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Identification and Purification of a Toxic Component to B Cell Hybridoma Cells in Fetal Calf Serum

Noritaka Nonaka[1,2] and Hidematsu Hirai (deceased)[1]

[1]*Tumour Laboratory, Higashitokura 1-15-3, Kokubunji, Tokyo 185 and* [2]*Department of Development, Shino-Test Corporation, Oonodai 2-29-14, Sagamihara, Kanagawa 229*

A component exhibiting toxicity to B cell hybridoma cells was isolated and purified from fetal calf serum (FCS) by immunoaffinity chromatography using a monoclonal antibody (mAb) which reacted with the high-molecular-weight glycoprotein (6B3·Ag) recognized by a mAb, 6B3, to human large cell lung carcinoma cells (HLC-2). The component (FCS-6B3·Ag) was a high-molecular-weight antigen (approximately 1,000,000), consisting mainly of 76,000 subunits. FCS-6B3·Ag showed the same mobility in the pre-β globulin region as that of 6B3·Ag on electrophoresis in 1.2% agarose gel. When FCS-6B3·Ag was analyzed by double immunodiffusion, it reacted with anti-6B3·Ag antiserum and the precipitin line fused partially with that formed between 6B3·Ag and anti-6B3·Ag antiserum. FCS-6B3·Ag was found to be toxic to hybridoma cells (anti-6B3·Ag, anti-α-fetoprotein, anti-carcinoembryonic antigen or anti-C-reactive protein mAb producing cells) specifically *in vitro* at 5 μg/ml. The antigen also strongly suppressed their growth. The toxic effect of FCS-6B3·Ag appeared immediately after addition, and death of the target cells was complete only after 36–48 h. However, the antigen exhibited only weak suppression of Ig-non-secretory mouse myeloma (P3U1), thymic lymphoma (EL4) or mastocytoma (P815) cell growth. Five lots of FCS contained 2.1 to 4.1 μg/ml of FCS-6B3·Ag.

Key words: Toxic component — Fetal calf serum — B cell hybridoma cell

In an attempt to identify tumor markers of lung cancer cells, monoclonal antibodies (mAbs) were raised against human large cell lung carcinoma cell line (HLC-2). Among them, one antibody designated 6B3 recognized a secretory-type antigen (6B3·Ag) present in various adenocarcinoma cells. The molecular weight of 6B3·Ag was approximately 1,000,000, and the antigen was a homodecamer of a 94,000 subunit.[1] The 94,000 subunit was labile and easily converted into two fragments of 66,000 and 27,000 after storage at 4°C for a month.[1] The 6B·3Ag, which was similar to L3 antigen reported by Linsley *et al.*,[2] was present in normal human serum at about 1 to 5 μg/ml and its serum level was elevated in patients with malignant diseases.[1] 6B3·Ag was partly present as a complex with IgG and/or IgM in human serum.[3]

We have found that hybridoma cells producing mAb to 6B3·Ag were easily injured when cultured in fetal calf serum (FCS)-containing culture medium as compared with other hybridoma cells producing mAbs to other antigens. This did not occur in a synthetic culture medium, suggesting the presence of a toxic component in FCS. We describe here the isolation, purification and partial characterization of this toxic component.

[2] To whom correspondence should be addressed.

MATERIALS AND METHODS

Reagents Bovine serum albumin (BSA, type V) was purchased from Sigma Chemical Co. (St. Louis, MO), Coomassie Brilliant Blue R-250 from Nakarai Chemical, Ltd. (Kyoto), RPMI-1640 medium and FCS from Gibco Ltd. (Grand Island, NJ), tissue culture flasks (750 ml, No. 3028) from Falcon Plastics (Cockeysville, MD), Sepharose 4B and molecular weight standards for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) from Pharmacia Chemicals AB (Uppsala, Sweden), membrane filter (pore size 0.22 μm, Millex-GV) from Millipore Corp. (Waltham, MA), ^{3}H-thymidine from New England Nuclear (Boston, MA), a synthetic medium for hybridoma cells or myeloma cells (S-Clone) from Sanko Junyaku Co. Ltd. (Tokyo), 96-well ELISA plate (Immunoplate II) and 96-well culture plate from Nunc (Wiesbaden, Germany), alkaline phosphatase (ALP, EIA grade) from Boehringer Mannheim Co. (Mannheim, Germany), gelatin from Difco Laboratories (Detroit, MI), penicillin and streptomycin from Meiji Seika Kaisha, Ltd. (Osaka).

