

Asian Fisheries Science 9 (1996): 183-189.

Asian Fisheries Society, Manila, Philippines

<https://doi.org/10.33997/j.afs.1996.9.3.005>

Generation and Characterization of Monoclonal Antibodies Against Grass Carp (*Ctenopharyngodon idellus*) Growth Hormone

S.-L. CHEN, F. YANG¹, L. HE, X.-H. CHEN, W.-T. DENG and Y.-C. WANG¹

*State Key Laboratory of Fish Germplasm Resources and Biotechnology
Chang Jiang Fisheries Institute
Shashi 434000, China*

¹*Biotechnology Research Center of CAAS
Beijing, China*

Abstract

Monoclonal antibodies (McAbs) directed against grass carp (*Ctenopharyngodon idellus*) growth hormone (gcGH) were generated by the fusion of myeloma cells (SP2/0) with spleen cells from mice that had been immunized with purified gcGH. Hybridomas were cloned by limiting dilution, and screened for McAb production using enzyme-linked immunosorbent assay (ELISA). Seven positive clones were produced from 167 clones tested, giving a positive rate of 4.1%. Two hybridoma cell lines were developed and used to produce ascitic fluids. Two McAbs to gcGH, designated 1D2B9 and 3E8A5, had a titer of about 1:1280000 and reacted specifically with gcGH, and did not react with salmon GH, bovine GH and black silver carp GH in ELISA. Western blotting showed that gcGH McAbs bound specifically with recombinant common carp GH transferred on nitrocellulose membrane. Immunocytochemistry study showed that McAbs specifically bound to GH cells in proximal pars distalis, and did not bind to cells in rostral pars distalis and pars intermedia.

Introduction

The development of monoclonal antibody (McAb) techniques, first reported by Kohler and Milstein (1975), permits a new approach to the generation of specific antibodies and the establishment of hormone immunoassays. Compared with polyclonal antisera (PCA), McAbs have several attractive features including higher specificity and the theoretically unlimited supply of antibodies with known and constant characteristics. There are also less stringent requirements for the purity of the antigen used for immunization since screening procedures ensure that only the antibody of chosen specificity is identified. However, in the generation of PCA, specificity is dependent upon the purity of the antigen preparation used for immunization. Even very pure hormone preparations give rise to a range of antibodies which recognize many antigenic sites with varying affinity. Fish growth hormone (GH) is a protein hormone synthesized and released by the anterior pituitary gland of teleostean species, its

major function is the promotion and regulation of growth and development in fish (Donaldson et al. 1979; Chen 1992). The development of McAbs with high specificity and titre against fish GH will be of great importance, and have potential application in the development of highly sensitive and specific immunoassays for fish GH, and in the purification of the corresponding GH from pituitary glands or recombinant expression products in *Escherichia coli*. So far, few reports on the development of McAbs against GH are available, mostly in salmonids (Furuya et al. 1988; Farbridge et al. 1990).

This paper reports the development of highly specific monoclonal antibodies against grass carp (*Ctenopharyngodon idellus*) GH and their immunohistochemical identification.

Materials and Methods

Hormones and Antiserum

Grass carp GH (gcGH) and prolactin (gcPRL) were isolated from pituitary glands of grass carp by Chen et al. (1995, 1996). Chinook salmon GH (sGH) and gonadotropin (sGtH) were also purified by Chen et al. (1991) and Chen (1992). Black silver carp gonadotropin (bscGtH) and its antiserum were kindly provided by Dr. Y.L. Yu of the Institute of Zoology, Taiwan. The expression products of recombinant common carp GH gene (rcGH) were kindly donated by Professor Y.K. Sun of Shanghai Biotechnology Center, Shanghai, China.

Immunization

Two 6-week old female BALB/c mice were immunized by four injections of gcGH. The first subcutaneous injection consisted of 200 μ l of a suspension of 100 μ g gcGH in 100 μ l saline plus 100 μ l Freund's complete adjuvant (Sigma). Two subsequent intraperitoneal (IP) injections were given with 200 μ l of a suspension of 100 μ g gcGH in 100 μ l saline plus 100 μ l Freund's incomplete adjuvant (Sigma) at 2-week intervals. One week later, an intravenous injection 100 μ g gcGH in 50 μ l distilled water was given.

