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Renewal of EBV-hybridoma method: Efficient generation of recombinant fully human neutralizing IgG antibodies specific for tetanus toxin by use of tetroma cells

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Abstract. We have generated tetroma cell lines by the fusion of newly developed parental 6JC5.2 cells with a human B cell line, which was transformed with Epstein-Barr virus (EBV) and enriched with antibody-forming cells that produce neutralizing antibodies to tetanus toxin (TT) by a limiting dilution method using IL-6. The resultant two tetroma cell lines stably produced different monoclonal antibodies (mAbs), TT1 (IgG1·λ) and TT2 (IgG4·κ) reactive with TT after three-times consecutive cell cloning. Although weak to almost nonexistent neutralizing activities against TT were detected in TT1 and TT2 mAbs, respectively, mixing of them resulted in a dramatic increase in the neutralizing activity and complete protection from the toxin was observed *in vivo*. Moreover, functional immunoglobulin (Ig) genes were cloned from at least 10 cells in the first cloning step of tetromas after the cell fusion. None of the endogenous Ig genes, derived from the parental cell that hinders functional Ig gene cloning, was amplified. In addition, the EBV genome derived from the B cells was eliminated from the antibody producing tetroma lines. This classical but revised EBV-hybridoma method using fusion partner 6JC5.2 may become one alternative method for production of fully human antibodies useful for prevention and treatment of infectious diseases and cancer.

Keywords: Fully human monoclonal antibody, tetroma cells, Ig gene-cloning, tetanus toxin

1. Introduction

Recently, antibody-based therapeutics have attracted attention, and more than ten monoclonal antibody (mAb) products have been approved as biopharmaceuticals by the FDA and are used currently in the treatment of tumors or transplant rejection [1,2]. Most of the therapeutic mAbs, which are currently available or in clinical studies, are fully or almost fully human antibodies derived from chimerization and humanization of rodent Abs, phage libraries, or transgenic mice bearing human immunoglobulin (Ig) loci [3,4]. Interestingly, none of them is produced thorough the selective and regulatory processes of the human immune system. For a more safe and effective therapeutic approach, it is desirable to use a fully human monoclonal or polyclonal antibody created by an intact human immune system.

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During the past three decades, there have been considerable efforts to produce a fully human mAb using native human B cell sources. In the 1970s, so as to immortalize the human B lymphocytes producing specific antibodies against the appropriate antigens, many researchers reported they could be transformed by *in vitro* Epstein-Barr Virus (EBV) infection [5,6]. However, it has been difficult to completely establish immortalized human B cell lines while maintaining stable antibody production, because of the low efficacy of immortalization, the arrest of cell growth, and the dominant immortalization of IgM producing cells. Recent reports have shown that most EBV-transformed B cells have shortened telomeres and limited life spans [7,8]. In 1975, a technological innovation, namely the hybridoma technology, was introduced by Köhler and Milstein [9]. In this method, the antibody-producing B cells which were recovered from the hyperimmunized mouse with target antigen were fused with the appropriate parental myeloma cell lines to be immortalized. This technology enabled the easy production of mAb in mouse models. The attempts to utilize this method in human models have been repeated for a long time; however, it was difficult to establish stable human mAbs-producing cell lines because: 1) there are no suitable fusion partner cell lines to immortalize human B cells, while maintaining the stable production of specific antibodies; and 2) there are few B cells which produce specific antibodies against target antigens, since it is ethically impossible for human to be hyperimmunized with any pathogenic antigens. The latter reason which results in the low efficiency of cell fusion was partially overcome by the EBV-hybridoma method devised by Kozbor in the 1980s [10]. Primary human B cells producing antigen-specific antibodies were expanded *in vitro* by EBV-transformation, followed by cell fusion with an appropriate fusion partner. This method was considered as the most effective method of human mAb generation. However, since there are no fusion partners that enable long-term maintenance of human chromosomes encoding Ig loci without production of non-specific and endogenous Ig, the EBV-hybridoma method has not become a major strategy to generate fully human mAbs to date. In each case, a key problem in the preparation of fully human mAbs from natural human sources was the lack of suitable parental cell lines to enable the stable production of human mAbs.