Materials Establishment of human large cell lung carcinoma cells (HLC-2) and the preparation of 6B3 mAb to HLC-2 were described previously.[4] The following mouse cell lines were kindly provided by Dr. N. Shinohara (Mitsubishi Kasei Institute of Life Science); a thymic lymphoma cell line (EL4), mastocytoma (P815) and B

cell tumor (A20.3). These cells were maintained in the RPMI-1640 medium containing 50 units/ml of penicillin, 50 μg/ml of streptomycin and 10% FCS. B cell hybridoma cells producing anti-α-fetoprotein (AFP), anti-C-reactive protein (CRP) and anti-carcinoembryonic antigen (CEA) were kindly given by Dr. M. Hatakeyama in this laboratory. The mAbs to 6B3·Ag (#K35A3, #O-10, #O-103 and #5H11) were prepared by a standard hybridoma technique.[5] B cell hybridoma cells were cultured in the S-Clone medium. Cell viability was determined by the trypan blue dye exclusion method.

The preparation of a rabbit antiserum to 6B3·Ag was described previously.[3]

Electrophoresis and western blot analysis SDS-PAGE of FCS-6B3·Ag was carried out according to the method of Laemmli.[6] The bands were stained with Coomassie Brilliant Blue R-250. Agarose gel electrophoresis was carried out in 1.2% agarose gel with 75 m*M* barbital buffer (pH 8.6, $\mu=0.05$), under a constant current of 2 mA/cm. For western blotting, proteins on an SDS-PAGE plate were transferred electrically to nitrocellulose membrane as described by Towbin *et al.*[7] using the electroblot system (Marysol Inc., Tokyo) by circulating cool water at 50 V for 16 h. The analysis of reactivity of mAb with 6B3·Ag was carried out according to the standard avidin-biotin peroxidase complex method at room temperature.[3]

Purification of 6B3·Ag and FCS-6B3·Ag The 6B3·Ag was purified from culture medium of HLC-2 by immunoaffinity chromatography using #K35A3 mAb to 6B3·Ag. HLC-2 cells (2×10^7) were cultured in a 750 ml tissue culture flask (Falcon No. 3028) for 6 days at 37°C in a 5% CO_2-95% humidified atmosphere, since 6B3·Ag was scarcely produced by HLC-2 cells cultured in a serum-free medium or a synthetic medium. The culture medium clarified by centrifugation at 10,000*g* for 10 min was applied to a #K35A3 mAb-coupled Sepharose 4B column (2.5×10 cm; 3 mg of mAb bound per ml of the gel). After washing with 10 m*M* phosphate-buffered saline, pH 7.2 (PBS), the 6B3·Ag was eluted with 3 *M* NaSCN, and dialyzed against PBS. The purity of 6B3·Ag was confirmed by SDS-PAGE. Since the FCS-6B3·Ag did not react with #K35A3 mAb, contamination of FCS-6B3·Ag in 6B3·Ag was completely avoided.

The FCS-6B3·Ag was purified from FCS by immunoaffinity chromatography using a #5H11 mAb (to 6B3·Ag)-coupled Sepharose 4B column (2.5×10 cm; 3 mg of mAb bound per ml of the gel). The procedures were almost the same as those for 6B3·Ag. Among 16 mAbs to 6B3·Ag only #5H11 mAb cross-reacted with FCS-6B3·Ag, and the reactivity of #5H11 mAb with 6B3·Ag was not much inhibited by the other 15 mAbs to 6B3·Ag in inhibition tests. Samples containing FCS-6B3·Ag were sterilized by filtration through a membrane filter (0.22 μm) before testing their suppressive activity on cell growth. The cross-reactivity of FCS-6B3·Ag with anti-6B3·Ag antiserum was determined by double immunodiffusion tests.[8]

