 myeloma (data not shown), the 5.0-kb gene must be related in synthesis of the Lm chain. Therefore, the 4.5-kb band is supposed to be related in synthesis of the Ls chain. Actually, 6 clones (#55–60) possessed only the 4.5-kb band in the southern blot analysis, and produce only mAb of Hs₂Ls₂ (Fig. 3B; Fig. 4, lanes 2, 3 and 6). On the other hand, 3 clones (#61–63) contain only the 5.0-kb gene and produce only mAb of Hs₂Lm₂ (Fig. 3C; Fig. 4, lane 5). The remaining 54 clones (#1–54) contain both 5.0- and 4.5-kb genes, and secrete three mAbs (Fig. 3A; Fig. 4, lane 4). The κ genes encoding Lm or Ls chains are deleted in the clones secreting one mAb species, namely the chromosomes derived from NS-1 myelomas or spleen lymphocytes are eliminated during cultivation of the parent hybridoma raised with NS-1 myeloma.

As has been mentioned above, the parent hybridoma is considered to contain chromosomes encoding Ls and Lm chains in the ratio of 3:2. Deletion mutants of the chromosomes encoding L chains occurs in 14% of the total clones separated from the parent hybridoma, and the mutants lacking the chromosome encoding Lm were obtained two times more frequently than those lacking the Ls-encoding chromosome. In other words, the Lm-encoding chromosome delete three times more frequently than the Ls-encoding one from the parent hybridoma. It is expected that the hybridoma mixture composed of 67% of the Hs₂Ls₂ producer and 33% of the Hs₂Lm₂ producer could be ultimately obtained after repeating cultivation of the parent hybridoma, if we assume that two types of chromosomes cannot be accommodated in a cell, and that one of them is forced to be deleted. However, the ratio of mAbs of Hs₂Ls₂, Hs₂LsLm and Hs₂Lm₂ obtained after 5 passages of cultivation is 1.9:1.2:1.0, being very close to that (2.0:1.3:1.0) obtained with the parent hybridoma, and 80–85% of the total clones produced three hybrid mAbs. Therefore, it is obvious that both chromosomes can exist stably in most of the hybridoma clones.

Concept of monoclonal antibody; structural or functional?

The immunoreactivities of the respective mAbs are considerably different, and that of Hs₂Lm₂ is almost

the half of that of Hs₂Ls₂ (Abe and Inouye, 1993). As long as we use mAbs produced by hybridomas raised with NS-1 myelomas, we must understand that the mAbs are the mixture of heterogeneous immunoglobulins. From the stand point of quality of mAbs, the mAb species Hs₂Ls₂ is the most appropriate and if the mixture of three species is used, the ratio of the species must be mentioned. Notwithstanding, it should be stated that the hybrid mAbs can be purified separately by using hydrophobic interaction HPLC as shown in Figs. 1 and 3. Although hydrophobic interaction HPLC has not been used much so far in the purification of mAbs (Morimoto and Inouye, 1992; Abe and Inouye, 1993; Inouye and Morimoto, 1993, 1994), it should be recommended for purifying mAbs especially for those produced by hybridomas raised with NS-1 myelomas and probably with other myelomas synthesizing L chains.

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