Therefore, we reasoned that if a parental cell line were to become available that stably produces mAbs after fusing with human B cells, the production of fully human mAbs might become technically easier. In fact, the efforts to create a suitable fusion partner have been carried out for a long time, and fusion partners derived from human myeloma and others have been reported recently [11,12]. We have developed a trioma cell line, 6JC5.2, a hybrid of mouse-human heterohybridoma with a human B cell, as such a parental cell line.

In this study, we revived the EBV-hybridoma method with the trioma cell line 6JC5.2, and investigated whether this method could be applicable to the development of therapeutic mAbs as an alternative method. We fused 6JC5.2 cells with B cells isolated from one of the co-authors (H.S) who received booster immunization with tetanus toxoid (Ttd) and transformed by EBV *in vitro*. We show that the revised EBV-hybridoma system could well be superior to the previously reported method both in the reproducibility in generating hybridomas and the stability of their antibody production and growth. Furthermore, we show that cloning of functional Ig genes was possible using only about 10 cells at the first cell-cloning step after selecting the mAb producing hybridomas after the cell fusion. In addition, we show that the EBV genome derived from the Ab-forming B cell reached undetectable levels in antibody-producing hybridomas.

2. Materials and methods

2.1. Trioma cell line 6JC5.2 as fusion partner

A fusion partner 6JC5.2 cell line was a subline of a 3HL3-6 cell line originally established by Dr. Nelson N. Teng and Kit S. Lam at Stanford University in 1986 [13]. The original 3HL series cell lines were produced by a fusion between heteromyeloma SHM-D3 and B lymphocytes from a lymph node of a patient with ovarian carcinoma. The 3HL3-6 cell line was one of the triple hybrid clones, which were human × (mouse × human) hybridomas backselected for HAT sensitivity and as a immunoglobulin nonproducer. The 3HL3-6 cell line was reported to have 11 human and 68 mouse chromosomes, grew rapidly, carried both ouabain and G418 resistant markers, and to be excellent fusion partners [13] but no further characterization of this cell line was carried out (Teng N. N., personal communication). The 6JC5.2 cell line, which we call hereafter triomas, was selected from the 3HL3-6 line after

cloning by limiting dilution and evaluation of the resultant subclones in fusion ability with an EBV-transformed cell line, TAPC-301-4 cl6, that stably produced anti-HBs IgG antibodies, by means of electronic cell fusion [14]. Nucleotide sequences of TAPC-301-4 cl6 determined by using tetromas have been reported (NCBI GenBank AF 027158 and AF 027159, 1994) [16]. Preliminary report of detailed studies with a variety of parental cell lines using mouse myelomas, heteromyelomas, etc. including 3HL3-6 (Usagawa et al., 1986) has been cited in the review by Dr. K. James and T. Bell [15]. 6JC5.2 was maintained in Iscove's modified Dulbecco's medium (IMDM, Sigma) containing 15% FCS, 10 μg/ml gentamicin (Sigma), 50 μM 2-mercaptoethanol (Sigma).

