Tumor Res. 18 (**Special Issue**), S 75-S 86 (1983)

Production of Monoclonal Anti-Basic Fetoprotein (BFP) and Usefulness of Monoclonal Anti-BFP for Immunodiagnosis of Human Cancer

M. ISHII[1], K. ARAI[1], Y. TAKEMORI[1], S. AKAZAWA[1], Y. KANDA[1], Y. KOMATSU[2], N. HATTORI[2], M. TANIGUCHI[3], Y. SHIMOZURU[4] and T. MORIMOTO[4]

[1] *Saitama Cancer Center, Saitama, 362, Japan*
[2] *Department of Internal Medicine, Kanazawa University, Kanazawa, 920, Japan*
[3] *Department of Immunology, Research Institute, Chiba University, Chiba, 280, Japan*
[4] *Tsukuba Research Institute, Eizai Co., 300-26, Ibaragi, Japan*

SUMMARY

Basic fetoprotein (BFP) is an oncofetal protein that is useful for immunodiagnosis of various types of human cancer. In order to enhance specificity of BFP for cancer, monoclonal anti-BFP was produced and evaluated by enzyme-linked immunoassay using monoclonal antibody for immunodiagnosis of human cancer. Monoclonal anti-BFP was obtained from hybridomas prepared by fusion of mice myeloma cell with spleen cell from mice immunized with BFP. Reactivity of monoclonal anti-BFP against BFP in sera of cancer patients, hepatitis patients and normal subjects were examined in comparison with reactivity of conventional anti-BFP against BFP in the same sera. A kind of monoclonal anti-BFP revealed lower reactivity against serum BFP in 5 of 6 normal subjects and 3 of 4 hepatitis patients, compared with reactivity of conventional antibody. However, the monoclonal antibody showed the same reactivity against serum BFP in 3 of 5 cancer patients and a hepatitis patient on convalescent stage as conventional antibody did. This result indicated that monoclonal anti-BFP is more useful than conventional anti-BFP for immunodiagnosis of human cancer.

Key words: Basic fetoprotein (BFP), Monoclonal anti-BFP, Enzyme-linked immunoassay (EIA), Oncofetal protein, Hybridomas

INTRODUCTION

Basic fetoprotein is an oncofetal protein which in 1974 was found in human

fetal serum and fetal gut and brain extracts as well as in various types of cancer tissue extracts (Ishii, 1978). In 1976, isolation of basic fetoprotein (BFP) from ascitic fluid of a patient with primary hepatocellular carcinoma was performed, and physicochemical and immunochemical characterization of BFP was analyzed (Ishii, 1978, Ishii, 1979a). BFP had a γ-mobility by electrophoresis. The molecular weight and isoelectric point of BFP were 73,000 and 9.3, respectively. From its basic property that is different from other oncofetal proteins, name of BFP was born. Aminoacid component of BFP was constituted of 606 mol aminoacids per 1 mol BFP. BFP contained rich arginine of basic aminoacid and poor glutamine of acidic aminoacid compared with those of α-fetoprotein (Nishi, 1972).

On the other hand radioimmunoassay of BFP was based on a coprecipitation inhibition technique using double antibody method (Ishii, 1978; Ishii, 1979a, b). Test for serum BFP from normal subjects and patients with non-cancerous disease and with cancer was performed to evaluate BFP for immunodiagnosis of human cancer by the radioimmunoassay. Of 105 normal subjects, 99% had serum BFP level under 100 ng/m*l*. From the result, cut off value of serum BFP was assigned as 100 ng/m*l*. Of 240 patients with non-cancerous disease, 27% was positive for serum BFP. On the other hand, 48% of 315 patients with cancer was positive for serum BFP. Positive cases in non-cancerous disease were occupied mostly by patients with liver injury (Ishii, 1979a). Imcompleteness in discriminating cancer from non-cancer, especially liver injury by oncofetal protein such as AFP (Ishii, 1973) and CEA (Moor *et al.*, 1972) was found in BFP too and decreased diagnostic value of BFP for human cancer.

Recently, we have demonstrated the presence of immune complex of BFP in serum of patients with cancer, but not in serum of normal subjects. The demonstration suggested that BFP produced by cancer cell differs in antigenic determinant from BFP produced by normal cell.

