Two Chicken B Cell Lines Resistant to Ouabain for the Production of Chicken Monoclonal Antibodies

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ABSTRACT. Two chicken B cell lines, termed MuH1 and MuH4, which are resistant to ouabain, were established as fusion partners for production of chicken monoclonal antibody from the parental cell lines R27H1 and R27H4 which were deficient in thymidine kinase activity. The MuH1 synthesized, but did not secrete μ chain, whereas the MuH4 secreted non-specific IgM. Fusions of the MuH1 and MuH4 with spleen cells from chickens immunized with human IgG resulted in successful preparations of hybridomas secreting antibodies specific to human IgG. Transformed cells insensitive to HAT appeared in many culture wells after cell hybridizations with R27H4, but on hybridization with MuH1 or MuH4 the appearance of such unfavorable cells could be avoided by the inclusion of ouabain in HAT medium. Thus, in comparison with the R27H4 line, the MuH1 and MuH4 lines did not provide higher fusion efficiencies, but gave increased opportunities to obtain hybridomas producing specific antibodies. Among a total of eight hybridomas, four derived from MuH1 and four derived from MuH4, seven secreted both IgG and IgM, and the remaining one derived from MuH1 secreted only IgG. In all eight hybridoma lines IgG was specifically reactive to human IgG. — **KEY WORDS:** chicken, fusion, monoclonal antibody, ouabain, thymidine kinase.

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IgG type antibodies raised in chickens have certain general properties that make them useful for immunochemical research and clinical application. For example, chicken IgG has very low cross-reactivity with mammalian IgGs [5], does not bind protein A and rheumatoid factor [4, 10], and does not activate the human complement system [7, 11].

In our previous studies, we have established four chicken fusion partner cell lines R27H1, R27H2, R27H3 and R27H4, lacking thymidine kinase (TK) suitable for production of chicken monoclonal antibodies (mAb) [13]. Of these cell lines, the R27H4 were hybridized with spleen cells from chickens immunized with keyhole limpet haemocyanin followed by growth of resulting hybrid cells in HAT medium. However, the R27H4 had some functional deficiencies. The cell line produced non-specific IgM and HAT-insensitive cells capable of growing in HAT medium often appeared in the fusion culture. To remove problems by these deficiencies, therefore, the additional improvement will be required of the fusion partner cell line.

Human B cell lines which are resistant to ouabain and TK-deficient have proved efficient for production of human

mAbs [2, 3, 6, 8, 9, 14, 15]. We have therefore attempted to prepare ouabain-resistant and TK-deficient cell clones from the R27H4 cell line. In addition, to separate the non-Ig-secreting clone, we used also the R27H1 cell line which only produced for μ chain [13]. Here, we describe the establishment of two ouabain-resistant and TK-deficient parental cell lines and their application for production of chicken mAb.

MATERIALS AND METHODS

Cell lines: Chicken TK-deficient B cell lines, R27H1 and R27H4 [13], were used in this study. These cell lines were routinely maintained in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal bovine serum (FBS), or in the same medium supplemented with 10 μ g/ml trifluorothymidine (TFT, Sigma, St. Louis, MO). Cells were grown in a 5% CO₂ incubator at 38.5°C.

Isolation of ouabain-resistant chicken cell line: The R27H1 and R27H4 cells were treated with the mutagenic agent, ethyl methanesulfonate (EMS, Ardrich, Milwaukee, WIS) for 24 hr, at a final concentration of 600 μ g/ml in RPMI 1640 supplemented with 5% FBS. Thereafter, the cells were cultured in medium containing 10% FBS for 4 days, and then maintained in IMDM supplemented with 10% FBS and 5 $\times 10^{-5}$ M ouabain. Finally, surviving cell clones were isolated from cultures containing 1 $\times 10^{-4}$ M ouabain and TFT (10 μ g/ml).