Suppression of tumor cell growth Hybridoma cells (#1, anti-6B3·Ag, #5H11; #2, anti-AFP; #3, anti-CRP; #4, anti-CEA mAb producing), immunoglobulin (Ig)-non-secretory myeloma cell line (P3-X63-Ag8-U1, P3U1), EL4, P815 and A20.3 cells were cultured at 3×10^4 cells/well in 0.25 ml in the presence of various concentrations of FCS-6B3·Ag for 16 h at 37°C. One μCi/well of ^{3}H-thymidine was added to each culture after 6 h incubation. The cultures were harvested on glass fiber filters, and the radioactivity was determined using a liquid scintillation counter.

Enzyme-linked immunosorbent assay (ELISA) and others The difference of antigenicity between 6B3·Ag and FCS-6B3·Ag was studied by ELISA for 6B3·Ag, as described in detail previously.[1] Two kinds of mAb to 6B3·Ag (#O-10 for the solid phase and #K35A3 for the ALP label) were used. The 6B3·Ag used as the ELISA standard was prepared on a dry weight basis. Total protein was determined by Lowry's method[9] using BSA as a standard. The concentration of FCS-6B3·Ag was measured by ELISA using #5H11 mAb for the solid phase and for the ALP label with 6B3·Ag as the standard.

RESULTS

Identification and characterization of a component reacting with mAb to 6B3·Ag from FCS A component reacting with mAb to 6B3·Ag was purified from 500 ml of FCS by immunoaffinity chromatography using #5H11 mAb to 6B3·Ag. Among 16 kinds of mAbs to 6B3·Ag only #5H11 mAb cross-reacted with this component. #5H11 mAb recognized a part of the 67,000 protein of 6B3·Ag (Fig. 1, lane c), and the other mAbs including #K35A3 mostly recognized a whole 67,000 protein of 6B3·Ag (Fig. 1, lane b). A component (approximately 1.5 mg as protein) was eluted from the #5H11 mAb-coupled Sepharose 4B column. The pass-through fraction of FCS from the #5H11 mAb immunoadsorbent column no longer showed toxicity to hybridoma cells producing mAb to 6B3·Ag. Five lots of FCS contained 3.4, 2.6, 2.1, 3.7 and 4.1 μg/ml of the component as determined by ELISA. From 500 ml of FCS (5 lots), 1 to 2 mg of the component was recovered as protein having antigenicity. The component was partly (approximately a tenth part) adsorbed on an anti-AFP mAb-coupled Sepharose 4B column. It appeared as a single band in the pre-β globulin region, the same region as that of 6B3·Ag, in 1.2% agarose gel electrophoresis (Fig. 2). This antigen from FCS was not completely detected by ELISA for 6B3·Ag using #O-10 and #K35A3 mAb to 6B3·Ag (Fig. 3b). It was eluted at the same fraction (approximately 1,000,000,