On consideration of diagnostic value of BFP for human cancer and on suggestion from result of immune complex of BFP, the present study of antigenic determinant of BFP was progressed by using monoclonal anti-BFP prepared by hybridoma.

MATERIALS AND METHODS

Purification of BFP

Primary hepatocellular carcinoma diagnosed by histological examination from a 45 years old man was used as original material for the purification. The mince of carcinoma tissue was homogenated in 0.15 M borate buffer at pH 8.0 in a VirTis '45' homogenizer at 45,000 rpm for 10 min in an ice bath. The homogenate was centrifuged at 5,000 rpm for 30 min at 4℃. The resultant supernatant in a cellulose tube was concentrated by 30% polyethylene glycol solution at 4℃ and adjusted to a

protein content of 70 mg/m*l*. This extract was stored at −30℃ until processed. Isolation of BFP was carried out as below.

Initial separation of the components of hepatoma tissue extract was performed by ion exchange column chromatography using QAE-Sephadex A-50 equilibrated with 0.15 M borate buffer at pH 8.0. BFP was passed the column in the same buffer. Further separation of BFP-rich pooled fractions was followed by gel filtration of Sephadex G-150 column chromatography. The 3rd step of the purification was proceeded by positive affinity chromatography using Sepharose 4B coupled with conventional anti-BFP prepared by immunizing antigen to rabbit. Furthermore, purified BFP fraction yielded by positive affinity chromatography was treated with passing through negative affinity chromatography using Sepharose 4B coupled with antibody against normal liver tissue extract excluded BFP and antibody against rabbit serum. The final step was performed by Sephadex G-150 column chromatography.

Production of Monoclonal Anti-BFP

The procedure for production of monoclonal anti-BFP is as follows. Animals and myeloma cell lines as below were employed for preparation of hybridomas secreting monoclonal anti-BFP. Eight week-old BALB/c mice for immunization of BFP, 4 week-old BALB/c mice as source of feeder cells and myeloma cell line of BALB/c origin, P3/X63Ag8U1 (P3U1) cell lines (Köhler and Milstein, 1975) that are resistant to 8-azaguanine were used.

Immunization of BALA/c mice was performed by injecting with 50 μg of purified BFP i. p., followed by doing with 10 μg of the antigen i. p. on days 14 and 21 and 50 μg of the antigen i. v. on days 28. On days 32, the spleen from a single mouse was minced with a fine scissors. The spleen cells suspended aseptically in a RPMI 1640 medium were centrifugated for 5 min at 1,000 rpm. The cell pellet was gently resuspended in the same medium. With the same treatment 3 times, single cell suspension was obtained for fusion with myeloma cell lines.

Lymphocyte hybridization technique employed was essentially the same as this described by Galfre *et al.* (1977). 10^7 spleen cells were mixed 10^6 P3U1 myeloma cells and the mixture was centrifugated at 1,400 rpm for 10 min at room temperature. The cells suspended in residual medium after discard of supernatant were incubated at 40℃ for 15 sec and then mixed gently with 1 m*l* of 50% polyethylene glycol 1540 in RPMI 1640 medium, followed by incubation at 37℃ for 1 min. Furthermore, 7 m*l* of RPMI 1640 medium was added slowly to the mixture during 3 min. The cell suspension was centrifugated at 1,400 rpm for 5 min at room temperature and the supernatant was discarded. The cell pellet was resuspended in 20 m*l* of RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 2 mM L-glutamine. The cell suspension was dispensed into 96 wells of microtiter

tissue-culture plate at approximately 10^5 tumor cells/well. After 24hrs. the medium was replaced with HAT medium (RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 μM hypoxanthine, 1 μM aminopterin and 16 μM thymidine). The HAT medium was removed and replaced with fresh HAT medium on days 2, 3, 5 and 11. On days 14, half of HAT medium in each wells was removed and then HT medium (RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 μM hypoxanthine and 16 μM thymidine) was added. All the cells surviving after HAT selection were transferred in 24 wells of microplate with 10^5 feeder cells prepared from mice thymocytes and allowed to grow after 2 weeks with change of medium 3 times.

Hybridomas were cultured at the single-cell level by limiting dilution in microtiter tissue-culture plates with different feeder cells obtained from 3-day culture of mouse thymocytes. After 2 weeks, clones became evident macroscopically and spent medium from clones was collected to use for analysis of monoclonal anti-BFP.

