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ESTABLISHMENT OF HYBRIDOMAS SECRETING HUMAN MONOCLONAL ANTIBODIES AGAINST TETANUS TOXIN AND HEPATITIS B VIRUS SURFACE ANTIGEN

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SUMMARY: Mouse-human heterohybrids (M·H) were constructed and compared with other cell lines (human or mouse) as parental cells to obtain hybrids secreting human monoclonal antibody (MoAb). One of the M·H lines, HM-5, was far superior to the others and useful for establishing hybrids secreting human MoAb. Using HM-5 as a parental cell line, we have obtained 2 hybrids secreting human anti-tetanus toxoid MoAb with neutralizing activity and a hybrid secreting human anti-hepatitis B virus surface antigen (HBsAg) MoAb which recognizes the a-determinant of HBsAg.

Human monoclonal antibodies (MoAb) are more useful than murine antibodies in clinical applications such as prophylactics and therapeutics for tumor and infectious diseases. Several experimental systems (1-12) have been developed to produce cell lines secreting human MoAb; however, each of these has a defect. Epstein-Barr virus transformants usually secrete a small quantity of antibody and tend to cease antibody production (1-3). In mouse-human hybrids, antibody production in general is unstable because of the preferential loss of human chromosomes (6-8). In the human-human system, the frequency of hybrid formation is low because of the absence of a suitable malignant fusion partner (11,12). Recently, Teng et al. demonstrated that stable human MoAb secreting hybrids could be obtained using mouse (myeloma)-human (myeloma) heteromyeloma as a parental cell line (13).

In the present study, we constructed and selected a mouse (myeloma)-human (normal lymphocyte) heterohybrid (M·H) with high fusion frequency,

Abbreviations : M·H, mouse-human heterohybrid; MoAb, monoclonal antibody; T.T., tetanus toxoid; HBsAg, hepatitis B virus surface antigen

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and used this M·H to obtain hybrids secreting human MoAb against tetanus toxoid (T.T.) and hepatitis B virus surface antigen (HbsAg).

MATERIALS AND METHODS

Cell lines: The 8-azaguanine (8-AG) resistant murine myeloma cell line, P3-X63-Ag8.UI (P3UI) (14) was obtained from Osaka University Medical School (Osaka, Japan). The 8-AG resistant human plasma cell leukemia derived cell line LICR-LON-HMy2 (LON) (11) was obtained from the Ludwig Institute for Cancer Research (London, U.K.).

Preparation of human peripheral blood lymphocytes (PBL): Human PBL were prepared from the heparinized blood of healthy donors by centrifugation on Ficoll-Hypaque solution (Litton Bionetics, MD, USA). The PBL were suspended in RPMI 1640 medium (Flow Labs. Ltd., VA, USA) containing 10% fetal calf serum (FCS) (M.A. Bioproducts, IL, USA), kanamycin (100 μg/ml) and $NaHCO_3$ (1.8 mg/ml) (growth medium) at a cell density of 2×10^6/ml and stimulated with pokeweed mitogen (PWM, 32 μg/ml) (P.L. Biochem. Inc., WI, USA). The PBL suspension was cultured for 5-7 days in a humidified atmosphere of 5% CO_2 in air and then used for cell fusion.

Fusion procedure: The human PBL were mixed with 8-AG resistant parental cells (P3UI, LON, or M·H) at a ratio of 2:1 and fused by a modification of the procedure described by Köhler and Milstein (15). The fused cells were suspended at 2×10^5 tumor cells/ml in RPMI 1640 medium supplemented with hypoxanthine 10^{-4}M, aminopterin 4×10^{-7}M, thymidine 1.6×10^{-5}M, and 10% FCS (HAT medium), and seeded in a volume of 1 ml to each well of Linbro 24-well multiplates (Flow Labs.).