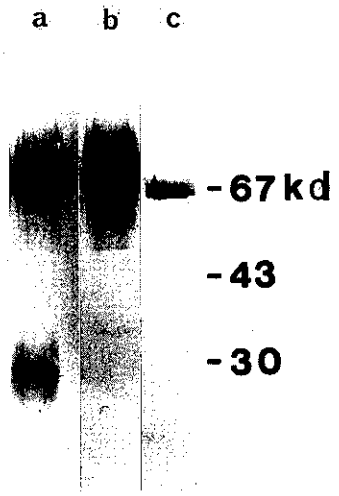

Fig. 1. Western blotting analysis of 6B3·Ag portion recognized by #5H11 mAb to 6B3·Ag. The 6B3·Ag from HLC-2 culture medium (denatured form of 66,000 and 27,000[1]) was used. After SDS-PAGE (12% gel), proteins in the gel were transferred to three sheets of nitrocellulose membranes (#a to #c). The membrane was blocked with 1% gelatin. The membranes #a, #b and #c were incubated with rabbit anti-6B3·Ag antiserum, #K35A3 mAb (anti-6B3·Ag mAb) and #5H11 mAb (anti-6B3·Ag mAb), respectively. After washing, #a membrane was incubated with biotinylated goat anti-rabbit IgG antibody, and #b and #c membranes were incubated with biotinylated horse anti-mouse IgG antibody for 2 h. After washing, the membranes (#a to #c) were incubated with avidin-biotin peroxidase complex for 2 h. After washing, the reactive bands were visualized by immersing the membrane in 0.03% 3,3′-diaminobenzidine solution in 20 m*M* Tris-HCl, pH 7.4, containing 0.005% H_2O_2. The other mAbs to 6B3·Ag (#O-10 and #O-103) recognized the whole 67,000 protein of 6B3·Ag, like #K35A3. a, rabbit anti-6B3·Ag antiserum; b, #K35A3 mAb; c, #5H11 mAb.

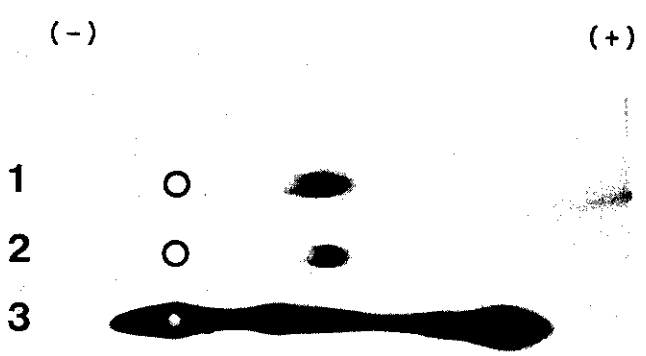

Fig. 2. Agarose gel electrophoresis of FCS-6B3·Ag. 1, 6B3·Ag from HLC-2 culture medium; 2, FCS-6B3·Ag; 3, normal human serum.

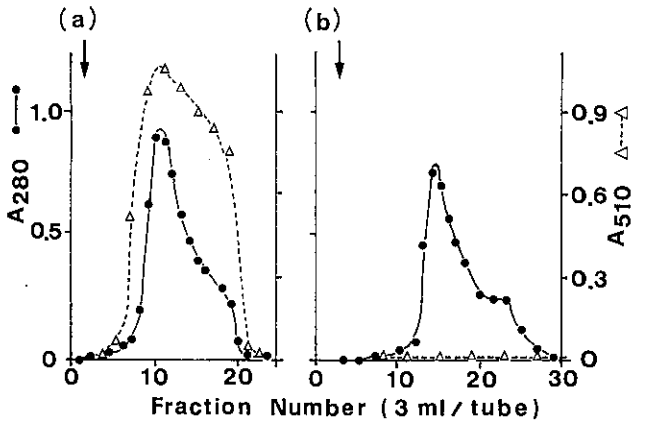

Fig. 3. The reactivity of FCS-6B3·Ag, eluted from the immunoaffinity column, with mAbs to 6B3·Ag on ELISA. (a) One liter of HLC-2 culture medium was applied to a #K35A3 mAb-coupled Sepharose 4B column (2.5×10 cm; 3 mg of mAb bound per ml of the gel). After washing with PBS, the 6B3·Ag was eluted with 3 *M* NaSCN and assayed by ELISA for 6B3·Ag using #O-10 mAb for the solid phase and #K35A3 mAb for the ALP label. (b) Five hundred ml of FCS was applied to a #5H11 mAb (to 6B3·Ag)-coupled Sepharose 4B column (2.5×10 cm; 3 mg of mAb bound per ml of the gel). After washing with PBS, FCS-6B3·Ag was eluted with 3 *M* NaSCN and assayed by the same ELISA as described above to establish whether FCS-6B3·Ag reacted with anti-6B3·Ag mAbs (#O-10 and #K35A3). FCS-6B3·Ag was not detected by the ELISA. Closed circles indicate absorbance at 280 nm. Triangles indicate absorbance at 510 nm on ELISA. Arrows indicate the fraction at which 3 *M* NaSCN was applied.