Solid-Phase Radioimmunoassay

Binding ability and specificity of monoclonal anti-BFP against BFP were examined by solid-phase radioimmunoassay. The solid-phase radioimmunoassay was developed with use of antibody-coated wells of disposable plastic microplate by a modification of the method of Catt and Tregear (1967). The radioimmunoassay was based on competitive binding of BFP in sample and ^{125}I-BFP to anti-BFP. The wells of microplate binding monoclonal anti-BFP were produced as below. Anti-mouse immunoglobulin was insolublized by binding to wells of microplates and then monoclonal anti-BFP was immunologically bound to the insoluble anti-mouse immunoglobulin. By using wells of microplate treated with anti-BFP, the radioimmunoassay was carried out. Mixture of 30 μl of ^{125}I-BFP (approximately radioactivity of 100,000 cpm) and 30 μl of sample were dispensed into wells bound with monoclonal anti-BFP and incubated at room temperature for 1h. After aspiration of reaction mixture, wells were washed with 0.9% saline solution and the radioactivity of ^{125}I-BFP bound to well was measured by autogamma scintillation counter.

Enzyme-Linked Immunoassay

Enzyme-linked immunoassay (EIA) using monoclonal anti-BFP produced by hybridomas and conventional anti-BFP produced by immunizing purified BFP to rabbit was used for determination of BFP in human serum. EIA was performed by a sandwich method which put antigen between insolublized antibody and enzyme-linked antibody (Engvall and Perlmann, 1971). Disporsable plastic microtiter plate was employed for the EIA. Two EIA systems were developed. One was direct method using microplate-wells which were coated with conventional anti

-BFP prepared by immunizing purified BFP to rabbit. The other was indirect method using microplate-wells which were coated with anti-mouse immunoglobulin and moreover coated indirectly with monoclonal anti-BFP bound immunologically to well-coated anti-mouse immunoglobulin. Preparation of enzyme-linked antibody was performed by conjugating conventional anti-BFP or monoclonal anti -BFP with alkaline phosphatase isolated from bovine intestine in glutaraldehyde. Procedure of EIA was as follow. One hundred microliters of a 1 : 11 dilution of test serum or standard BFP were pipetted into each wells coated with anti-BFP and incubated at 37℃ for 1 h. After aspiration of content in wells, the wells were washed with 0.9% saline solution 3 times and 100 μl of enzyme-linked anti-BFP were dispensed into each wells, followed by incubation at 37℃ for 1h. After removal of well content and wash of well with 0.9% saline solution 3 times, 100 μl of enzyme substrate, p-nitrophenyl phosphate was pipetted into wells and then incubated at 37℃ for 1h. Thereafter, reaction of enzyme activity was stopped by addition of 100 μl of 1N NaOH in wells and dose of p-nitrophenol produced by which enzyme catalyzed substrate was measured as absorbance at 405 nm by spectrophotometer. Standard BFP was prepared using purified BFP.

RESULTS

Preparation of Hybridoma Secreting Anti-BFP

After fusion of each spleen cells from 5 mice immunized with BFP with myeloma cells P3U1 was performed, the immune spleen cells hybridized were cultured in HAT selection wells of 96 microtiter tissue-culture plates. On 14 days after initiation of hybridization, approximately one fourth of wells developed colonies macroscopically. When spent culture media in all wells were tested for anti-BFP activity binding to ^{125}I-BFP by a solid-phase radioimmunoassay, 73 of 806 (9.1%) wells were found to contain the antibody activity. Figure 1 illustrates the results from one successful culture plate. Of 96 spent media, 27 (28%) contained anti-BFP antibodies as determined in a solid-phase radioimmunoassay. ^{125}I-BFP binding greater than radioactivity of 1,000 cpm was taken as an indication of antibody production. The negative control was HAT medium exhibiting radioactivity under 500 cpm. The antibody which had high activity exhibiting ^{125}I-BFP bound rate greater than 50% was found in two spent media of this plate.