2.2. Generation of tetromas

Peripheral blood samples were collected from a volunteer vaccinated with Ttd. PBMCs were isolated by Ficoll-paque density gradient centrifugation (density 1.077), and B cells were immortalized by cultivation in an IMDM medium containing 20% FCS, 10 μg/ml gentamicin, 50 μM 2-mercaptoethanol, 5 μg/ml bovine insulin (Sigma), and 8 μg/ml human transferrin (Boehringer Mannheim) containing EBV derived from an EBV-positive marmoset cell line B95-8 [10]. EBV-transformed B cell lines that produce anti-Tetanus Toxin (TT) antibodies were expanded to one well of a 24-well culture plate and stored frozen in liquid nitrogen. Enrichment of B cells producing TT-specific antibodies was carried out with the limiting dilution method in the same culture medium containing 1 U/ml or 4 U/ml of IL-6. The EBV-transformed B cell line was seeded at a ratio of 5, 10 and 50 cells per well, in 180, 120 and 60 wells, respectively. Specific reactivity to TT was screened by ELISA using Ttd as described below. 4.0×10^7 cells of the enriched EBV-transformed B cell line were fused with 1.0×10^7 cells of 6JC5.2 in the presence of PEG using the standard method [17]. The fused cells were seeded to the wells of 96-well culture plates (Coaster) at a ratio of 2.5×10^5 cells per each well, and selected by HAT (Sigma) and 0.5 nM ouabain (Sigma) [10]. Anti-TT antibodies-positive clones were cloned by three-times consecutive cell cloning with the limiting dilution method.

2.3. Specific reactivity to TT

TT-specific antibodies were detected with standard ELISA methods. Screening of culture supernatants of tetromas was carried out as below. Wells of microtiter plates (Coster) were coated with 0.5 μg/ml Ttd (Kaketsuken, Kumamoto, Japan) in a 50 mM sodium carbonate buffer (pH 9.6). Following an antigen coating overnight at 4 °C, the wells were blocked for 3 h at room temperature with 0.5% (w/v) gelatin in PBS(-). After washing with PBS(−) containing 0.05% (v/v) Tween 20 (PBS-Tween), each culture supernatant was added to the wells and the plates were incubated for 2 h at room temperature. Antigen-specific human antibodies were detected by incubation for 1 h at room temperature with alkaline phosphatase (ALP)-conjugated goat anti-human Igs (Biosource). The enzyme reaction was started by adding 1 mg/ml of *p*-nitrophenylphosphate in 1 M diethanolamine (pH9.8) containing 0.5 mM $MgCl_2$. The absorbance at 405 nm was measured with an AUTO READER III (Sanko Junyaku, Tokyo, Japan). As a positive control, 1,000-fold diluted serum of human infants vaccinated with DPT was used. Specific reactivity of mAbs to TT and Ttd was determined using a similar ELISA. Wells of microtiter plates were coated with 3.0 μg/ml purified TT (NIID, Tokyo, Japan) or inactivated TT fixed with 0.5% formalin for 13 h in 0.05 M $NaHCO_3$ buffer, pH 9.6. After blocking, four-fold serial dilutions of each of the purified mAbs were added to the wells and the plates were incubated for 1.5 h at room temperature. The detection of bound human mAbs and the enzyme reaction were carried out as described above. The experiments were performed in triplicate for each sample, and repeated independently three times. The data are presented as means ± standard deviations of one experiment.

2.4. Purification, isotype determination and quantification of human mAbs

Human mAbs were purified from culture supernatants by $(NH_4)_2SO_4$ precipitation followed by affinity chromatography using a HiTrap rProtein A FF column (Amersham Biosciences). Each tetroma was cultured in serum-free medium E-RDF (Kyokuto, Tokyo, Japan) containing RD-1 supplement (Kyokuto), and 10 μg/ml gentamicin. The purified antibodies were dialyzed against PBS (−), and the purity was determined by SDS-PAGE with silver staining. Isotypes and concentrations of human mAbs were determined by a sandwich ELISA using microtiter plates coated with a goat anti-human immunoglobulins antibody (Cappel). The IgG subclasses and light chain subtypes

were detected using the biotinylated mouse anti-human IgG1 (clone HP6069), IgG2 (clone HP6002), IgG3 (clone HP6047), IgG4 (clone HP6025) (ICN Biomedicals), and the biotinylated goat anti-human kappa or lambda chain (Biosource). The bound biotinylated antibody was detected with ALP-conjugated streptavidin (Gibco-BRL). The immunoglobulin isotypes were detected using murine ascites specific for human IgA, IgM (BRL), followed by detection with ALP-conjugated goat anti-mouse IgG + IgM (Biosource). In the estimation of antibody concentration, purified human IgG1 and IgG4 (Chemicon International) were used to generate standard curves. Bound antibodies were detected with ALP-conjugated goat anti-human immunoglobulins (Biosource). The enzyme reaction was carried out as described above.