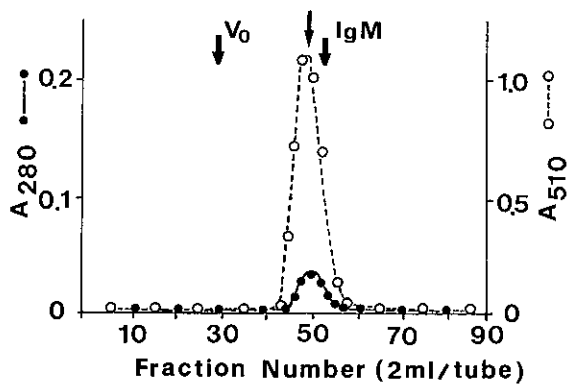

Fig. 4. Elution profile of FCS-6B3·Ag on a Sepharose 4B column. FCS-6B3·Ag (0.4 mg) was applied to a Sepharose 4B column (2.5×90 cm) equilibrated with 0.1 *M* acetate buffer, pH 6.0, containing 0.5 *M* NaCl. Closed circles indicate absorbance at 280 nm. FCS-6B3·Ag was detected by ELISA using #5H11 mAb for the solid phase and for the ALP label (○). The molecular weight of IgM was 900,000. V_0 indicates the position of the void volume.

Fig. 4) as 6B3·Ag from HLC-2 culture medium on a Sepharose 4B column (2.5×90 cm) and a main 76,000 band was seen on SDS-PAGE (12% gel) under reducing conditions (Fig. 5). On double immunodiffusion, the antigen from FCS (FCS-6B3·Ag) reacted with anti-6B3·Ag antiserum and the precipitin line fused partially with that formed between 6B3·Ag and anti-6B3·Ag antiserum (Fig. 6).

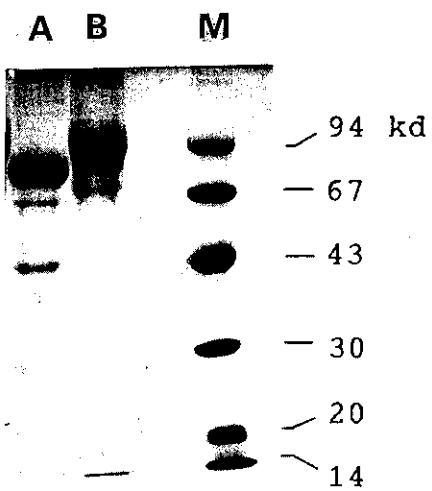

Fig. 5. SDS-PAGE (12% gel) of FCS-6B3·Ag under reducing conditions (2% 2-mercaptoethanol). A, FCS-6B3·Ag; B, 6B3·Ag from freshly prepared HLC-2 culture medium. Marker proteins (M) were as follows: 94,000 (94 kd), rabbit muscle phosphorylase *b*; 67 kd, bovine serum albumin; 43 kd, ovalbumin; 30 kd, bovine erythrocyte carbonic anhydrase; 20 kd, soybean trypsin inhibitor; 14 kd, bovine milk α-lactalbumin.

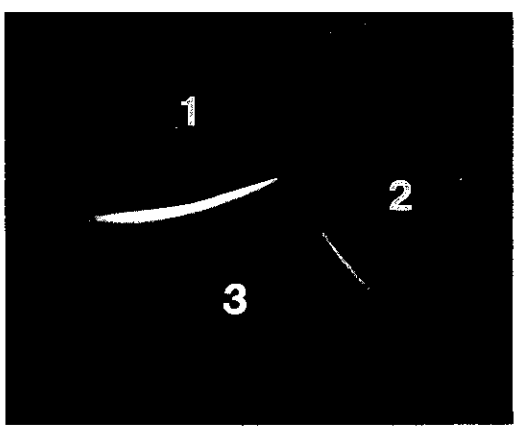

Fig. 6. Double immunodiffusion test of FCS-6B3·Ag and 6B3·Ag. 1, 6B3·Ag from HLC-2 culture medium; 2, FCS-6B3·Ag from FCS; 3, rabbit anti-6B3·Ag antiserum.