Cloning of Hybridoma

Sixteen of original hybrid cell lines secreting anti-BFP antibodies were selected for cloning in consideration of antibody activity, grade of growth and distinction of different hybridization. The hybrid cells were cloned successfully by limiting dilution. Of 311 cloned hybrids, 78 (25.1%) had ability to secrete anti-BFP and of these 63 could be maintained growth at single cell level of

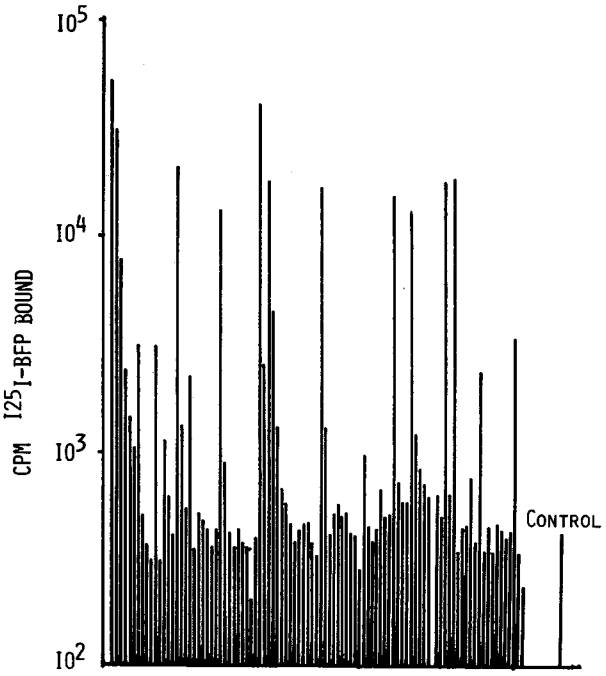

Fig. 1 Production of anti-BFP antibody by hybrid cells resulting from the fusion of immunized splenocytes of BALB/c mouse with P3U1 myeloma cells. The results from one successful culture plate showed that 27 (28%) out of 96 spent media from hybrid cell culture contained anti-BFP antibodies as determined in a solid-phase radioimmunoassay using ^{125}I labeled BFP. The ^{125}I labeled BFP binding greater than radioactivity of 1,000 cpm was taken as an indication of antibody production. The negative control was HAT medium exhibiting radioactivity under 500 cpm.

hybridomas keeping anti-BFP secretion.

Isotyping of Anti-BFP

Isotype analysis of anti-BFP antibody was performed on the 48 cloned cell lines which produced anti-BFP with high antibody activity. In this experiment, solid-phase radioimmunoassay based on a sandwich method which put monoclonal anti-BFP between insolublized anti-mouse immunoglobulin and ^{125}I labeled antibodies against mouse IgGl, IgG2a, IgG3 or IgM using wells of disposable plastic microplate was used. Most of the anti-BFP resulted in isotype of mouse IgGl or IgG2a.

Specificity of Anti-BFP

Specificity of monoclonal anti-BFP was examined by competitive binding inhibition technique using a solid-phase radioimmunoassay. The same highly purified BFP as used for immunizing mouse splenocytes was employed as standard BFP. The BFP-free fraction eluted through positive BFP-affinity column in process of BFP isolation was concentrated to a protein content of 65 mg/m*l* as determined according to the method of Lowry *et al.* (1951) and the concentrated fraction was used as BFP-negative control. The HT medium was control of anti-BFP. Figure 2 illustrates several results of test for specificity of anti-BFP as examples. The findings that binding of ^{125}I-BFP to antibody was inhibited by standard BFP level greater than 0.01 μg of BFP/m*l* such as the results of examples in Figure 3 were observed in all of spent media from the cloned hybrids culture. On

