RAPID COMMUNICATION Jpn. J. Cancer Res. 81, 967–970, October 1990

Alloimmunization with Cultured Human Stomach Cancer Cell Lines and the Establishment of Human-Human Hybridomas Producing Monoclonal Antibodies

Kotaro Koyama,[1] Koichi Akiyama,[2] Hiroharu Kawahara,[2] Akira Egashira[3] and Hiroki Murakami[3]

[1]*Biotechnology, Technical Research Center, Marubeni Feed Co., 1292 Kotare, Shibu-cho, Ono-shi, Hyogo 675-13,* [2]*Food Science and* [3]*Technology Institute, Graduate School of Genetic Resources Technology, Kyushu University, 6-10-1, Hakozaki, Higashi-ku, Fukuoka 812*

Alloimmunization with the cultured human stomach cancer cell lines MKN-45 and MKN-28 was conducted and human-human hybridomas were established. Living MKN-45 cells (10^7 cells per injection) were injected monthly 34 times into one of the authors (K.K.) over 2 years and living MKN-28 cells (10^7 cells per injection) were co-injected 14 times after the 20th injection of MKN-45 alone. Cultured stomach cancer cells injected subcutaneously into K.K.'s forearm were rejected completely, although they grew transiently at the injection site. All the injections were made voluntarily at the direction of K.K. himself. Two human-human hybridomas producing monoclonal antibodies reactive with MKN-45 and MKN-28 cells, respectively, were separated from a hybridoma of human B-lymphoblastoid cell line named HO 323/WIL2-NS and lymphocytes in K.K.'s peripheral blood. One cell line of the hybridoma FMK-H3 produced IgM-type monoclonal antibody, and the other line, EMK-F7, produced IgG-type antibody. These two human-human monoclonal antibodies were highly reactive with MKN-45 and MKN-28 cells, weakly reactive with MKN-74 stomach cancer cell line, and not reactive with the other stomach cancer cell lines tested (MKN-1, MKN-7, TMK-1, NUGU-2, NUGC-3, NUGC-4 and KATO-III).

Key words: Human monoclonal antibody — Human stomach cancer cell line — Alloimmunization

Human monoclonal antibodies reactive with cancer cell lines have been isolated from human-human hybridomas of human B lymphoma cell lines and lymphocytes of cancer patients,[1-4] and therapeutic application of human monoclonal antibodies (MoAbs) has been attempted in cancer patients.[5] No study, however, has been reported on MoAbs obtained from hybridomas of human B-lymphoma cell lines and human B-lymphocytes produced by injecting living human cancer cells as an antigen.

Tumor cells transplanted into humans are generally rejected, since the tumor transplant and the recipient are immunologically allogenic to each other. Induction of antibodies to various tumor-associated antigens in response to the injected tumor cells can be expected. We attempted to separate a human-human MoAb for a tumor-associated antigen of human stomach cancer cells by injecting living tumor cells into an allogenic host. The cultured MKN-45 cell line used for the injection was established from a poorly differentiated adenocarcinoma of the human stomach.[6] The culture medium was serum-free RDF-ITES[4] (a 2:1:1 mixture of RPMI 1640, Ham's F12 and Dulbecco's modified Eagle's media containing 10 μg of human-type insulin per ml, 10 μg of human-type transferrin per ml, 10 μmol of ethanolamine and 2.5 nmol of sodium selenite) supplemented with 100 μg each of streptomycin, kanamycin and penicillin per ml. The cells were subcultured 10 times in this medium and were free of mycoplasmas. The cells cultured in the medium were then harvested with a cell scraper. After being washed and suspended in phosphate-buffered saline (PBS), they were injected subcutaneously into the forearm or upper-arm of one of the authors (K.K.). All injections of the living cancer cell lines were performed voluntarily at the insistence of K.K. himself. Numerous animal experiments suggested that no substantial risk to the volunteer could be expected to be involved in this study. In addition, this experiment was not expected to expose other people in the community to any risk. The details of the injection schedule are as follows: first, 10^6 cells were suspended in PBS and injected subcutaneously weekly 5 times. Then 10^7 cells were injected subcutaneously monthly 34 times in order to stimulate the immune response. In addition, to obtain antibody with a high titer for stomach cancer, another human stomach cancer cell line, MKN-28 (differentiated adenocarcinoma)[6] was selected. MKN-28 was cultured in the RDF medium containing 1% K.K.'s serum and 10^7 cells were injected subcutaneously along with the MKN-45 cells as a mixture. The injection of MKN-28 cells was started after 20 of the monthly injections of MKN-45 cells had been given.

