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ESTABLISHMENT OF STABLE MOUSE/HUMAN-HUMAN HYBRID CELL LINES PRODUCING LARGE AMOUNTS OF ANTI-TETANUS HUMAN MONOCLONAL ANTIBODIES WITH HIGH NEUTRALIZING ACTIVITY

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To establish stable hybrid cell lines producing human anti-tetanus antibody with high toxin-neutralizing activity, peripheral lymphocytes from humans hyperimmunized with tetanus toxoid were, after in vitro antigen stimulation, fused with a mouse/human heteromyeloma or human lymphoblastoid cell line and cloned. Unlike the IgM secretors (six clones), the IgG secretors we obtained (six clones) produced anti-tetanus human monoclonal antibodies with high neutralizing activity (the highest one, cell line G2, 4.3 IU/100 μg IgG).

Appropriate combinations of three or four kinds of monoclonal antibodies of the IgG type resulted in markedly increased neutralizing activity comparable with that of anti-tetanus human polyclonal immunoglobulin preparations currently used clinically on the basis of toxin-specific IgG content. Five of these cell lines produced 10~20 μg of antibody per ml for more than 3 months. The cell line G2 produced 6 mg of antibody per day in serum-free medium in a 500-ml bioreactor in perfusion culture and 13-104 mg in a nude mouse. These cell lines satisfied, for the first time, the minimal requirements for applying human monoclonal antibodies to clinical use.

INTRODUCTION

The potential of monoclonal antibodies (MAbs) produced by cell hybridization for prophylaxis and therapy of infections and malignancy has been amply shown using murine antibodies (18, 25, 28). For clinical use in immunotherapy, however, human MAbs are better than murine MAbs because their use does not cause the problem of sensitization to foreign proteins resulting from administration of heterologus (murine) immunoglobulins.

Cell hybridization to produce human MAbs for immunotherapy has in general been hampered by the difficulty of obtaining sufficient human B cells producing specific antibody to the antigen involved in the disease aimed for study, because sufficient number of B cells appear in the peripheral blood only in humans who have been hyperimmunized with the particular antigen. However, for production of antibody against tetanus, immunized lymphocytes of defined specificity can easily be obtained because tetanus toxoid is a potent immunogen in humans, and healthy volunteers can safely be given booster doses of it. Moreover, tetanus toxin is a protein antigen,

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whose reaction with antitoxin, including not only binding activity, but also neutralization of toxicity (14, 17), has been extensively studied. Thus the establishment of hybrid clones to produce monoclonal anti-tetanus antibodies of human origin will not only be a significant advance in the immunotherapy of tetanus but will also provide a useful model of production of MAbs for immunotherapy of other human diseases. Accordingly, attempts to develop hybridomas secreting human anti-tetanus MAbs are currently in progress in several laboratories (10). But, to date only limited success has been reported: the levels of neutralizing activity of the MAbs secreted by hybridomas have been much lower than those of human polyclonal anti-tetanus antibodies, and the amounts of MAbs produced by these hybridomas have tended to decline during continued cell culture. Thus from both qualitative and quantitative standpoints, no hybridomas secreting human MAbs are yet available for production of MAbs for clinical use in prevention or treatment of tetanus. One reason that it has been difficult to generate favorable hybridomas is that suitable myelomas or lymphoblastoid cell lines have not been available as fusion partners. Recently, several experimental systems have been developed to establish cell lines secreting human MAbs: mouse-human heterohybridomas (2, 4, 5, 11), mouse/human-human heterohybridomas (7, 8, 26), human-human hybridomas (2, 13, 32) and Epstein-Barr virus (EBV) transformants of immunized human B lymphocytes (9, 30, 33).

In the present study, we examined stable hybrid cell lines that secrete anti-tetanus human MAb with high neutralizing activity, generated by fusion of immunized peripheral blood lymphocytes (PBL) with a human lymphoblastoid cell line, a mouse/human heteromyeloma that we developed or a commercially available mouse/human heteromyeloma to increase the fusion efficiency and to stabilize human chromosomes. We also tested conditions aimed at increasing the number of specific antibody-producing lymphocytes, including in vitro antigen stimulation of immunized PBL and EBV transformation of B lymphocytes.

As described here, we established several stable hybrid cell lines which continuosly secrete anti-tetanus antibodies of the IgG type with high toxin-neutralizing activity. Appropriate combinations of three or four of these human MAbs had neutralizing activities comparable with those of the human anti-tetanus polyclonal IgG preparations currently used clinically for prophylaxis and therapy of tetanus.

MATERIALS AND METHODS

Cell lines and cell culture

A mouse/human heteromyeloma cell line, SHM D-33 (ATCC CRL 1688, American Type Culture Collection, Rockville, Md.) (29), was purchased from ATCC. Another heteromyeloma line, RF-S1, was newly prepared by fusing a human myeloma line, RPMI 8226, with a mouse myeloma line, FO, as follows: Heteromyelomas were selected in HAT medium [RDF medium (a mixture of RPMI 1640, Dulbecco's modified MEM and Ham F12 medium in a ratio of 2:1:1) supplemented with 15% fetal calf serum (FCS), 10^{-4} M hypoxanthine (H), 4 x 10^{-7} M aminopterin (A) and 1.6 x 10^{-5} M thymidine (T)] containing 10 μM ouabain. Then 8-azaguanine-resistant mutants of the heteromyelomas were selected in RDF medium containing 15% FCS (RDF/FCS medium) and 8-azaguanine (100 μg/ml). The concomitant change to HAT sensitive cells of the resultant 8-azaguanine-resistant mutants was confirmed. From these mutants, a cell line, RF-S1, was chosen as a heteromyeloma fusion partner because of its high fusion and cloning efficiencies. The human lymphoblastoid cell line HO-323 was established by Ohashi et al. (23). The cell lines were cultured in RDF/FCS medium. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Lymphocytes