Preparation of chicken mAb: Four-months-old CB inbred line chickens that were obtained from Dr. Yamamoto of

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Hiroshima University were used in this study. The chickens were immunized intramuscularly with 100 μ g human IgG (PROTOGEN AG, Laufelfingen, Switzerland) in 0.5 ml phosphate buffered saline (PBS) emulsified with an equal volume of Freund's complete adjuvant. Thirty days later, they received 200 μ g human IgG in 0.5 ml PBS by intravenous injection. Chicken fusion partner cell lines were hybridized with the immune spleen cells three days after boosting by the method described previously [10], except for the use of polyethylene glycol 1500. Fusions were carried out at a parental cell/spleen cell ratio 1:5. The fused cells were washed gently, suspended in IMDM supplemented with 10% FBS, and distributed to 96-well culture plates. After 24 hr incubation at 38.5°C, HAT medium (1×10^{-4} M hypoxanthine, 0.8×10^{-7} M aminopterin and 1.6×10^{-5} M thymidine) was added to each well. After 4 days of cell fusion, half the culture supernatant was removed and replaced with HAT medium containing 2×10^{-5} M ouabain. After 10 days of cell fusion, culture medium was changed to HT medium containing 2×10^{-5} M ouabain. Specific hybridomas were subcloned by the soft agar culture method as described previously [13].

Determination and specificity of chicken mAb: Specificity of chicken mAb was determined by an enzyme-linked immunosorbent assay (ELISA) using human IgG-coated ELISA plates. Briefly, ELISA plates were filled with 50 μ l/well of human IgG at a concentration of 1 μ g/ml in PBS and incubated overnight at 4°C. The plates were then blocked with 200 μ l/well of PBS containing 1% BSA for 1 hr at room temperature, and rinsed once with PBS containing 0.05% Tween 20 (PBST). After 1 hr incubation with 50 μ l of culture supernatant, the plates were washed twice with PBST, and then incubated with peroxidase (PO)-conjugated goat anti-chicken IgG (H+L) (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The reaction was visualized by the addition of 0.4 mg/ml *o*-phenylenediamine dihydrochloride in 0.1 M phosphate-citrate buffer, pH 5.6, containing 0.02% H_2O_2 . The color development was terminated with 4 N H_2SO_4 . Optical density measurements were made at 492 nm using a Microplate Reader MPR A4 (TOSOH, Japan).

Determination and quantification of chicken

immunoglobulin: Chicken hybridoma clones obtained in this study were cultured in FBS-free IMDM for 24 hr. IgG and IgM secreted into the culture supernatants during that time were determined by ELISA and Western blotting analysis according to the methods described previously [13]. In these experiments, we used three kinds of mouse mAbs specific for chicken Ig chains, L, μ and γ , respectively. These mAbs were developed from fusion with SP2/O-Ag14 cells [16] and BALB/c mouse spleen cells immunized with purified chicken IgG (Cappel, West Chester, PA) or IgM (Inter-Cell Technologies, Hopewell, NJ).

RESULTS

Establishment and characteristics of new parental cell lines: In order to obtain ouabain-resistant clones from the R27H1 and R27H4 lines, these cells were treated with the chemical mutagen EMS, followed by stepwise addition of ouabain at concentrations increasing from 5×10^{-5} to 1×10^{-4} M. Finally, two ouabain-resistant and TK-deficient clones, termed MuH1 and MuH4, were obtained from R27H1 and R27H4, respectively. These clones survived in medium containing ouabain, but not in HAT medium. The biological properties of these two clones are shown in Table 1 and Fig. 1. The characteristics of these new partner cell lines were not so different from those of their parent lines. MuH1 synthesized, but did not secrete, immunoglobulin μ chain (Table 1 and Fig. 1). While, MuH4 synthesized μ (MW: 84,500) and L (MW: 31,500 and 28,000) chains and secreted IgM (Table 1 and Fig. 1). Neither of these cell lines synthesized γ chain.