Six hours after the injection of cells, flares (outside diameter 50–78 mm) and swelling appeared in the subcutaneous area of the forearm, but these diminished after

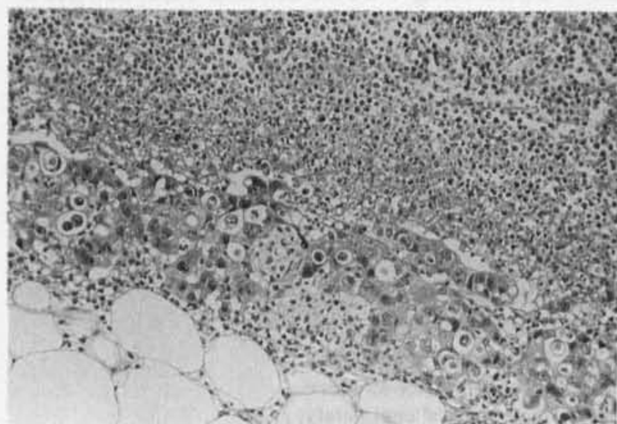

Fig. 1. A biopsied tissue sample, 5 days after subcutaneous injection of 10^7 living MKN-45 cultured cells. HE stain, ×200. Proliferation of tumor cells with lymphocytic infiltration and necrosis in the dermis and subcutaneous tissue is seen.

48 h. Sclerotic swelling and flares (outside diameter 20 mm) occurred after 3 days and completely disappeared after 20–30 days. Figure 1 shows a biopsy specimen 5 days after the injection; living tumor cells are evident. These observations, after the injection of MKN-45 and MKN-28 cells, were almost the same throughout the course of repeated injections. Cytotoxicity to MKN-45 and MKN-28 cells was determined by an *in vitro* assay using the inactivated serum of K.K. sampled after the injection of MKN-45 and MKN-28 cells. The assay procedure was as follows: the serum samples were added to RPMI medium containing 5% fetal calf serum and 2 units of complement for the hemolytic system (guinea pig serum). MKN-45 cells (1×10^4) were added to 5 ml of medium in the plates. The plates were then incubated at 37°C in a CO_2 incubator for 3 days, and the number of living cells which survived was determined by means of trypan blue staining. No toxicity was observed even when 50% serum samples were assayed.

Isolation of hybridomas which produce monoclonal antibodies for MKN-45 and MKN-28, from K.K.'s B-lymphocytes and human lymphoblasts was then attempted. The cell line used as a fusion partner was a 6-thioguanine-resistant human B-lymphoblastoid cell line, HO 323/WIL2-NS.[7] The lymphocyte preparations were obtained by Ficoll-Paque gradient centrifugation of K.K.'s peripheral blood, which was sampled 4 days after each injection of 10^7 MKN-45[6] and 10^7 MKN-28[6] cells. Cell fusion was performed by using polyethylene glycol (MW 4,000). Selection of the hybridomas was conducted in HAT medium (ERDF/15% FCS medium supplemented with 100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine)[4] and antibodies were screened by using MKN-45 and MKN-28 as target cells by enzyme-linked immunosorbent assay (ELISA). The hybridomas were cloned by the limiting dilution method. Immunoglobulins were detected by Houghton's ELISA method.[8] The MoAbs were examined for reactivity with stomach cancer cell lines MKN-1,[6] MKN-7,[6] MKN-28, MKN-45, MKN-74,[6] TMK-1,[9] NUGU-2,[10] NIGC-3,[10] NUGC-4[10] and KATO-III.[11]

By means of these procedures, 1,478 hybridomas were produced by 6 separate cell fusion experiments (30 months after), and two human-human hybridomas cell lines were obtained, each of which produced a human type antibody: FMK-H3 (IgM type) and EMK-F7 (IgG type).