Human peripheral blood lymphocytes (PBL) were prepared from heparinized blood from five healthy donors who had been hyperimmunized with tetanus toxoid (TT). Peripheral blood obtained after immunization was diluted two-fold with RDF medium, and the PBL were separated by Ficoll Hypaque (Pharmacia LKB Biotechnology, Uppsala, Sweden) density gradient centrifugation.

In vitro antigen stimulation

The PBL were suspended in RDF/FCS medium containing pokeweed mitogen (PWM, 1:10,000, Gibco Inc., Grand Island, N.Y.) and various concentrations of TT (0 to 125 ng/ml). They were then cultured in a 24-well plate at a cell density of 5 x 10^5 cells/ml in 1 ml of medium per well for 5 to 7 days. The immunoglobulin levels of the culture supernatants were measured by ELISA as described below.

Epstein-Barr virus (EBV) transformation

B cells were isolated from post-immunization PBL using 2-aminoethyl iso-thiouronium bromide hydrobromide (AET)-treated sheep red blood cell (SRBC) rosettes, as described by Saxon et al. (27). The isolated B cells were transformed by EBV in the supernatant of a B95-8 cell culture. After absorption of EBV at 37°C for 3h, the B cells were pelleted by centrifugation, resuspended in RDF/FCS medium containing TT (5 ng/ml) and introduced into 96-well U-bottom microtiter plates at a cell density of 2 x 10^4 cells/200 μl per well. The U-bottom microtiter plates were used to separate the transformants from the other cells that collected densely in the U-shape bottom of the plates (19).

Fusion procedure

The fusion procedure to generate hybridomas was essentially the same as that described by Murakami et al. (21). Briefly PBL prestimulated with antigen in vitro or EBV transformed B cells were mixed with SHM D-33, RF-S1 or HO-323 cells at a ratio of 2:1 in RDF medium. The cells were fused by adding 50% polyethylene glycol (PEG 1500, Boehringer Mannheim, Federal Republic of Germany) in RDF medium. Ten to 15 days after fusion, MAb-positive wells were screened by enzyme-linked immunosorbent assay (ELISA) as described below. Hybridomas secreting anti-TT human MAbs were cloned by the limiting dilution method. Cloning procedures were repeated at least three times for each hybridoma.

Enzyme-linked immunosorbent assay (ELISA)

The reactivity of antibodies with TT was assayed by ELISA according to the method described by Hashizume et al. (6), except that TT (10 µg/ml) was used for coating and phosphate buffered saline containing 0.1% bovine serum albumin and 0.05% Tween 20 was used to prevent non-specific adsorption.

The immunoglobulin concentrations of the culture supernatants were determined by ELISA using the procedure described above except that plates coated with 10 µg/ml of goat anti-human IgG or IgM (Tago, Burlingame, Cal.) were used. Human IgG (Sigma Chemical Co., St. Louis, Mo.) and/or IgM solutions (Green Cross Co., Osaka, Japan) of known concentrations were used as standards.

IgG subclass was determined by ELISA with a human immunoglobulin G subclass identification kit (The Binding Site LTD, Birmingham, England).

The light chain type of IgG was determined by ELISA as described above using goat anti-human kappa or lambda chain (Tago, Burlingame, Cal.).

Domains of the tetanus toxin molecule to which MAbs bind specifically were also determined by ELISA using fragments of tetanus toxin [A-B] and [C] (24) and tetanus toxin (16).

Production of human MAbs by hybridoma cells in perfusion culture

Perfusion culture was performed using a culture stirring system, Model SHC-1 (Shimadzu, Co., Kyoto, Japan). Hybridoma cells were cultured in RDF medium containing 15% FCS in thirty to fifty 10-cm culture dishes for 3 days. Actively growing cells were collected by centrifugation, washed three times with RDF medium without serum and inoculated at a density of 2 x 10^5 cells/ml into 500 ml of serum-free RDF medium containing insulin (5 µg/ml), transferrin (35 µg/ml), ethanolamine (20 µM), selenium (2.5 nM) and human serum albumin (2 mg/ml), and incubated at 37°C with stirring at 60 rpm, keeping the medium at pH 7.2 and the concentration of dissolved oxygen at 5.2 ppm.

Production of human MAb by a hybridoma in nude mice

Hybridoma cells were washed twice with RDF medium and were injected intraperitoneally at 1 x 10^7 cells per mouse into nude mice (BALB/c origin) that had been treated with pristane (2, 6, 10, 14 - tetramethylpentadecane) 2 weeks previously. Ascites fluid was collected 2 to 8 weeks after the inoculation.