Enzyme linked immunosorbent assay (ELISA): Human antibodies (IgG and IgM) and anti-T.T. antibodies were determined by the ELISA procedure (16). One hundred microliters of culture supernatant of a hybrid was added to each well of the microplate (Nunc Intermed, Denmark) coated with goat anti-human IgG, IgM (1 μg/well, Cappel Labs. Inc., PA, USA) or purified T.T. (3.3 Lf/well) and the plate was incubated for 3 hr at 24°C. After the plate was washed with saline, 100 μl of horseradish peroxidase (HRP) conjugated goat anti-human IgG or IgM (Cappel Labs.) was added to it. The plate was then incubated for 3 hr at 24°C and washed again. The HRP conjugate bound was detected by incubating the plate at 24°C with a substrate solution containing orthophenylendiamine (2.2 mg/ml) and 0.1% H_2O_2 in 0.1M citrate buffer (pH 5.5). The reaction was stopped by adding 4N H_2SO_4, and optical densities at 492 nm were read with Titertek multiscan (Flow Labs.).

Determining the anti-HBsAg antibody: Anti-HBsAg antibody was determined by the AUSAB enzyme immunoassay kit (Abbott Labs., IL, USA). The polystyrene beads coated with HBsAg were added to a well containing 200 ul of the culture supernatant of hybridomas and the beads were incubated for 2 hr at 40°C. After the beads were washed with saline, 200 μl of the conjugate of HRP and HBsAg was added. Then the beads were incubated for 2 hr at 40°C and washed again. The HRP conjugate bound was detected by the same method as described above.

Chromosomal analyses: Chromosomes were analysed by the following two methods.

1) Giemsa method (17): Approximately 2×10^6 cells were suspended in 10 ml of growth medium containing colchicin (2 μg/ml) (Wako Pure Chem., Osaka, Japan). After they were incubated for 1.5 hr at 37°C, the cells were harvested by centrifugation at 250 x g for 10 min. The cell pellet was suspended in 3 ml of 75 mM KCl solution and kept at 24°C for 15 min. The cells were then washed by centrifugation twice in a fixative (methanol/

acetic acid, 3:1), suspended in a few drops of the fixative, air dried on microscopic slides, stained with Giemsa solution, and examined microscopically.

2) Trypsin-Giemsa method (18): The specimen prepared by the Giemsa method described above was decolorized with the fixative and treated with phosphate buffered saline (PBS, pH 5.8) containing trypsin (0.02%) (GIBCO Labs. Inc., MI, USA) for 6 min at 0 ℃. After they were washed with PBS, the cells were restained with the Giemsa solution and examined microscopically.

RESULTS

Construction of parental heterohybrids useful for cell fusion: Six 8-AG resistant mouse (P3UI)-human (normal PBL) heterohybrids (HM-1 to HM-6) which showed rapid growth, died in HAT medium, and secreted no human immunoglobulin were constructed. To obtain hybrids secreting human MoAb these heterohybrids were fused with normal human PBL and compared with other reported parental cells (human or mouse). One of the M·H lines, HM-5, was far superior to the human (LON) or mouse (P3UI) parental cells in the following points (data not shown). First, the frequency of obtaining hybrids between normal human PBL (1.66 per 10^6 human PBL) was about 2 times and 8 times higher than those when P3UI and LON cells were used as parental cells, respectively, and the frequency of obtaining human MoAb-secreting hybrids (0.38 per 10^6 human PBL) was about 13 times higher than that when LON cells were used as parental cells. Second, hybrids (M·H-H) between HM-5 and human PBL secreted human antibody (in this case, antigen specificity is unknown) for more than 7 months, whereas human antibody was not detected in hybrids between P3UI and human PBL after 3 passages. Third, clones were easily obtained from M·H-H by limiting dilution.

Establishment of hybrids secreting antigen specific human MoAb: To obtain hybrids secreting antigen specific MoAb, fusion experiments between the HM-5 cells and human PBL from anti-T.T. or anti-HBsAg antibody positive donors were carried out repeatedly. As shown in Table 1, two anti-T.T. MoAb-secreting hybrids (I12-22 and F12-24) from 2 independent fusion experiments and an anti-HBsAg MoAb-secreting hybrid (HBIII-43) have been established. The representative clones I12-22.25 and HBIII-43.1 obtained

Table 1. Establishment of hybridomas secreting antigen specific human MoAb using HM-5 as a parental cell line

Expt. No.	Stimulation of human PBL*1	Wells with viable hybrids	Established cell line	Binding specificity of MoAb
I12	PWM (32μg/ml), 3 days T.T. (1ng/ml)	33/34	I12-22	T.T.
F12	PWM (32μg/ml), 3 days	24/24	F12-24	T.T.
HBIII	PWM (32μg/ml), 2 days	56/80	HBIII-43	HBsAg

*1 PBL from anti-T.T. or anti-HBsAg antibody positive donor were used.

by cloning I12-22 and HBIII-43 hybrids, respectively, were selected and examined for the kinetics of growth and antibody production. As shown in Fig. 1, the I12-22.25 cells doubled every 22 hr and produced IgG1 antibody at the rate of 2.4 g/ml per 10^6 cells per day. The HBIII-43.1 cells doubled every 26 hr and produced IgG1 antibody at the rate of 0.6 g/ml per 10^6 cells per day.