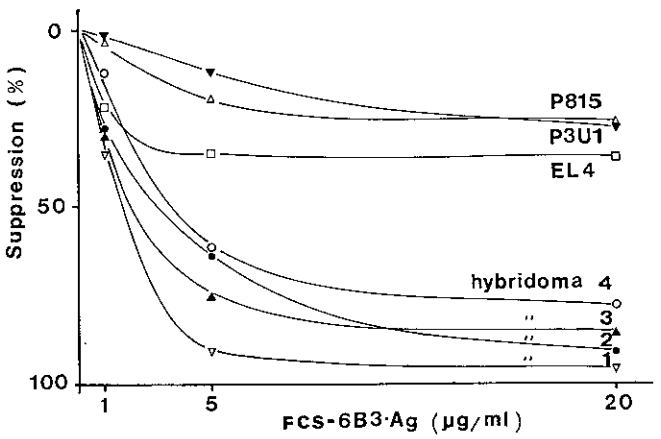

Fig. 7. Effect of FCS-6B3·Ag on tumor cell growth. The FCS-6B3·Ag was added to hybridoma (#1 to #4), EL4, P815 or P3U1 cells at a final concentration of 0, 1, 5 or 20 μg/ml, and the cells were cultured for 16 h at 37°C. Cultures were finally harvested after a further 6 h of incubation with 1 μCi of ^{3}H-thymidine. The per cent suppression was calculated in the following way: % suppression = $100 \times (Cm - Ct)/Cm$, where Cm represents incorporated radioactivity of control cultures and Ct stands for that of corresponding experimental cultures where FCS-6B3·Ag was added. Hybridoma #1, anti-6B3·Ag, #5H11; #2, anti-AFP; #3, anti-CRP, #4, anti-CEA mAb producing cells. EL4, a thymic lymphoma; P3U1, Ig-non-secretory myeloma; P815, mastocytoma.

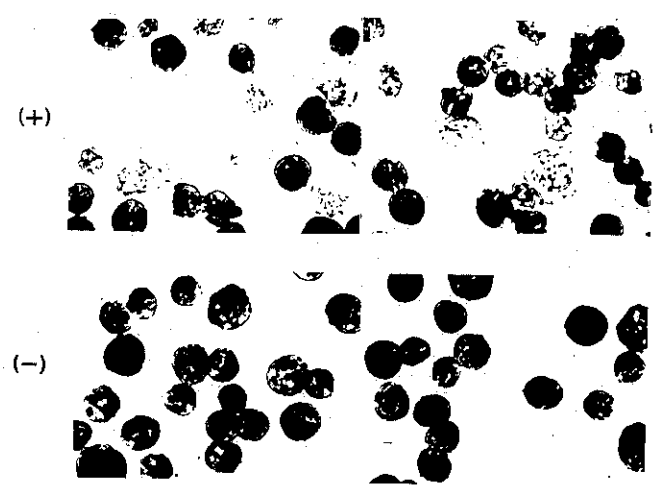

Fig. 8. Giemsa staining of hybridoma cells treated with FCS-6B3·Ag. FCS-6B3·Ag was added to hybridoma #2, anti-AFP mAb producing cells at a final concentration of 0 or 5 μg/ml, and the cells were cultured for 6 h at 37°C. The cells were applied to a glass plate, dried in air and fixed with methanol solution. They were stained with Giemsa and observed under a microscope. (+), FCS-6B3·Ag 5 μg/ml; (−), FCS-6B3·Ag 0 μg/ml.