Toxin-neutralizing activity

The toxin-neutralizing activity of human MAbs was measured using OF1 mice of both sexes weighing 22 to 26 g. Test samples were injected intramuscularly into the hind legs of the mice. Standard tetanus test toxin (Lot TA-4B) and horse National Standard Tetanus Antitoxin (Lot B, 1 International Unit (IU) = 0.33898 mg), used as standards, were gifts from the National Institute of Health, Tokyo, Japan. The minimal lethal dose (MLD) of the standard test toxin was determined from the dose-response (time until death) curve as described previously (15). Times until death were determined by extrapolating the curve of progression of symptoms as described previously (15). When necessary, toxin, antitoxin, the samples of MAbs and human immune sera were diluted with PBS containing 0.2% gelatin (Difco Laboratories, Detroit, Mich.).

(i) Screening of toxin-neutralizing activity of MAbs

For highly sensitive detection of low toxin-neutralizing activity in the culture fluids of hybridomas, 4 volumes of the test sample containing MAb was mixed with 1 volume of standard test toxin solution (100 MLD/ml) and the mixture was incubated at 37°C for 60 min. Then, 0.5 ml of the mixture (containing 10 MLD of the standard test toxin per mouse) was injected. In the absence of any neutralizing activity, 10 MLD of the test toxin killed a mouse 36h after its injection, but the presence of toxin-neutralizing activity delayed death. In the range of toxin doses of 10 MLD to 1 MLD, the dose-response (time until death) curve (15) changed almost linearly and remarkably from 36 h to 96 h, and a significant increase in the time until death definitely showed the presence of toxin-neutralizing activity.

(ii) Quantification of toxin-neutralizing activity

The test MAb solution was diluted appropriately in $10^{0.5}$-fold steps and the diluted MAb solutions were mixed with standard test toxin solution. The mixtures were incubated and injected into mice as described above, except that in this case toxin solution containing 200 MLD/ml was used. If the test sample did not contain any neutralizing activity, the 20 MLD

(per mouse) of test toxin in the sample injected killed mice 26 h after its injection. Under these conditions, neutralizing activity was assayed at very diluted concentrations of antitoxin (MAb) and toxin to make sure that the MAbs obtained had high avidity and neutralized the toxin with high affinity. A human polyclonal anti-tetanus immunoglobulin preparation (Tetanobulin Lot 076, Green Cross Co., Osaka, Japan), of known International Units (IU) was used as a standard. Comparison of the curve of progression of symptoms seen with the mixture of the standard antitoxin and the test toxin, with that seen with the test MAb and the standardized test toxin, allowed us to determine the mixture of toxin and appropriately diluted MAb that killed mice 96h after its injection and to calculate the neutralizing activity of the test MAb sample from the corresponding IU of the standard antitoxin immunoglobulin. Groups of 6 to 13 mice were used for determination of each value. Experiments were repeated at least three times.

(iii) Assay of antitoxin titers of human polyclonal antitoxin sera

The neutralizing activities of human immune sera were determined by the method routinely used for human polyclonal antisera, using test doses of toxin at $L_+/10$ and $L_+/100$ levels which had been standardized using standard horse antitoxin. A volume of 0.4 ml of mixtures containing appropriately diluted antiserum and the standardized toxin (1 test dose) was injected per mouse.

Purification of human MAbs

MAbs produced in culture supernatants of serum-free medium were purified by affinity column chromatography on a Protein A-Cellulofine (Seikagakukogyo Co., LTD., Tokyo, Japan) column (1.4 x 12.5 cm) and by ion-exchange chromatography on a prepacked column of Mono S HR5/5 (Pharmacia LKB Biotechnology, Uppsala, Sweden). To the culture supernatant (500 to 950 ml), glycine (final concentration 1.5 M) and NaCl (final concentration 3 M) were added and the pH of the mixture was adjusted to 8.9 with NaOH. The mixture was then applied to a Protein A-Cellulofine column equilibrated with 1.5 M glycine-NaOH buffer (pH 8.9) containing 3 M NaCl. Material was eluted with 0.1 M citrate buffer (pH 3.0). The fraction containing MAb was collected and dialyzed against 10 mM acetate buffer (pH 6.0) (buffer A) and further applied to a Mono S column equilibrated with buffer A. Material was eluted with a linear gradient of NaCl (0.01 M NaCl increase per min) formed using buffers A and B (buffer B is buffer A supplemented with 0.5 M NaCl) at a flow rate of 0.5 ml/min in a fast-protein liquid chromatography (FPLC) system (Pharmacia LKB Biotechnology, Uppsala, Sweden). Then the fraction containing MAb was dialyzed against PBS. The purity of the MAb preparations was examined by SDS-polyacrylamide gel electrophoresis (12) and silver staining (22).

Tetanus toxoid (TT)

For immunizing human volunteers, an alum-precipitated TT preparation (Kanonji Institute, the Research Foundation for Microbial Diseases of Osaka University, Kanonji, Kagawa, Japan) was used. For in vitro stimulation of PBL and for ELISA, TT prepared by formalin-treatment (17) of tetanus toxin that had been highly purified by the method described previously (16) was used.

Fragments of tetanus toxin

Fragmants [A-B] and [C] of tetanus toxin were prepared as described previously (24).

Examination of mycoplasma

Contamination of the cell culture with mycoplasma was evaluated using a mycosplasma detection kit, Mycotrim-TC™ (Hana Media, Inc., Berkeley, Cal.).