The MoAb FMK-H3 (IgM) showed strong reactivity with cultured MKN-45, MKN-45/K (cultured in medium containing no serum), and MKN-28, while it showed weak reactivity with TMK-1 and the other cultured stomach cancer cell lines (MKN-1, MKN-7, MKN-74, NUGU-2, NUGC-3, NUGC-4, and KATO-III). The MoAb EMK-F7 (IgG) was also strongly reactive with MKN-45, MKN-45/K MKN-28, and was not reactive with the other stomach cancer cell lines tested (Table I and Fig. 2).

These results indicate the appearance of B-lymphocytes which produce antibodies against MKN-45 and MKN-28 cells in K.K.'s blood. Hybridoma/FMK-H3

Table I. Reactivity of Human Monoclonal Antibodies with Human Cell Lines by ELISA

Stomach cancer cell line	Monoclonal antibodies from hybridomas	
	FMK-H3[a]	EMK-F7[b]
MKN-1	−	−
MKN-7	−	−
MKN-28	++	+++
MKN-45	++	+++
MKN-45/K	+++	+++
MKN-74	+	−
TMK-1	−	−
NUGU-2	−	−
NUGC-3	−	−
NUGC-4	−	−
KATO-III	−	−

The strength of the MoAb reactivity was expressed as follows: the absorbance at 405 nm was below 0.1 (−); 0.1–0.2 (+); 0.2–0.3 (++), and over 0.3 (+++).
a) IgM type, *b*) IgG type.
The reaction was initiated by the addition of 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt as a substrate. After the mixture was held at 37°C for 15 min, the reaction was terminated by the addition of oxalic acid. Absorbance at 405 nm was then determined with a microplate electrometer.