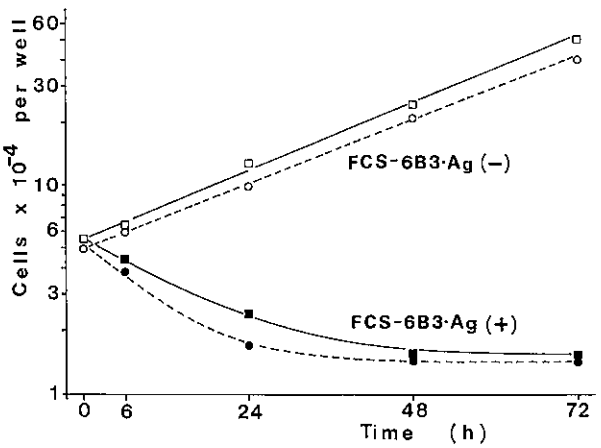

Fig. 9. Time course of suppression of hybridoma cell proliferation by FCS-6B3-Ag. Hybridoma cells producing anti-6B3-Ag mAb (#5H11 and #O-103) were cultured at 5×10^4 cells/well in 0.5 ml of the S-Clone medium in the presence of FCS-6B3-Ag at 0 or 5 μg/ml for 0 to 72 h. The number of live cells was measured at various times, and compared with that of the control. Standard deviation for cell counts was generally less than 10% (expressed as variation coefficient). #5H11, □; #O-103, ○.

Effect of FCS-6B3-Ag on B cell hybridoma cells and other tumor cell lines We next examined whether FCS-6B3-Ag had a toxic or growth-suppressive effect on B cell hybridoma or other tumor cells using ^{3}H-thymidine uptake as an index. The FCS-6B3-Ag suppressed hybridoma cell proliferation by 70–90% at above 5 μg/ml (Fig. 7). When hybridoma cells treated with FCS-6B3-Ag for 6 h were stained with Giemsa stain, a toxic effect was revealed (Fig. 8). A kind of B cell tumor, A20.3, was also affected by FCS-6B3-Ag in the same manner as B cell hybridoma cells. On the other hand, FCS-6B3-Ag showed only marginal effects on the growth of P3U1, EL4 or P815 cells at the same FCS-6B3-Ag level, and had no toxic effect on these cells. When B cell hybridoma cells producing anti-6B3-Ag mAb (#5H11 and #O-103) were cultured with FCS-6B3-Ag at a final concentration of 0 or 5 μg/ml, viable cells almost disappeared by 48 h (Fig. 9). The other lots of FCS-6B3-Ag derived from 5 lots of FCS had similar effects.

DISCUSSION

An antigen (FCS-6B3-Ag) analogous to 6B3-Ag and exhibiting toxicity to B cell hybridoma cells was found in FCS. FCS-6B3-Ag suppressed the growth of various tumor cell lines, and had a toxic effect on B cell hybridoma cells and a B cell tumor cell line (A20.3). Since FCS-6B3-Ag was not detected by sandwich ELISA for 6B3-Ag using 2 kinds of mAb to 6B3-Ag (Fig. 3b), FCS-6B3-Ag is different from 6B3-Ag. However, FCS-6B3-Ag reacted with anti-6B3-Ag antiserum, and the precipitin line fused partially with that formed between 6B3-Ag and anti-6B3-Ag antiserum, suggesting that FCS-6B3-Ag is an analogous protein to 6B3-Ag, and that 6B3-Ag has the same activities as FCS-6B3-Ag.

The 6B3-Ag was secreted by HLC-2 cells and was also present in normal human serum. An analogue of 6B3-Ag was reported to be a novel antigen and a minor protein in human serum.[2, 10] FCS-6B3-Ag, an analogous protein to 6B3-Ag, seems to be a novel protein in FCS.

The content of FCS-6B3-Ag in 5 lots of FCS was determined to be 2.1 to 4.1 μg/ml by ELISA using #5H11 mAb as the solid phase and enzyme-labeled antibody, when 6B3-Ag was used as a standard. The concentration of FCS-6B3-Ag which exhibited toxicity or suppressed the growth of B cell hybridoma cells was above 5 or 1 μg/ml, respectively (Fig. 7). Usually, FCS was added as a tenth part with respect to the culture medium for culture of hybridoma cells, suggesting that the growth of hybridoma cells might not be directly affected by FCS-6B3-Ag, despite the lot variation of FCS. Since anti-6B3-Ag mAb producing cells were prepared in our case and FCS-6B3-Ag in FCS bound to anti-6B3-Ag mAb, the FCS-6B3-Ag might be concentrated on the hybridoma cell surface, resulting in the toxic effect. Further study on the FCS-6B3-Ag toxicity to B cell hybridoma cells may yield a new mechanism of toxicity.

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