RESULTS

In vitro antigen stimulation of PBL before fusion

To determine the optimal antigen concentration for in vitro stimulation of immune PBL in the presence of pokeweed mitogen, we examined the effect of the concentration of TT on the production of immunoglobulins by the PBL. Fig. 1 shows that, when PBL were incubated with various concentration of TT for 5 days, their secretion of IgG specific for TT was highest in the presence of TT at 5 ng/ml, but that their secretion of total IgG was not influenced much by the concentration of TT. In contrast, the concentration of TT had no effect on their secretion of either IgM specific for TT or total IgM. During incubation, colonies of PBL induced to blast formation continued to increase in size for 5 days. However, in vitro antigen stimulation for more than 6 days resulted in a gradual decrease in the viability of the PBL, as judged from change in their morphological appearance. Similar results on optimal conditions were obtained for PBL derived from different individuals. Therefore, we stimulated PBL with 5 ng TT/ml for 5 days before fusing them with fusion partners.

Human anti-tetanus of IgM type MAbs produced by human-human bybrid cell lines

Human anti-tetanus MAbs produced by hybrid cell lines obtained by fusion of the PBL with the human lymphoblastoid line HO-323 were all found to

be of the IgM type (Table 1). No neutralizing activity was detected in the culture supernatants of these hybrid cell lines although TT-binding activity was detected by ELISA. The neutralizing activity was examined further after concentrating the culture supernatants by ammonium sulfate fractionation to increase the sensitivity of the assay. Table 1 summarizes the findings on the toxin-neutralizing activities of the concentrated culture supernatants of these hybrid cell lines. These human anti-tetanus MAbs showed little, if any, toxin-neutralizing activity. Only one hybrid cell line, M2, produced human anti-tetanus MAb with significant neutralizing activity (Table 1). The combinations of two to four of these

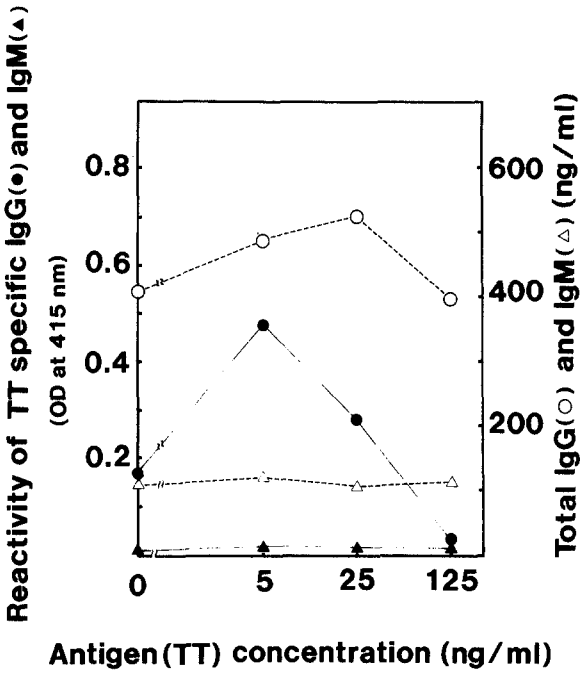

Figure 1. - Effect of concentration of tetanus toxoid (TT) on in vitro stimulation of PBL. The PBL of a volunteer (KU) were cultured in RDF medium containing 15% FCS, pokeweed mitogen (1:10000) and various concentrations of TT (0 to 125 µg/ml). Culture supernantants were harvested after incubation for 5 days and their total IgG (○) and IgM (△) concentrations were assayed by ELISA with purified human IgG and IgM as standards. TT-specific IgG (●) and IgM (▲) levels were assayed by ELISA as described in the Materials and Methods.

TABLE 1. - Toxin-neutralizing activity of human anti-tetanus monoclonal IgM type antibodies.

Antibody	Concentrated culture supernatant*	
	Neutralizing activity (IU**/ml)	IgM content*** (µg/ml)
MAb produced by hybrid cell line		
M1	< 1/3000	50
M2	1/300	50
M3	< 1/3000	50
M4	< 1/3000	30
M5	< 1/3000	84
M6	< 1/3000	150
Mixture**** of MAbs		
[M1, 2]	1/300	50
[M1, 3]	< 1/3000	50
[M1, 2, 3]	< 1/3000	50
[M4, 5, 6]	< 1/3000	88
[M2, 4, 5, 6]	1/300	79

* Culture supernatants were concentrated by ammonium sulfate fractionation (0 to 40% saturated fraction). Neutralizing activity and IgM content were expressed by those per ml of the ammonium sulfate concentrates.

** IU, International Unit.

*** Assayed by ELISA as described in the Materials and Methods.

**** Mixture of equal volumes of the concentrated culture supernatants of each MAb.

antibodies did not significantly increase the neutralizing activity on the basis of the IgM content (Table 1).

High toxin-neutralizing activities of IgG type MAbs produced by mouse/human-human hybrid cell lines

Six human anti-tetanus MAb-secreting hybrid cell lines were obtained by fusion of human PBL with mouse/human heteromyelomas. One hybrid cell line (G1) was obtained by fusion of cell line SHM D-33 with EBV-transformed B cells (designated as the E series), four hybrid cell lines (G2, G3, G4 and G5) were obtained by fusion of immunized PBL with cell line SHM D-33 (HM series), and one hybrid cell line (G6) by fusion of immunized PBL with cell line RF-S1 (RF series) (Table 2). No fried egg-like colonies characteristic of mycoplasma were observed on examination of the culture of the hybrid lines by a mycoplasma detection kit. All these hybrid cell lines

TABLE 2. - Toxin-neutralizing activity of human anti-tetanus monoclonal IgG type antibodies.