Development of McAbs

Three days after the intravenous injection, splenocytes were mixed in a ratio of 5:1 with log-phase SP2/0 myeloma cells. The fusion was mediated by 45% (v/v) polyethylene glycol MW 1540. Hybridomas were selected in a medium containing 100 μ M hypoxanthine, 0.4 μ M aminopterin and 16 μ M thymidine (HAT medium, Sigma) and approximately 5×10^5 thymocytes \cdot ml⁻¹. Fused cells were distributed into 96-well culture plates in HAT medium. Two weeks later, the cells were incubated in HT-DMEM medium for one week, then incubated in DMEM medium. Hybridomas were cloned by the method known as limiting dilution. Ascites fluid was prepared by IP injection of hybridoma cells into the pristane-primed male BALB/c mouse. The isotypes of McAbs were identified by double diffusion in agarose gel using rabbit antisera to mouse immunoglobulin isotypes (IgM, IgG1, IgG2a, IgG2b and IgG3) (Sigma).

Direct ELISA for Binding Assay

The generation and specificity of McAbs were examined by direct enzyme-linked immunosorbent assay (ELISA). The plates were coated with 100 μ l of various hormones in coating buffer overnight at 4°C, washed twice, incubated with 100 μ l of 0.5% bovine serum albumin for 1 h at 37°C, washed twice, incubated with 100 μ l culture supernatants of hybridomas (or ascites fluid or purified McAbs) for 2 h at 37°C, washed thrice, incubated with 100 μ l peroxidase-labelled rabbit anti-mouse immunoglobulin (Beijing Biological Products Institute, China) for 1 h at 37°C, washed four times, and incubated with 100 μ l orthophenylene-diamine (OPD) (Sigma) solution for 20 minutes at room temperature. The reaction was stopped with 2 Mol·l⁻¹ H₂SO₄, and the absorbance read at 490 nm.

SDS-PAGE and Western Blotting

Immunoaffinity-purified rcGH was subjected to sodium dodecyl sulfate-polyacryl amide gel electrophoresis (SDS-PAGE) at pH 8.6 using 12.5% homogeneous gels. Proteins were either stained in Commassie blue R250 solution or were blotted by the classical electroblotting procedure of Towbin et al. (1979) for 45 minutes at room temperature on nitrocellulose membranes. The membranes were immunostained with gcGH McAbs as primary antibody, using HRP-labelled sheep anti-mouse IgG as second antibody and 4-chloro-1-naphthol as substrate.

Immunohistochemistry

Grass carp pituitary glands were fixed in freshly prepared Bouin-Hollande sublimate, then embedded in paraffin and sectioned by routine procedures. Immunohistochemical staining of GH-secreting cells was carried out by peroxidase-antiperoxidase (PAP) (Sino-American) method. After being deparaffinized and returned to water, the sections were first incubated with gcGH McAbs or rabbit anti-sGH serum; then incubated with goat anti-mouse (or anti-rabbit) IgG and mouse (or rabbit) PAP successively. Finally, diamine-benzidine (DAB) (Sino-American) was used for color development.

Results

Development of McAbs

Two BALB/c mice were immunized with purified gcGH, and two cell fusions were made. Fused cells were distributed into three 96-well culture plates. The percentage of fused hybrid cells was about 90%. Seven positive clones were obtained from 167 clones tested by direct ELISA, of which two strongly positive clones reacted only with gcGH and rcGH (Table 1). After three limiting dilutions, cells from the seven hybridomas were injected IP into mice to generate ascites fluid. Finally, two stable McAb lines, designated 1D2B9 and 3E8A5, were obtained. The isotypes of the both McAbs, 1D2B9 and 3E8A5, were identified to be IgG2b by the double diffusion method.