Cell hybridization using three kinds of fusion partner cell lines: Spleen cells (1×10^7 cells) from chickens immunized with human IgG were hybridized with 2×10^6 cells of the fusion partner lines, R27H4, MuH1 and MuH4 and then plated in three 96-well plates. The number of wells with cell growth was much higher in R27H4 (100%) compared with MuH1 (67.4%) or MuH4 (18.8%) (Table 1). In the fusion experiment using R27H4, many of HAT-insensitive and smaller lymphoblastoid cells other than hybrid cells appeared after hybridization and disturbed the isolation of hybridoma producing specific antibody. In case of MuH1 or MuH4, the appearance of such cells was not observed,

Table 1. Biological property and fusion efficiency of parental cell lines

Cell line	Ig expression				Generation time (hr)	No. of wells with cell growth	
	Cytoplasmic Ig ^{a)}			Secretion ^{b)}		/288 wells	
	L	μ	γ			Exp. 1	Exp.2
R27H1	–	+	–	μ	17.29	ND ^{c)}	ND
R27H4	+	+	–	IgM	13.80	288	ND
MuH1	–	+	–	–	20.89	175	213
MuH4	+	+	–	IgM	15.72	59	50

a) Examined by indirect immunofluorescence test using either anti-chicken L, μ or γ chain, and fluorescein-conjugated second antibody.

b) Determined by Western blotting analysis.

c) Not done.

Fig. 1. Western blotting analysis of immunoglobulin chains from MuH1 and MuH4 cells. Supernatants from MuH1 (lane 1) and MuH4 (lane 2) were subjected to 10% SDS-PAGE followed by immunoblotting using mouse mAbs against chicken L (a), μ (b) and γ (c) chains, respectively.

due to the addition of ouabain to the HAT medium. Fusion efficiency was higher in MuH1 rather than in MuH4. Finally, in two fusion experiments, 29 hybridomas producing specific antibody to human IgG were selected. Of these, 18 were from the MuH1-derived hybridomas and 9 were from the MuH4. The remaining 2 were from the R27H4.

Quantification of antibody and immunoglobulin typing of their specific antibody: From 27 hybridomas producing anti-human IgG mAb obtained in fusion trials using HuM1 and HuM4, four MuH1-derived hybridomas and four MuH4-derived hybridomas were selected and subcloned for characterization of their antibodies. These hybridomas were, CHM1-HuIg1, 2, 3 and 4 (MuH1-derived), and CHM4-HuIg1, 2, 3 and 4 (MuH4-derived).

The amounts of immunoglobulin secreted by the two fusion partner lines MuH1 and MuH4 and their 8 hybridomas were measured by ELISA (Table 2). Both IgG and IgM were not detectable in the supernatant fluids of the MuH1 cells, but a low level of IgM was produced by the MuH4 cells. In 8 hybridomas, IgG production was higher in the MuH1-derived hybridomas (0.24 to 5.22 $\mu\text{g/ml}$) than the MuH4-derived hybridomas (0.45 to 0.75 $\mu\text{g/ml}$). Only small amounts of IgM were produced by the hybridomas (<3.2 ng/ml). The CHM1-HuIg3 did not produce IgM but produced IgG at a relatively high level (5.22 $\mu\text{g/ml}$).

In order to examine the specificity of mAb produced by these hybridomas, immunoglobulin reactive for human IgG

Table 2. Immunoglobulin concentrations in two parental cell lines and their hybridomas

Cells	IgG ($\mu\text{g/ml}$)	IgM (ng/ml)
MuH1	0.00	0.00
MuH4	0.00	0.26
CHM1-HuIg1	2.38	3.12
CHM1-HuIg2	3.96	3.18
CHM1-HuIg3	5.22	0.00
CHM1-HuIg4	0.24	0.24
CHM4-HuIg1	0.72	1.53
CHM4-HuIg2	0.45	1.98
CHM4-HuIg3	0.65	0.78
CHM4-HuIg4	0.75	2.00

Table 3. Immunoglobulin classification of antibody against human IgG produced by hybridomas