Series	Antibody	Ig class		Neutralizing activity*
		H-chain	L-chain	(IU**/100 µg IgG***)
	MAb produced by hybrid cell line			
E	G1	γ1	λ	0.43
HM	G2	γ1	λ	4.3
	G3	γ1	λ	0.043
	G4	γ1	κ	0.014
	G5	ND*****	ND	0.0043
RF	G6	γ1	κ	1.4
	Mixture**** of MAbs			
	[G1, 2, 3, 4, 6]			43
	[G 2, 3, 4, 6]			22
	[G1, 2, 4, 6]			22
	[G1, 2, 3, 6]			22
	[G1, 2, 3, 4,]			14
	[G 2, 4, 6]			43
	[G1, 2, 6]			43
	[G 2, 3, 6]			14
	[G1, 2, 3,]			14
	[G1, 2, 4,]			14
	[G 2, 3, 4]			0.014
	[G1, 2]			14
	[G 2, 4]			14
	[G 2, 3]			4.3
	[G1, 3]			1.4
	[G1, 4]			1.4
	[G 3, 4]			0.043

* Activities are for ammonium sulfate concentrates (0 to 40% saturated fraction of culture supernantants). Groups of 6 to 13 mice were used for determination of each values. The values of all antibody formulations were titrated in the same experiment.

** IU, International Unit.

*** IgG contents were assayed by ELISA as described in the Materials and Methods.

**** Mixture of equal amounts of each MAb.

***** ND, not done.

were found to secrete human MAbs of the IgG type. Therefore, the antibodies produced were designated as MAb-G1 to MAb-G6, respectively. All of these MAbs had heavy chains of γ1 type (MAb-G5, not determined) (Table 2). Table 2 shows that these human anti-tetanus MAbs of the IgG type had much higher toxin-neutralizing activity (on the basis of IgG content) than those of the IgM type (on the basis of IgM content). Of these IgG type MAbs, MAb-G2, secreted by the hybrid cell line G2, showed the highest neutralizing activity (4.3 IU/100 μg IgG) which was very high (Table 2). Mixture of IgG type MAbs showed marked synergistic neutralizing activities. The best combinations were the mixture of MAbs-G1, G2, G3, G4 and G6, the mixture of MAbs-G2, G4 and G6 and the mixture of MAbs-G1, G2 and G6. The neutralizing activities of these mixtures were all 43 IU/100 μg IgG (Table 2). As single reagents, MAb-G2, G6, G1, G3 and G4 at sufficient doses protected mice completely against the effect of tetanus toxin: e.g. these individual MAbs prevented death due to tetanus toxin (20 MLD) in mice at doses of 0.028, 0.089, 0.28, 0.89 and 2.8 μg, respectively.

Table 3 shows that all of these hybridomas were obtained using PBL from donors whose sera had very high neutralizing titers after booster injections with TT. No hybridomas secreting anti-TT antibody were obtained using PBL from donors who showed low responses to injected TT. The establishment of hybridomas secreting anti-TT antibody was not dependent on the time after the last injection of TT (Table 3), as shown by the finding that two hybrid cell lines, G2 and G6, which secreted MAbs with high neutralizing activities, were obtained from the same donor, KU, using PBL prepared 8 and 17 months, respectively, after the last injection of TT.

Stable production of human anti-tetanus IgG type MAbs by hybrid cell lines of the G-series

The six hybrid cell lines obtained were all cloned more than three times. Even after repeated cloning, the culture supernatants obtained from all wells with growing cells reacted with TT in ELISA. Moreover, these cloned cell lines produced human anti-tetanus MAbs for at least 3 months, and during this period the neutralizing activity did not change significantly on the basis of IgG content. Table 4 shows the productions of human anti-tetanus MAbs of the IgG type by various hybrid cell lines of the G-series. Concentrations of 10~20 μg of IgG/ml were obtained in the culture supernatants of 5-day cultures of all the cell lines except G5, which secreted much less IgG (Table 4).

Production of human anti-tetanus MAb by hybrid cell line G2 in serum-free medium and in nude mice

Since cell line G2 secreted MAb with the highest toxin-neutralizing activity on the basis of IgG content, we further examined the antibody production by this cell line in serum-free medium and in nude mice. Figure 2 shows the growth of hybrid cell line G2 and the kinetics of its MAb production in dishes containing medium with and without FCS. Cell line G2 grew as well in serum-free medium as in medium containing serum and produced similar amounts (ca. 12 μg IgG/ml) of anti-TT IgG antibody in 5 days in the two media (Fig. 2). Hybrid cell line G2 also produced MAb with culture stirring in a 500-ml bioreactor in serum-free medium: the concentration of MAb increased to a plateau 6 days after the start of the culture with a perfusion rate of 800 ml per day. The

TABLE 3. - Neutralizing titers of human anti-tetanus immune sera of donors.

Donors from which PBL were obtained	Neutralizing titer* (IU/ml)	Hybrid cell line generated	Time of bleeding for PBL after last immunization (month)
KU	20	G1	7
		G2	8
		G3	8
		G6	17
IW	20	G4	1
		G5	1
KM	1	-**	1, 3, 5, 8
TK	0.3	-	5
KR	< 0.03	-	1

* Neutralizing titer of immune serum determined two weeks after the last immunization. IU, international Units.

** Hybridomas were obtained, but no hybrid cell lines that secreted significant amounts of neutralizing antibody were obtained.

TABLE 4. - Production of human anti-tetanus monoclonal antibodies of the IgG type.