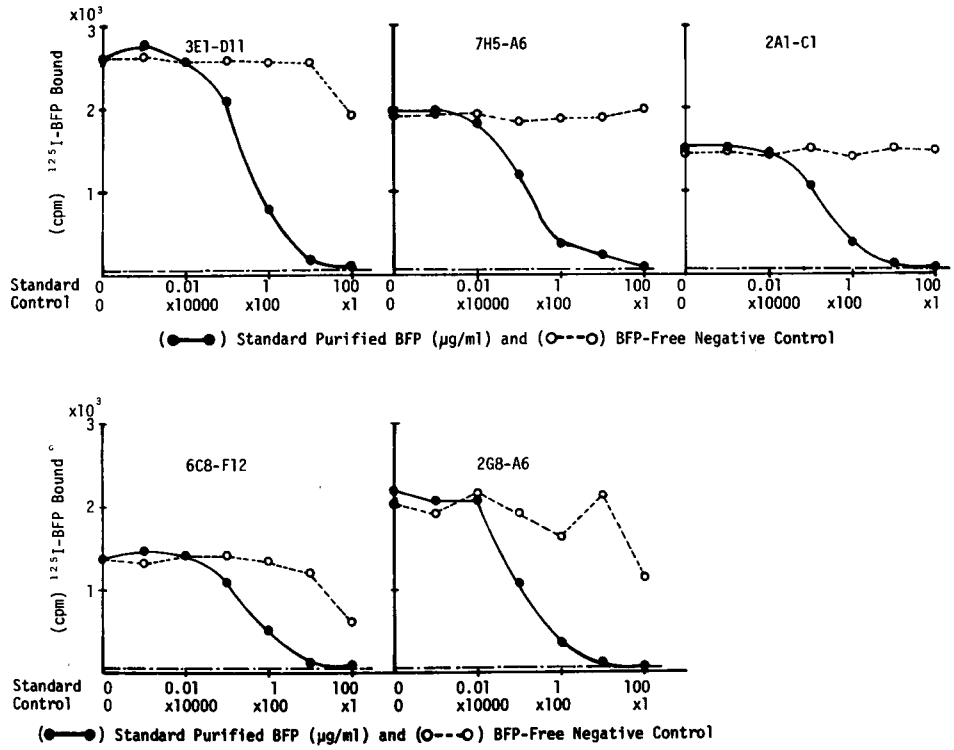

Fig. 2 Results of specificity of monoclonal anti-BFP secreting from the cloned hybrid cells by solid-phase radioimmunoassay based on a competitive binding inhibition technique. The binding of ^{125}I labeled BFP to antibody was inhibited in all of spent media from 5 cloned hybrids culture. Effect of inhibition by non-diluted BFP-free negative control on the binding of ^{125}I labeled BFP to antibody was observed in antibody from 3E1-D11, 6C8-F12 and 2G8-A6 cloned hybrid cells, but did not in antibody from 7H5-A6 and 2A1 -C1 cloned hybrid cells.

the other hand, effect of inhibition by non-diluted BFP-negative control on binding of ^{125}I-BFP to antibody as resulted in antibody from 3El-D11, 6C8-F12 and 2G8-A6 cloned hybrids in Figure 2 was found in other several antibodies. However, most of monoclonal antibody was not inhibited by BFP-negative control such as antibody from 7H5-A6 and 2A1-C1 in Figure 2.

Enzyme-linked Immunoassay Using Monoclonal Anti-BFP

Development of enzyme-linked immunoassay by sandwich method using wells of disposable plastic plate was performed by using monoclonal antibody from the cloned hybrid cells. For the EIA of direct method, conventional anti-BFP raised by immunizing BFP to rabbit as insolublized antibody and monoclonal antibody from hybrids of single clone as enzyme-liked antibody were employed. For the EIA of indirect method, the conventional anti-BFP as enzyme-linked antibody and the

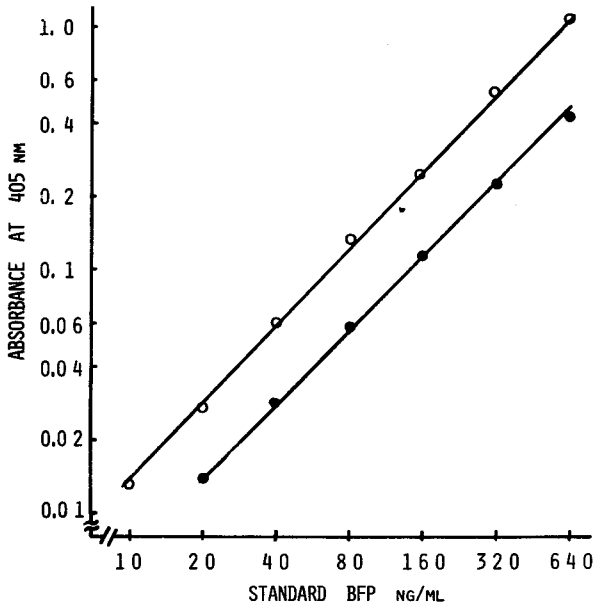

Fig. 3 Standard BFP curves obtained by an enzyme-linked immunoassay (EIA) of indirect sandwich method (○—○) employing monoclonal anti-BFP bound to anti-mouse γ-globulin and enzyme-linked conventional anti-BFP and by an EIA of direct sandwich method (●—●) employing insolublized conventional anti-BFP and enzyme-linked monoclonal anti-BFP. Two different standard curves showed close parallel. The sensitivity of EIA of indirect and direct sandwich method could allow reproducible detection of 10 ng and 20 ng of BFP/m*l*, respectively.