Hybridoma	ELISA value (OD 492 nm)		
	Anti-L	Anti- μ	Anti- γ
CHM1-HuIg1	0.680	-0.011	0.892
CHM1-HuIg2	0.712	-0.003	0.861
CHM1-HuIg3	0.703	-0.009	0.842
CHM1-HuIg4	0.544	-0.011	0.769
CHM4-HuIg1	0.502	-0.014	0.632
CHM4-HuIg2	0.354	-0.017	0.525
CHM4-HuIg3	0.672	-0.015	0.694
CHM4-HuIg4	0.397	-0.013	0.655

was typed by ELISA analysis using mouse mAbs specific for chicken Ig chains. The eight antibodies tested were reactive with mouse mAbs to chicken Ig chains L and γ but not with anti- μ mAb (Table 3), indicating that all specific mAbs were IgG type.

DISCUSSION

In a previous study, we established the chicken parental B cell lines, termed R27H1, R27H2, R27H3, and R27H4, for production of chicken mAb [13] and succeeded in hybridization between R27H4 cells and spleen cells from chickens immunized with defined antigens followed by hybridoma selection and production of several mAbs [1, 12, 13]. Since the above partner cell lines are characterized by deficiency in TK activity, they make it possible to select hybridomas from unfused cells by using HAT medium. In the process of hybridoma selection, however, HAT-insensitive lymphoblastoid cells smaller than hybridomas appeared frequently and grew rapidly. The viability of these cells was not as good as that of hybridomas in conditions of low cell density, but they inhibited the initial growth of hybridomas and decreased the chances of selection of desirable clones. The appearance of HAT-insensitive cells might be due to the genetic mis-matching between immune spleen cells and the fusion partner cells, since the partner lines were originally established from outbred chickens.

In the experiments preliminary to this current study,

R27H1 and R27H4 cells were highly sensitive to the addition of ouabain in their cultures (data not shown). Therefore, to avoid growth of HAT-insensitive cells, an ouabain-resistant character was introduced in two HAT-sensitive parental lines R27H1 and R27H4. We thus established two new parental cell lines, MuH1 and MuH4. By using these cell lines the appearance of unfavourable HAT-insensitive cells could be ruled out by addition of ouabain in HAT medium. Consequently, they enhance selection and isolation of hybridomas producing specific antibody.

The new cell lines MuH1 and MuH4 were hybridized with spleen cells from chickens immunized with human IgG. Fusion efficiencies with the new cell lines were lower than with R27H4, but stable hybridomas producing specific antibodies were obtained (Table 1). The MuH1 line gave higher fusion efficiency and developed more specific hybridomas than MuH4. In addition, the antibody production of MuH1-derived hybridomas was higher than that of the MuH4-derived hybridomas (Table 2). The specific mAb from the established hybridomas was of the IgG isotype.

Since the MuH4 itself secretes μ and L chains (IgM-type Ig), the MuH4-derived hybridomas are expected to secrete IgM preferentially. In fact, four MuH4-derived specific hybridomas produced both IgM and IgG. By contrast, the MuH1 line synthesized, but not secrete μ chain, and was expected not to produce IgM. Surprisingly, three hybridomas derived from this fusion partner (CHM1-HuIg1, 2 and 4) secreted both IgM and IgG, while the CHM1-HuIg3 line secreted only IgG (Table 2). Therefore, the IgM from the above three hybridomas derived from the MuH1 was predicted to the recombinant Ig arising from MuH1-derived μ chain and spleen-derived L chain. However, the establishment of the CHM1-HuIg3 which was productive for IgG but not IgM indicates that the MuH1 cell line is a more suitable chicken fusion partner line than the R27H4 and MuH4 lines. Further characterization of eight chicken mAbs specific for human IgG obtained in this study is in progress.

It is pointed out that what used to be called chicken IgG is not an IgG [17]. That is, the current majority usage terminology is IgY, with heavy chains ν , and this is used for the similar molecule occurring in reptiles and amphibia. In our previous reports with regard to chicken mAb [1, 12, 13], IgG as terminology of the chicken low molecular weight Ig was used. Therefore, here we used habitually IgG but not IgY.