Table 1. Cross-reaction of several monoclonal antibodies with various pituitary hormone preparation.

Hormone preparation ¹	Monoclonal antibodies						
	1C1A4	1A2E6	1D2B9	2G10F3	2E5H8	3B11D7	3E8A5
gcGH	+	+	++	+	+	+	++
rcGH	-	-	+	-	-	+	+
sGH	-	-	-	-	-	-	-
sGtH	-	-	-	-	-	-	-
bGH	-	-	-	-	-	-	-

+ positive, ++ strongly positive, - negative

¹gcGH = grass carp growth hormone GH; rcGH = recombinant common carp GH; sGH = chinook salmon GH; sGtH = chinook salmon gonadotropin; bGH = bovine GH.

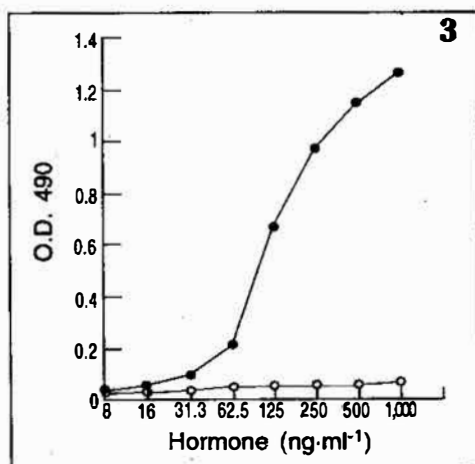
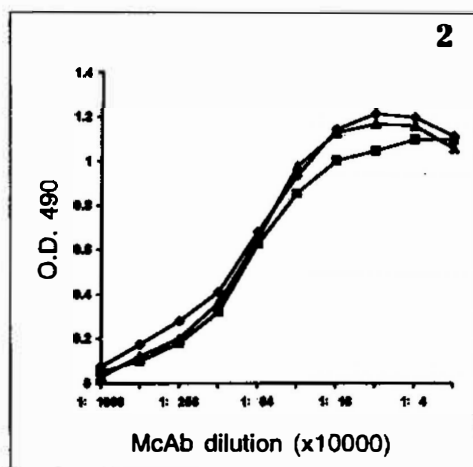
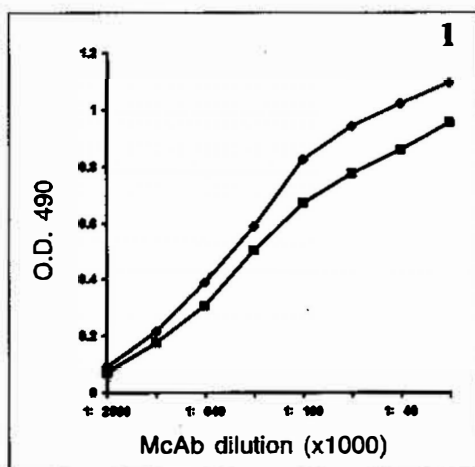


Fig. 1. McAb titration curve. Plates are coated with 0.5 μg gcGH $\cdot\text{ml}^{-1}$.

(\blacklozenge) 3E8A5 (\blacksquare) 1D2B9

Fig. 2. Complementary effect of McAb 1D2B9 and 3E8A5. Plates are coated with 0.5 μg gcGH $\cdot\text{ml}^{-1}$.

(\blacksquare) 1D2B9 (\blacklozenge) 3E8A5 (\blacktriangle) 1D2B9+3E8A5

Fig. 3. Specificity of the gcGH monoclonal antibody in direct ELISA. The wells of plates were coated with gcGH ($\bullet\bullet$), sGH, sGtH, bGH or bscGtH ($\circ\circ$). Ascites fluid of gcGH McAbs was used for measurement at 10⁻⁴ dilution.

Titers and Binding Specificities of McAbs

Direct ELISA showed that two McAbs, 1D2B9 and 3E8A5, had a titer of 1:1280000 (Fig. 1). There was no complementary effect between McAb 1D2B9 and 3E8A5 (Fig. 2). The specificities of McAbs were also examined by direct ELISA using gcGH, sGH, sGTH, bGH and bscGTH as antigens. The McAbs reacted only with gcGH, but did not react with the antigens of other hormones (Fig. 3).