monoclonal anti-BFP as antibody binding to insolublized anti-mouse immunoglobulin were used. As shown in Figure 3, both EIAs of direct and indirect methods were accomplished successfully and the sensitivity could allow reproducible detection of 20 ng of BFP/m*l* of serum. The EIA of sandwich method using only a monoclonal anti-BFP produced by identical single cloned hybrids failed of success. However, the EIA using different kind of monoclonal anti-BFP produced by different single cloned bybrids could be developed.

Clinical Study of monoclonal Anti-BFP

Immunoreactivity of monoclonal anti-BFP against BFP in sera of nomral subjects, patients with hepatitis and patients with cancer (stomach cancer and primary liver cell cancer) was investigated by EIA of indirect sandwich method, compared with immunoreactivity of conventional anti-BFP against BFP in the same sera by EIA of direct sandwich method. In this investigation, BFP in human sera was determined by 44 different EIAs using each anti-BFP antibodies produced by 44 different single cloned hybrids. The standard BFP was same in EIAs using monoclonal and conventional anti-BFP and the same purified BFP as used for immunizing splenocytes. The monoclonal antibody produced by a cloned hybridoma, 6C8-F1-7A5 was found to reveal lower reactivity against serum BFP in 5 of 6 normal subjects and 3 of 4 patients with hepatitis, but same reactivity against BFP in a patients with stomach cancer and 2 of 4 patients with primary liver cell cancer in comparison with the reactivity of conventional anti-BFP against the same serum BFP. The detailed results are shown in Table 1. Serum of a hepatitis patient, which showed no difference of reactivity between the monoclonal antibody and the conventional antibody was taken on convalescent stage of hepatitis. Hence, blood was collected twice from a patient with acute hepatitis, case No. 16 as shown in Table 2 at two points on aggravating stage and convalescent stage. Serum α-fetoprotein level was 5 ng/m*l* on aggravating stage and rose up to 228 ng/m*l* on convalescent stage. Serum BFP on aggravating stage was estimated as 4,600 ng/m*l* by EIA using conventional anti-BFP, but as low level of 1,140 ng/m*l* by EIA using monoclonal anti-BFP. However, serum BFP level on convalescent stage did not show significant difference between serum BFP value as determined by different EIAs using conventional antibody and monoclonal antibody.

DISCUSSION

In the present study, we describe the production of monoclonal antibody which reacts with BFP and the initial clinical evaluation of the monoclonal antibody for immunodiagnosis of human cancer. The monoclonal anti-BFP could be produced using the cell fusion techniques described by Köhler and Milstein (1975). The highly purified BFP was employed for immunization of BALB/c mouse. The

Table 1. *Comparison of Serum BFP Value as Determined by EIA of Direct Sandwich Method Employing Conventional Anti-BFP and by EIA of Indirect Sandwich Method Employing Monoclonal Anti-BFP Bound to Anti-Mouse γ-Globulin*

Diagnosis	Cases	Serum BFP with Conventional Anti-BFP	Serun BFP with Monoclonal Anti-BFP	Mo. Anti-BFP/Co. Anti-BFP
Normal Subjects	1 N-1	73	12.6	0.17
	2 N-2	86	51	0.59
	3 N-3	91	59	0.65
	4 N-4	68	45	0.66
	5 N-5	63	52	0.83
	6 N-6	70	49	0.70
Stomach Cancer	7 K. T	110	128	1.16
Hepatoma	8 I. M	410	390	0.95
	9 O. T	82	51	0.62
	10 O. S	196	160	0.82
	11 T. K	252	232	0.92
Hepatitis	12 I. U	145	95	0.66
	13 S. S	390	205	0.53
	14 K. O	520	410	0.79
	15 K. T	113	137	1.21
	16 S. M	(A)4,600	1,140	0.25
		(C) 205	220	1.07

(A) in case 16 S. M is serum from a patient with acute hepatitis on aggravating stage. (C) in case 16 S. M is serum from the same patients on convalescent stage.

employment of the highly purified BFP facilitated to obtain more easily hybridoma producing monoclonal antibody specific to BFP than the employment of impure BFP.