Hybrid cell line	MAb in culture supernatant	
	IgG content* (µg/ml)	Production rate** (pg/cell/d[illegible])
G1	12	6.5 ± 1.8
G2	12	6.4 ± 0.6
G3	11	12 ± 3.3
G4	20	23 ± 5.4
G5	0.8	0.076 ± 0.072
G6	12	8.4 ± 3.8

* Hybrid cell lines were suspended at 2 x 10^5 cells/ml in medium containing serum and volumes of. 2.5 ml of suspension were introduced into 35-mm culture dished and incubated for 5 days. IgG contents of culture supernatants of 5-day cultures were assayed by ELISA as described in Materials and Methods.

** Cell numbers and IgG contents of the cultures were determined daily. Production rate is the increase in IgG content divided by the geometrical mean of the initial and final cell numbers for each period (one day). Average production rates were given with SD.

concentration in the spent medium was continuously about 7 µg of IgG/ml from day 6 (Fig. 3). Therefore, cell line G2 produced 5 to 6 mg of MAb per day in the 500-ml bioreactor. Cell line G1 produced a much larger amount (26~39 mg per day) of MAb than cell line G2 in serum-free medium in the 500-ml bioreactor in perfusion culture.

Cell line G2 produced a large amount of MAb (13 to 104 mg of anti-TT IgG in 7 to 20.8 ml of ascitic fluid per mouse) in the ascites of nude mice.

Purification of MAbs

MAbs produced by these hybrid cell lines in the culture supernatants of serum-free medium were purified by affinity column chromatography on a Protein A-Cellulofine column. Purified preparations of MAbs showed almost single protein bands of mol. wt. ca. 150 K on SDS-gel electrophoresis. The recovery was 70 to 90%. They showed the same toxin-neutralizing activity as that of the corresponding culture supernatant on the basis of IgG content. MAbs-G2 and G6, which showed very high neutralizing activity among these antibodies, were further purified by ion-exchange column chromatography on a Mono S column. The recovery at this step was 69 to 74%. Figure 4 shows the electrophoretic patterns of the purified MAb-G2 and G6 preparations on SDS-polyacrylamide gel electrophoresis under reduced conditions. The

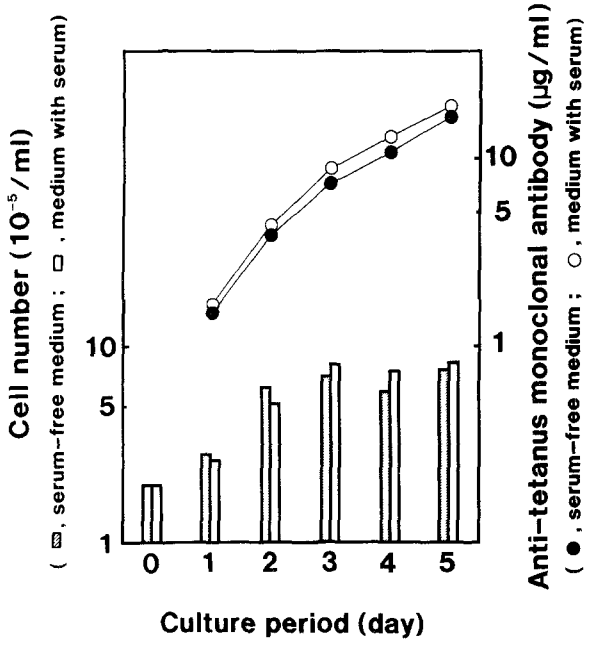

Figure 2. - Production of MAb in medium with (○) and without (●) serum by cell line G2. Cells were washed three times with RDF medium without serum and suspended at 2 x 10^5 cells/ml in medium with or without serum and 2.5 ml aliquots of each cell suspension were incubated in 35-mm culture dishes for the indicated periods. Open and hatched bars indicate cell growth in medium with and without serum, respectively.

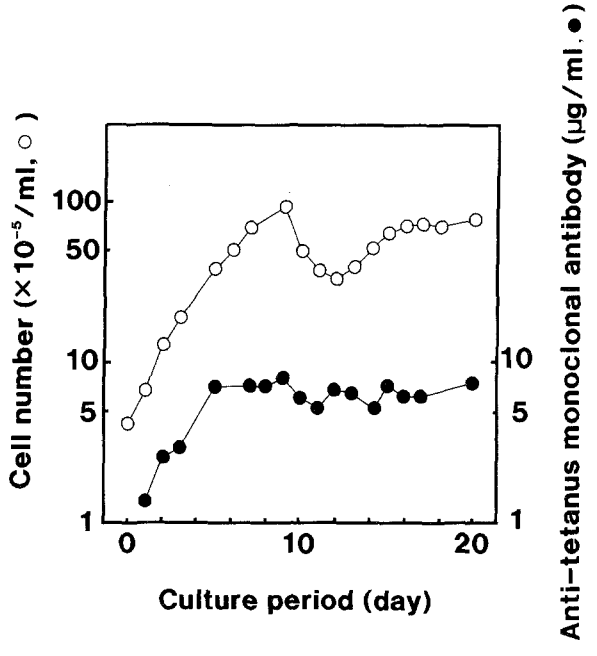

Figure 3. - Growth (○) of hybrid cell line G2 and its production (●) of MAb in perfusion culture in a SHC-1 (Shimadzu) stirring culture system. Cells were inoculated into 500 ml of serum-free medium at a cell density of 4 x 10^5 cells/ml. Details of culture conditions are described in the Materials and Methods.