SDS-PAGE and Western Blotting

The specificities of McAbs were confirmed by SDS-PAGE and western blotting. As shown in Fig. 4, partially purified rcGH migrated as a single band (Fig. 4A) with an MW of 22000 and strongly reacted with McAbs against gcGH (Fig. 4B).

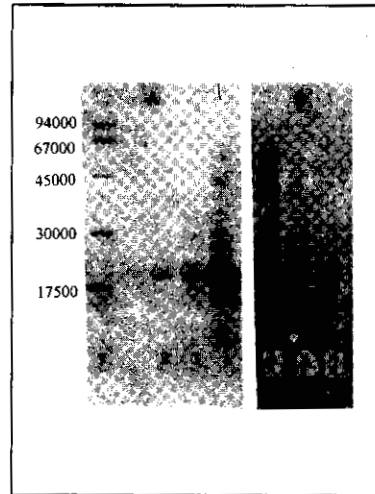


Fig. 4. SDS-PAGE pattern of (A) recombinant common carp GH (rcGH) and Western blotting pattern (B) developed with McAbs against grass carp GH. 1. Molecular weight marker; 2. and 3. Purified rcGH; 4. Nonpurified *E. coli* expression of rcGH gene.

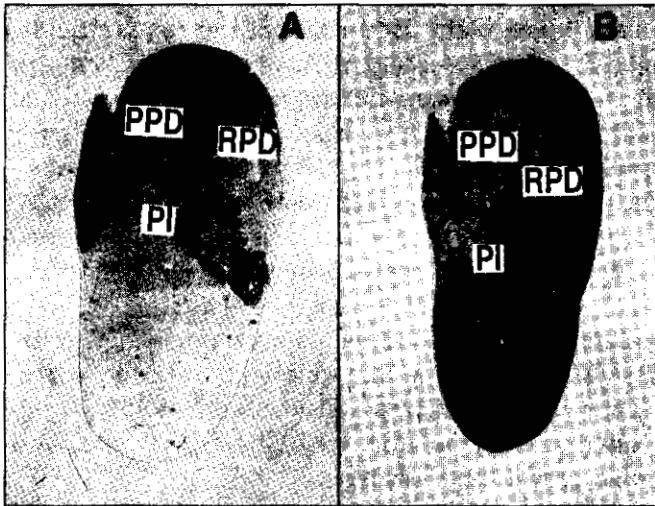


Fig. 5. Immunohistochemical staining of GH-secreting cells in grass carp pituitary. A. Staining with gcGH monoclonal antibodies at a concentration of 1/1000. B. Staining with rabbit anti-sGH serum at a concentration of 1/1000. RPD = rostral pars distalis; PPD = proximal pars distalis; PI = pars intermedia.

Immunohistochemistry

Immunohistochemical staining of pituitary gland sections from grass carp revealed that the McAbs specifically bound to GH-secreting cells in the proximal pars distalis (PPD), and did not bind to cells in the rostral pars distalis (RPD) and pars intermedia (PI) (Fig. 5A). A similar staining pattern in the PPD was observed in sections stained with rabbit anti-sGH serum (Fig. 5B).

Discussion

There is increasing interest in the development of McAbs against fish hormones in view of their high specificity, smaller amount of antigen needed for immunization, and great potential for application in the fields of fish endocrinology and genetic engineering of fish peptide hormones. Many fish endocrinologists tried to develop McAbs against teleost GH but, so far, only Farbridge et al. (1990) have successfully generated GH McAb using recombinant SGH as immunogen. In the present study, two hybridoma cell lines secreting specific McAbs against purified gcGH were developed. Direct ELISA showed that the gcGH McAbs had high specificity and titer. This is the first report on the development of McAbs against cyprinid GH.