Hybridization of spleen cells of BALB/c mouse immunized with BFP with myeloma cell lines, P3X63/Ag8U1 was performed 5 times. In the second hybridization, hybrid cells producing anti-BFP could be obtained at most offective rate of 22.4%. However, we could not find reliable technique to enhance success of hybridization. Hybridomas were cloned on relatively early time, 14 days after start of fusion, while on the time macroscopical development of hybrid colony was observed in only approximately one fourth of wells. The time was chosen in order to obtain the cloned cells having high durability.

The results from analysis of isotyping for monoclonal anti-BFP indicated that

most of antibodies was isotype of IgG1 or IgG2a. A single clone producing antibody of IgM type was found, but died in short time. Several antibodies which were doubtful of specificity to BFP were indicated by EIA based on a competitive binding inhibition technique. We could not clear completely the specificity from the reason that inhibition of ^{125}I-BFP binding was affected by protein content in reaction mixture and BFP-negative control of high protein content was used.

Accomplishment of EIA using monoclonal anti-BFP produced by hybridoma is thought to be useful for mass screening tests of cancer in near future. On the other hand, we found a monocolonal anti-BFP which revealed low reactivity against serum BFP of normal subjects and hepatitis patients on aggravating stage, but the same reactivity against serum BFP of cancer patients and hepatitis patients on convalescent stage in comparison with conventional anti-BFP. The results from the reactivity of the monoclonal antibody suggest to be difference in antigenic determinants between BFP produced by normal cell and by cancer cell or regenerated liver cell.

ACKNOWLEDGMENT

This work was supported by a grant from the Japanese Ministry of Health and Welfare.

REFERENCES

CATT, K. and TREGEAR, G. W.: Solid-phase radioimmunoassay in antibody-coated tubes. Science **158,** 1570 (1967).

ENGVALL, E. and PERLMANN, P.: Enzyme linked immunosorbent assay (ELISA) quantitative assay of IgG. Immunochemistry **8,** 871-874 (1971).

GALFRE, G., HOWE, S. C., MILSTEIN, C., BUTCHER, G. W. and HOWARD, J. C.: Antibodies to major histocompatibity antigens produced by hybrid cell lines. Nature **266,** 550 (1977).

ISHII, M.: Radioimmunoassay of α-fetoprotein. GANN Monogr. **14,** 89-98 (1973).

ISHII, M.: A new carcinoembryonic protein characterized by basic property. Scand. J. Immunol. **Suppl. 8,** 611-620 (1978).

ISHII, M.: Characterization of basic fetoprotein and clinical usefulness of BFP for immunodiagnosis of human cancer. In: Carcino-Embryonic Proteins. Chemistry, Biology. Clinical Application, Lehmann, F.-G. (Ed.) Elisevier/North-Holland Biomedical Press, Amsterdam, Vol. I, pp. 333-340 (1979a).

ISHII, M.: Clinical usefulness of basic fetoprotein for immunodiagnosis of human cancer. In: Compendium of Assay for Immunodiagnosis of Human Cancer, Development in Cancer Res., Herberman, R. B. (Ed.) Elisevier/North-Holland Biomedical Press, New York, Vol. I, pp. 45-50 (1979b).

KÖHLER, G. and MILSTEIN, C.: Continuous cultures of fused cells secreting antibody of predefined specificity. Nature **256,** 495-497 (1975).

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J.: Protein measurement

with the Folin phenol reagent. J. Biol. Chem. **193,** 265–275 (1951).

MOOR, T., DHAR, P., ZAMCHECK, N., KEELY, A., GOTTLIEB, L. and KUPCHIK, H. Z.: Carcinoembryonic antigen(s) in liver disease. I. Clinical and morphological studies. Gastroenterology **63,** 88–94 (1972).

NISHI, S.: Isolation and Characterization of a human fetal -globulin from the sera of fetuses and a hepatoma patient. Cancer Res. **30,** 2507–2513 (1970).