Neutralizing activity of anti-T.T. MoAb against tetanus toxin (T. toxin): To examine whether the anti-T.T. antibodies neutralize T. toxin, 5 weeks-old ddH mice were injected subcutaneously with a mixture of 0.2 ml of various doses of T. toxin and the same volume of the concentrated (about 10-fold) culture supernatant of I12-22.25 or F12-24.6 (the representative

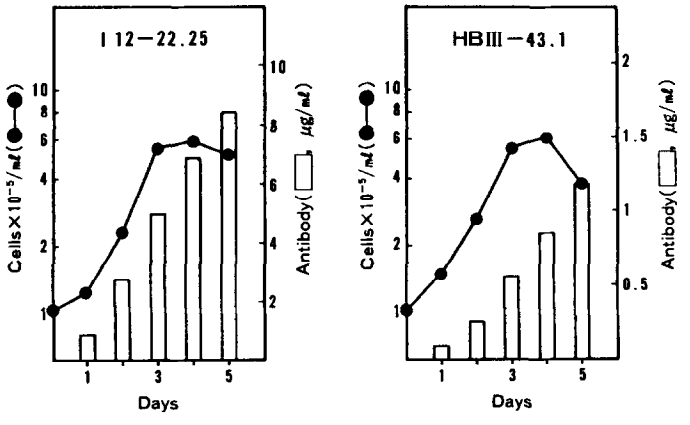

Figure 1. Kinetics of growth of, and antibody secretion by hybrids. Each hybrid was suspended at 10^5 per ml and seeded in a volume of 2 ml to the well of 24-well multiplate. Viable cells were counted in a hemocytometer by means of trypan blue exclusion. Culture supernatants were assayed for IgG antibody by ELISA method as described in MATERIALS AND METHODS.

Table 2. Neutralizing activity of human MoAb against tetanus toxin

Toxin*1 (U/ml)	Antibody			
	I12-22.25	F12-24.6	Mixture*2	-
2430	0/4	0/4	0/4	0/4
810	0/4	0/4	4/4	0/4
270	0/4	0/4	4/4	0/4
90	0/4	0/4	4/4	0/4
30	4/4	4/4	4/4	0/4

Two hundred microliters of each MoAb sample was mixed with the same volume of various doses of toxin and injected to mice subcutaneously. Results were indicated as the ratio of surviving mice (at day 4)/total mice injected.

*1 1U = LD_{50}.

*2 The mixture of equal volume of I12-22.25 and F12-24.6 MoAb.

clone of F12-24). As shown in Table 2, each of these MoAb had neutralized T. toxin, and a synergistic effect was observed when these MoAb were mixed.

Determinant recognized by anti-HBsAg antibody: To know which determinant of HBsAg was recognized by the HBIII-43.1 antibody, a blocking experiment was performed. Plasmas from 26 HBsAg positive donors (adr, 10; adw, 10; ayr, 1; and ayw, 5 donors) were used as blocking agents, and the binding of HBIII-43.1 antibody to sheep red blood cells coated with the mixture of adr, adw, ayr, and ayw of HBsAg was examined. The binding was blocked by all 26 plasmas (Yoshida et al., personal communication). The results indicated that the HBIII-43.1 MoAb recognized the a-determinant of HBsAg.

Chromosomal analyses: The chromosomes of P3UI, HM-5, and HBIII-43.1 cells were analysed. Representative patterns are shown in Fig. 2. HM-5 cells had 113 chromosomes with 6 metacentrics of which 4 were from P3UI cells; the other 2 were determined to be human chromosome No. 17 by the trypsin-Giemsa method. HBIII-43.1 cells had, in addition to human chromosome No. 17 from HM-5 cells, 13 metacentric human chromosomes including No. 14 and 22 on which the genes for the H chain and λ chain of human immunoglobulin are located, respectively.