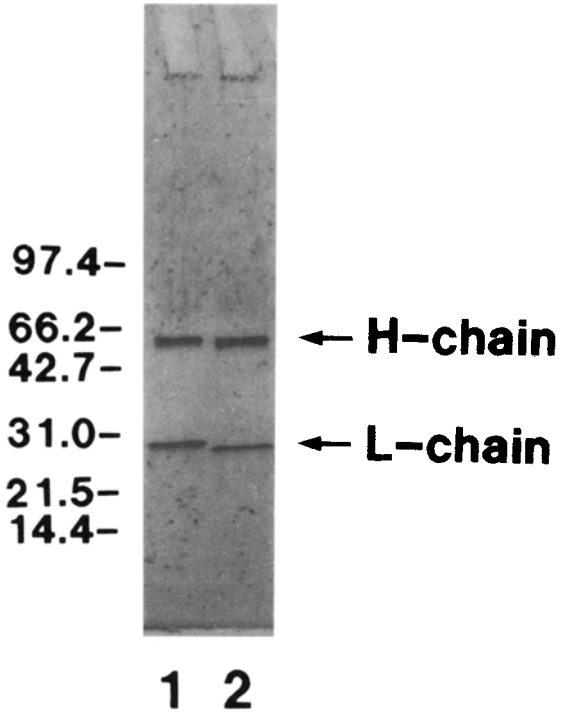

Figure 4. - SDS-polyacrylamide gel electrophoresis (4~20% gradient gel) of purified anti-tetanus human MAb: lane 1, reduced MAb-G2; and lane 2, reduced MAb-G6. Molecular weights (X 10^3) are shown on the left. H-chain, heavy chain; and L-chain, light chain. Silver stain.

TABLE 5. - ELISA values of monoclonal antibodies versus toxin and toxin fragments.

Monoclonal antibody*	ELISA titer versus:		
	Fragment [A-B]	Fragment [C]	Toxin
G1	1.041	0.376	1.795
G2	0.557	1.819	1.842
G3	1.215	0.123	1.805
G4	1.574	0.553	1.909
G5	ND**	ND	ND
G6	1.395	0.438	1.818
Tetanobulin***	1.505	1.562	1.830

* 0.1 µg IgG/ml

** ND = not done

*** 5 µg IgG/ml

purified MAb preparations each gave two bands corresponding to heavy and light chains of immunoglobulin respectively, demonstrating that the preparations were highly purified (Fig. 4).

Antibody characterization

Table 5 shows the reaction profile of each MAb preparation against tetanus toxin and toxin fragments in the ELISA. All MAb preparations showed strong reactivity with toxin. The preparations fell into two group: (i) MAb-G2 preparation which showed strong reactivity to Fragment [C] but reacted poorly with Fragment [A-B]; and (ii) other preparations which reacted strongly with Fragment [A-B] but poorly with Fragment [C] (Table 5).

DISCUSSION

In the present study we established stable hybrid cell lines that secreted human anti-tetanus MAbs with high toxin-neutralizing activity. These hybrid cell lines produced the human MAbs in serum-free medium in a perfusion culture system as well as in ascites of nude mice. These cultured hybridomas provide the less limited source of human antibody than human donor blood plasma.

Since the discovery of antitoxin therapy, horse polyclonal antitoxin serum preparations have long been used for immunotherapy of tetanus. But in humans horse antitoxin is a foreign antigen and may induce serum sickness. Therefore, homologous, human polyclonal antitoxin antibody in the form of an immunoglobulin preparation is currently usually used for prevention and therapy of human tetanus. However, the human antitoxin preparations now available are obtained from the plasma of hyperimmunized donors, and their supply is thus limited, while there is an increasing need for human antitoxin against tetanus (1). Moreover, use of donor blood plasma as a source of antitoxin involves the risk of possible infection with viruses such as HIV present in the donor plasma. Therefore, hybrid cell lines that secrete human MAbs against tetanus in vitro should be new and better sources of antibody for clinical use.

If MAbs are to be used clinically, at least six requirements must be satisfied for the hybrid cell lines obtained: (i) these lines must secrete human MAbs, (ii) they must produce MAbs stably, (iii) their antibodies must have a high neutralizing activity for toxin, comparable with that of the human polyclonal antitoxin preparation currently used clinically, (iv) they must produce at least the same amount of antibody in serum-free medium that they produce in serum-containing medium, (v) the amounts of the antibody they produced in vitro must be sufficiently high for their large scale production in continuous culture and (vi) the antibodies must be readily purified. No hybrid cell lines that meet all these requirements have so far been reported.