The most tedious, yet most important, step in McAb generation is to determine whether or not a hybridoma is secreting a McAb to the relevant hormone. The screening procedure must be rapid, accurate and simple because hundreds of hybridomas must be tested during the development of McAbs. In this paper, to screen positive hybridomas, we first used direct ELISA which is rapid and capable of testing many hybridomas at the same time. Because the gcGH preparation used in direct ELISA is the same as that tested for the initial immunization, and is not absolutely pure, there is a low incidence of false positives during the screening of hybridomas. It is necessary to use different tests for determining the specificity of positive hybridomas. Immunohistochemistry and western blotting techniques were used to meet the requirements. Immunohistochemical staining revealed that gcGH McAbs bound specifically to GH-secreting cells in PPD of grass carp pituitary, but did not stain GtH-secreting cells in PPD and cells in both RPD and PI. These results are similar to those observed in other teleosts species (Cook et al. 1983; Moons et al. 1991). Western blotting showed that gcGH McAbs had strong binding with purified rcGH. Such stringent tests could completely rule out the possibility of false positives, and fully confirmed that the McAb obtained here was truly directed against the gcGH molecule.

McAbs are powerful reagents for use in studying pituitary physiology in the development of immunoassays and in the affinity purification of the corresponding peptide. Using McAbs against gcGH, a sensitive and specific sandwich ELISA has been developed in our laboratory (published elsewhere) and applied in research on cyprinid growth endocrinology.

Acknowledgment

This research was supported by the National Natural Sciences Foundation of China.

References

- Chen, S.L. 1992. Fish growth endocrinology and aquaculture. *Journal of Fisheries of China* 16: 91-100.
- Chen, S.L., B. Breton and P.Y. LeBail. 1991. Separation and purification of pituitary thyrotropin and gonadotropin of chinook salmon (*Oncorhynchus tshawytscha*). *Acta Zoologica Sinica* 37: 263-270.
- Chen, S.L., W.T. Deng, X.T. Liu, X.H. Chen and H. Long. 1995. Isolation and purification of growth hormone from grass carp, *Ctenopharyngodon idellus*, and preparation of antibody against the gcGH. *Acta Zoologica Sinica* 41: 40-50.
- Chen, S.L., W.T. Deng, X.H. Chen and S.Q. Xia. 1996. Purification and identification of prolactin from grass carp (*Ctenopharyngodon idellus*). *Chinese Biochemical Journal* 12: 109-111.
- Cook, H., A.F. Cook, R.E. Peter and S. Walker-Wilson. 1983. Ultrastructural immunocytochemistry of growth hormone cells in the goldfish pituitary gland. *General and Comparative Endocrinology* 50: 348-353.
- Donaldson, E.M., U.H.M. Fagerlund, D.A. Higgs and J.R. McBride. 1979. Hormonal enhancement of growth. In: *Fish physiology*, Vol. 8. Bioenergetics and growth (eds. W.S. Hoar, D.J. Randall and J.R. Brett), pp. 455-597. Academic Press, New York.
- Furuya, A., M. Ohtomo, T. Inada and H. Yoshida. 1988. Generation and application of monoclonal antibodies against somatotropin and prolactin. *Agricultural and Biological Chemistry* 51: 2331-2335.
- Farbridge, K.J., G. McDonald-Jones, C.L. Mclean, P.J. Lowry, R.J. Etches and J.F. Leatherland. 1990. The development of monoclonal antibodies against salmon pituitary hormones and their immunohistochemical identification. *General and Comparative Endocrinology* 79: 361-371.
- Kohler, G. and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of pre-defined specificity. *Nature* 256: 495-497.
- Moons, L., L.R. Berghman and F. Vandesande. 1991. Immunoaffinity purification and partial characterization of sea bass (*Dicentrarchus labrax*) growth hormone. *General and Comparative Endocrinology* 83: 265-275.
- Towbin, H., T. Staehelin and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylic gels to nitrocellulose sheets: procedures and some applications. *Proceedings of the National Academy of Sciences of the United States of America* 76: 4350-4356.