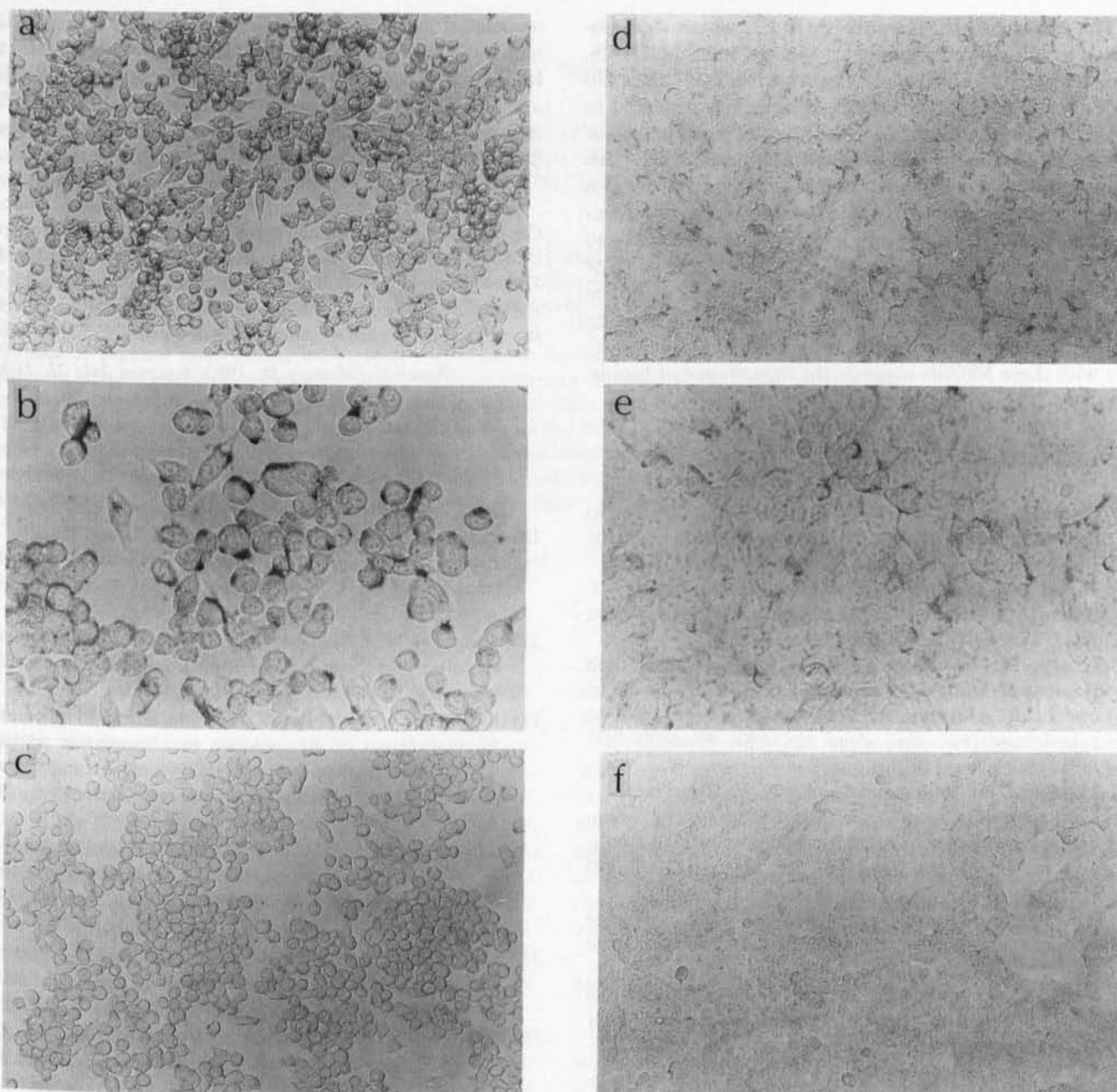

Fig. 2. Reactivity of MoAb/EMK-F7 (IgG) with MKN-45 (a, b, c) and MKN-28 (d, e, f) cultured cells. The cultured cells were fixed with 0.06% glutaraldehyde and ELISA was performed. MoAb/EMK-F7 (1.3 mg/ml) was purified before the test. The photographs were taken after 15-min incubation at 37°C initiated by the addition of the substrate solution. a) MKN-45 cells after the reaction (×100), b) (×200), and c) without the reaction (×100); d) MKN-28 cells after the reaction (×100), e) (×200), and f) without the reaction (×100). The stained regions in a), b), d) and e) correspond to the reacted antigens.

and FMK-F7 so far made could be cultured in a serum-free medium (RDF-ITES) supplemented with egg yolk lipoprotein. Hybridomas were grown in the serum-free medium by the use of a stirred culture vessel equipped with a perfusion system which allowed the cells to grow to 10^7/ml. MoAb productivity by the hybridomas continued throughout the stationary phase and was 80–120 μg/ml, representing a moderate production. The MoAb produced was easily purified from the serum-free spent medium.[4]

The MoAbs FMK-H3 and EMK-F7, which were obtained by alloimmunization with MKN-45 and MKN-28, had strong and specific reactivity with these cell lines. No toxicity for MKN-45 or MKN-28 cells was observed in the serum after immunization with the two cell lines, nor was antibody for the cell lines detected by ELISA. This result may suggest that the antibody titer in the serum is low. It is important to know whether the specific reactions of MoAb FMK-H3 and EMK-F7 for MKN-45 and MKN-28 cell lines are targeting allogenic antigens or tumor-associated antigens. Further studies on the antigen reactivity for tissues and purification of the antibodies will be required. The identification of the antigens reactive with these MoAbs suggests the importance of tumor-associated antigens in human cancer.[12-17] The establishment of human monoclonal antibodies applicable to humans has been difficult and very few such antibodies are available. In this study, we have established useful human-human MoAbs by the experimental procedures described in the text.

We thank Dr. T. Sugimura, Dr. M. Terada (National Cancer Center) and Dr. K. Nomoto (Medical Institute of Bioregulation, Kyushu University) for helpful discussions, and Dr. E. Tahara (School of Medicine, Hiroshima University), Dr. T. Watanabe (School of Medicine, Nagoya University), and the Japan Cancer Research Resources Bank for providing cell lines.