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REFERENCES

1. Asaoka, H., Nishinaka, S., Wakamiya, N., Matsuda, H., and Murata, M. 1992. Two chicken monoclonal antibodies specific for heterophil Hanganutziu-Deicher antigens. *Immunol. Letters* 32: 91–96.
2. Fount, S. K. H., Perkins, S., Raubitschek, A., Larrick, J., Lizak, G., Fishuill, G., Engleman, E. G., and Grumet, F. C. 1984. Rescue of human monoclonal antibody production from EBV-transformed B cell line by fusion to a human-mouse hybridoma. *J. Immunol. Methods* 70: 83–90.
3. Goldsby, R. A., Osborne, B. A., Simpson, E., and Herzenberg, L. A. 1977. Hybrid cell lines with T-cell characteristics. *Nature (Lond.)* 267: 707–708.
4. Guss, B., Eliasson, M., Olsson, A., Uhlen, M., Frej, A. K., Jorvall, H., Flock, J. I., and Lindberg, M. 1986. Structure of the IgG-binding regions of streptococcal protein G. *EMBO J.* 5: 1567–1575.
5. Hodge, D. and Ambrosius, H. 1984. Evolution of low molecular weight immunoglobulin. IV. IgY-like immunoglobulins of birds, reptiles and amphibians, precursors of mammalian IgA. *Mol. Immunol.* 21: 699–707.
6. Haskard, D. O. and Archer, J. R. 1984. The production of human monoclonal autoantibodies from patients with rheumatoid arthritis by the EBV-hybridoma technique. *J. Immunol. Methods* 74: 361–367.
7. Jonsson, L., Larsson, A., and Thomander, L. 1987. Immune-complexes and complement components in Bill's palsy. *O.R.L.* 49: 294–301.
8. Kozbor, D. and Roder, J. C. 1984. *In vitro* stimulated lymphocytes as a source of human hybridomas. *Eur. J. Immunol.* 14: 23–27.
9. Kozbor, D., Trippuuti, P., Roder, J. C., and Croce, C. M. 1984. A human hybrid myeloma for production of monoclonal antibodies. *J. Immunol.* 133: 3001–3005.
10. Larsson, A. and Sjoquist, J. 1988. Chicken antibodies: a tool to avoid false positive results by rheumatoid factor in latex fixation tests. *J. Immunol. Methods* 108: 205–208.
11. Larsson, A., Jonsson, L., and Sjoquist, J. 1988. Determination of circulating immune-complexes by chicken anti-human C3 and anti-human C1q micro ELISA. *J. Immunol. Methods* 113: 93–99.
12. Lillehoj, H. S., Sasai, K., and Matsuda, H. 1994. Development and characterization of chicken-chicken B cell hybridomas secreting monoclonal antibodies that detect sporozoite and merozoite antigens of *Eimeria*. *Poultry Sci.* 74: 1685–1693.
13. Nishinaka, S., Suzuki, T., Matsuda, H., and Murata, M. 1991. A new cell line for the production of chicken monoclonal antibody by hybridoma technology. *J. Immunol. Methods* 139: 217–222.
14. Teng, N. N. H., Kaplan, H. S., Hebert, J. M., Moore, C., Douglas, H., Wunderlich, A., and Braude, A. I. 1985. Protection against Gramnegative bacteria and endotoxemia with human monoclonal IgM antibodies. *Proc. Natl. Acad. Sci. U.S.A.* 82: 1790–1794.
15. Teng, N. N. H., Lam, K. S., Riera, F. C., and Kaplan, H. S. 1983. Construction and resting of mouse-human heteromyelomas for human monoclonal antibody production. *Proc. Natl. Acad. Sci. U.S.A.* 80: 7308–7312.
16. Shulman, M., Wilde, C. D., and Kohler, G. 1978. A better cell line for making hybridomas secreting specific antibodies. *Nature (Lond.)* 276: 269–270.
17. Warr, G. W., Magor, K. E., and Higgins, D. A. 1995. IgY: clues to the origins of modern antibodies. *Immunol. Today* 16: 392–398.