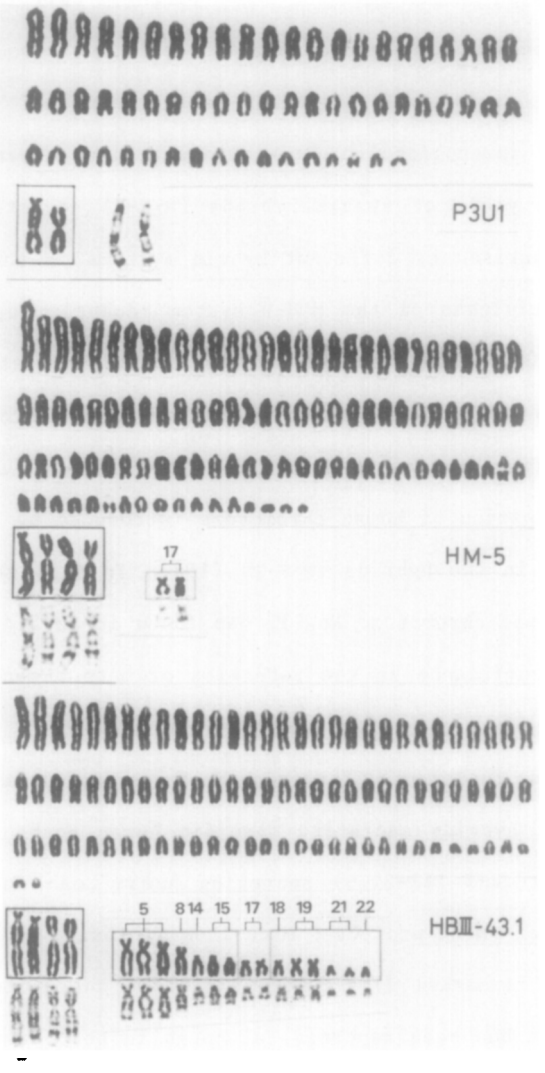

Figure 2. Representative chromosomal patterns of P3U1, HM-5, and HBIII-43.1 cells. The chromosomes were analysed by the Giemsa method. To make a fine analyses and differentiate human and mouse chromosomes, metacentric chromosomes were analysed by the trypsin-Giemsa method.

DISCUSSION

In order to establish useful parental cell lines to obtain hybrids secreting human MoAb, several kinds of fusion systems were compared in the present study. Normal human PBL were fused with either one of the following parental cell lines, LON (human), P3UI (mouse), or M·H (HM-1 to HM-6). As a number of previous reports (6-8, 11, 12) have pointed out, we also found in

repeated fusion experiments that the human parental cell line had a defect in fusion efficiency and the mouse parental cell line also had a defect in the stability of hybrids. On the other hand, the use of HM-5, one of the HAT-sensitive and immunoglobulin nonsecreting M·H, as a parental cell line resulted in high yield of stable hybrids (M·H-H) secreting human MoAb. Thus, direct comparison of different hybrid systems in the present study confirmed the usefulness of the M·H-H system recently reported by three groups (13, 19, 20). The reason for stable antibody secretion in M·H-H is unknown but it is speculated that human chromosome or chromosome-fragment retained by the first fusion (M·H) modifies the intracellular environment so that the elimination of human chromosome introduced by a second fusion may be inhibited in the hybrids (M·H-H) (19). In this sense, it is interesting that human chromosome No. 17 was found in HM-5. The chromosome may have some significance in the retention of more human chromosomes in HBIII-43.1.

Based on these fundamental findings, HM-5 was useful as a parental cell line to construct hybrids secreting specific human MoAb. First, two of hybrids (I12-22.25 and F12-24.6) secreting human MoAb to T.T. were established. Each of these protected mice from T. toxin, and the mixture of the two exhibited a marked synergystic effect on the protection. Such a cooperative effect has been reported, although in that case the MoAb were mouse origin (21). Second, a hybrid (HBIII-43.1) secreting human MoAb to HBsAg was established. This may be the first published report that a hybrid secreting human MoAb to HBsAg has been established. However, several such MoAb may be required for prophylactic and therapeutic use in HBV infection. Trials to obtain more hybrids secreting anti-HBsAg MoAb, using HM-5 as a parental cell line, are under way.

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