(Received February 28, 1990/Accepted July 21, 1990)

REFERENCES

1) Olsson, L. and Kaplan, H. S. Human-human hybridomas producing monoclonal antibodies of predefined specificity. *Proc. Natl. Acad. Sci. USA*, **77**, 5427–5431 (1980).
2) Sikora, K., Alderson, T., Phillips, J. and Watson, J. Human hybridomas from patients with malignant disease. *Br. J. Cancer*, **47**, 135–145 (1983).
3) Glassy, M. C., Handley, H. H., Hagiwara, H. and Royston, I. UC729-6, a human lymphoblastoid B-cell line useful for generating antibody-secreting human-human hybridomas. *Proc. Natl. Acad. Sci. USA*, **80**, 5429–5431 (1980).
4) Murakami, H., Hashizume, S., Ohashi, H., Shinohara, K., Yasumoto, K., Nomoto, K. and Omura, H. Human-human hybridomas secreting antibodies specific to human lung carcinoma. *In vitro Cell. Dev. Biol.*, **21**, 593–596 (1985).
5) Irie, R. F., Matsuki, T. and Morton, D. L. Human monoclonal antibody to ganglioside GM2 for melanoma treatment. *Lancet*, **i**, 786–787 (1989).
6) Hojo, H. and Oboshi, S. Establishment of cultured cell lines of human stomach cancer origin and their morphological characteristics. *Niigata Med. J.*, **91**, 737–763 (1977) (in Japanese).
7) Ohashi, H., Hashizume, S., Murakami, H., Aihara, K., Shinohara, K. and Omura, H. HO-323, a human B-lymphoblastoid cell line useful for making human-human hybridomas. *Cell Biol. Int. Rep.*, **10**, 77–83 (1986).
8) Houghton, A. N., Brooks, H., Cote, R. J., Taormina, M. C., Oettgen, H. F. and Old, L. J. Detection of cell surface and intracellular antigens by human monoclonal antibodies. *J. Exp. Med.*, **158**, 53–65 (1983).
9) Ochiai, A., Yasui, W. and Tahara, E. Growth-promoting effect of gastrin on human gastric carcinoma cell line TMK-1. *Jpn. J. Cancer Res.*, **76**, 1064–1071 (1985).
10) Uchida, T., Hirabayashi, Y., Watanabe, T. and Kondo, T. Analysis of cell surface antigen of gastric cancer by autologous typing. *Clin. Immunol.*, **17**, 858–868 (1985) (in Japanese).
11) Sekiguchi, M., Sakakibara, K. and Fujii, G. Establishment of culture cell lines derived from a human gastric carcinoma. *Jpn. J. Exp. Med.*, **48**, 61–68 (1978).
12) Koyama, K., Nudelman, E., Fukuda, M. and Hakomori, S. Correlation of glycosylation in a membrane protein with a molecular weight of 150,000 with tumorigenic property of rat fibrosarcoma variants. *Cancer Res.*, **39**, 3677–3682 (1979).
13) Koyama, K., Tamayama, C., Tomono, Y. and Watanabe, K. Glycosphingolipids in clonal variants of rat fibrosarcoma cells with different transplantability. *Gann*, **74**, 517–523 (1983).
14) Hakomori, S. and Kannagi, R. Glycosphingolipid as tumor-associated and differentiation markers. *J. Natl. Cancer Inst.*, **71**, 231–251 (1983).
15) Hakomori, S., Nudelman, E., Levery, S. B. and Patterson, C. M. Human cancer-associated gangliosides defined by a monoclonal antibody (IB9) directed to sialosyl 2-6 galactosyl residue: a preliminary note. *Biochem. Biophys. Res. Commun.*, **113**, 791–798 (1983).
16) Tai, T., Paulson, J. C. and Irie, R. F. Ganglioside GM_2 as a tumor antigen (OFA-1). *Proc. Natl. Acad. Sci. USA*, **80**, 5392–5396 (1983).
17) Carubia, J. M., Yu, R. K., Macala, L. J., Kirkwood, J. M. and Varga, J. M. Gangliosides of normal and neoplastic melanocytes. *Biochem. Biophys. Res. Commun.*, **120**, 500–504 (1984).