In the present study, we isolated six hybrid cell lines that secrete tetanus toxin-neutralizing human MAb of the IgG type. In our previous studies, hybrid cell lines producing human MAbs often showed decreased production after repeated cloning. However, the hybrid cell lines reported here were cloned more than three times without any decrease in their MAb production, and have secreted human MAbs constantly for more than three months. Therefore, these cell lines are considered to be stable producers of MAbs. All these IgG type MAbs had much higher toxin-neutralizing activity than those of the IgM type MAbs we first isolated. Of these IgG type MAbs, those produced by the hybrid cell line G2 were found to have the highest neutralizing activity, which was as high as 4.3 IU per 100 μg of IgG (Table 2), and the antibody by itself completely neutralized the effect of tetanus toxin. The neutralizing activity of 43 IU per 100 μg of IgG obtained with an appropriate combination of the MAbs produced by cell line G2 and those of two or three other cell lines was comparable with that of the human polyclonal antitoxin preparation currently used clinically on the basis of toxin-specific IgG content and was considered to reach the theoretically maximal neutralizing activity for the following reason. The molecular weight of tetanus toxin (ca. 150,000) is similar to that of the IgG molecule and 1 IU of tetanus antitoxin determined for polyclonal antitoxin antibody is equivalent to approximately 3 μg of tetanus toxin. Therefore, 3 μg of polyclonal IgG specific for tetanus toxin is considered to have a neutralizing activity of 1 IU. Thus, 100 μg of polyclonal IgG antibodies specific for tetanus toxin is calculated to have an average neutralizing activity of approximately 33 IU. The apparently low synergistic effects among our MAbs (e.g. only 10-fold that of the antibody produced by cell line G2 alone) in comparison with the very high synergism (~100-fold) of the MAbs reported previously [31, 32] was due to the fact that our MAb-G2 alone already had very high neutralizing activity without cooperation with other antibodies. Our "oligoclonal" antibody preparations composed of mixtures of the MAbs described above showed the same kinetics of toxin neutralization as human polyclonal antibody (detailed data to be published elsewhere). The maximal neutralizing activity, on the basis of IgG content, obtained with an appropriate combination of the anti-tetanus MAbs (which are composed only of TT-specific IgG molecules) reported here accordingly enables us to minimize the amount of immunoglobulin for administration to humans. In contrast to MAbs, the human anti-tetanus polyclonal immunoglobulin preparations contain antibodies against many other antigens besides tetanus toxin. In fact we found that the human polyclonal anti-tetanus immunoglobulin preparations currently used require 50 to 100 times as much IgG as that of the mixture of the MAb preparations described above to achieve the same neutralizing potency.

The present study shows that it has now become possible to continuously produce as much as 5 to 6 mg of IgG of MAbs per day in a 500-ml spinner flask in perfusion culture in serum-free medium (e.g. by cell line G2) (Fig. 3). These MAbs could be readily purified by a Protein A-Cellulofine column and further purified with ion-exchange chromatography on a Mono S column (Fig. 4) without decrease in specific neutralizing activity. We also found that as much as 104 mg MAb per mouse could be produced by the hybrid cell line G2 in nude mice. Thus, both perfusion culture in serum-free medium and preparation in ascites of nude mice are useful for production of MAbs for practical application. Simple preliminary calculations of the costs of medium and cultivation in a perfusion system to produce a mixture of MAbs with the same potency as that of anti-tetanus human polyclonal immunoglobulin preparations currently used clinically showed that the former were about one-fifth the commercial price of producing the latter. However the development costs, scale-up difficulties after the laboratory experiment and regulatory issues related to gaining approval must also be calculated. Some hybrid cell lines (e.g. G1, G4) produce much larger quantities of MAbs than cell line G2 in perfusion culture. Thus synergism of the MAbs including MAbs-G1 and G4 will further reduce the cost of production of the MAbs for optimal mixture.

The establishment of hybrid cell lines suitable for clinical use seemed to be dependent on the immune status of the lymphocyte donors, because all the hybrids reported here were obtained from donors with high serum titers of neutralizing antibody after boosting (Table 3). The period after boosting apparently did not affect the generation of desirable hybrid cells, because hybridomas were obtained using lymphocytes prepared from the same donors at various time (7 to 17 months) after boosting. This is in contrast to a report (9) of the importance of the time after the last immunization in preparing the lymphocytes for generation of favorable hybridomas. Further investigations are required on the critical factors for generation of suitable hybridomas.

The synergistic effects of combinations of the MAbs described above, together with the additive effects of individual MAbs on a protein basis (Table 2) indicate that all these six MAbs of IgG type are directed against different epitopes in the tetanus toxin molecule. All of these MAbs belong to subclass γ1 (Table 2). Thus the difference in the neutralizing activity of the MAbs could not be related to the difference in subclass of IgG. Preliminary tests on the reactivity in ELISA of the MAbs with tetanus toxin and toxin fragments showed that MAb-G2, which showed the highest toxin-neutralizing activity, recognized fragment [C] of tetanus toxin, while all of the other MAbs, with various degrees of neutralizing activity, reacted with fragment [A-B] complementary to fragment [C]. Therefore the antitoxin epitopes were distributed over the domains of the tetanus toxin molecule. The exact relationship between the

neutralizing activity and the localization of the epitopes for the MAbs remains to be investigated. Precise comparative analysis using labeled antibodies and detailed study on the localizations of these epitopes in the domains of the tetanus toxin molecule are now in progress in our laboratory.

Antibodies suitable for human use must not only neutralize toxin but must persist in the host. Anti-tetanus human polyclonal antibody, which is a homologous protein to humans, has been reported to have a much longer half life in humans than the heterologous horse antitoxin antibody (3). Thus human MAbs are expected to have half lives similar to those of the human polyclonals in humans, although this aspect remains to be investigated.

The present results show that our hybrid cell lines are the first to meet all the requirements described above for clinical use of human MAbs. Therefore, the establishment of these hybrid cell lines is a definite step towards the ultimate goal of administration of human MAb for prevention and therapy of tetanus. The present system should also be useful as a model for development of human MAbs for prevention and treatment of other infectious diseases and tumors.

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