2.5. Competitive binding assay to TT

Five hundred μg of each purified mAbs were biotinylated with 100 μg of biotinyl N-hydroxysuccinimid ester (NHS-LC-Biotin, Pierce), according to the manufacturer's instructions. The biotinylated mAbs were dialyzed against PBS (−). For the competitive binding assay, 50 μl of two-fold serial dilutions of unlabeled human mAbs as a competitor were added to the wells coated with TT and plates were incubated for 1 h at room temperature. Subsequently, 50 μl of 0.5 μg/ml biotinylated mAb were added to the wells and plates were kept for 1 h at room temperature to allow mixing. The detection of bound biotinylated antibodies and the enzyme reaction were carried out as described above. The experiments were performed in five points for each sample, and repeated independently three times. The results were expressed as the relative value to the average absorbance in the absence of the competitor.

$$\text{Relative value of binding} = \frac{\text{The average absorbance in the presence of competitor}}{\text{The average absorbance in the absence of competitor}}$$

2.6. Affinity determination of human mAbs

The binding affinity of the human mAbs against TT was determined using Biacore T100 (Biacore) on the basis of the procedure of indirect capture of mAbs [18]. Rabbit anti-human IgG Fc polyclonal Abs (Jackson Immuno Laboratories) were immobilized to a CM5 sensor chip (Biacore) as the capture antibody. Two μg/ml of each human mAb was applied at a flow rate of 10 μl/min within the level ranging from 25 to 500 resonance units. After binding stabilization for 300 sec, TT as the analyte was injected at a flow rate of 30 μl/min to reduce mass transport limitations for 120 sec, and dissociation of TT was observed for 180 sec. Injections of TT were performed with two-fold dilutions from 80 μg/ml in HEPES buffered saline (pH7.4) with 3 mM EDTA and 0.05% Tween 20. The surface was regenerated with 50 mM phosphoric acid to disrupt the interaction between the capture antibody and the human mAb. The affinity constant of each human mAb was estimated with Biacore T100 evaluation software (Biacore), according to the manufacturer's instructions. The experiments were repeated independently two times, and the data are presented as means ± standard deviations of two experiments.

2.7. In vivo TT neutralization assay

The ability of human mAbs to neutralize TT was evaluated in mice, using tetanus working test toxin (NIID, Tokyo, Japan) and tetanus antitoxin standards (NIID, Tokyo, Japan). The 50% lethal dose (LD_{50}) of the test toxin was calculated with the method of Reed and Muench [31]. For initial screening of the toxin-neutralizing activity of antibodies in culture supernatants of EBV-transformed B cells, the culture supernatant was mixed with 2 LD_{50} of TT. Mice surviving one week after the challenge without any symptoms were considered protected. The toxin neutralizing activities of each mAb produced by established tetromas were determined using doses of toxin at L+/100 levels [19]. Purified mAb and the mixture of both mAbs at a ratio of 1:1 were titrated and mixed with 400 mouse LD_{50} toxin, followed by 30 min incubation at room temperature. Complexes of mAb and TT were injected into the femoral s.c. of ddY mice (SLC, Shizuoka, Japan), age 4 weeks at the time of inoculation. Controls included the administration of TT alone. An *in vivo* neutralization assay using mice was carried out in accordance with the Guides for Animal Experiments Performed at NIID and approved by the Animal Care and Use Committee of the National Institute of Infectious Diseases.

2.8. Sequencing of full-length cDNA of the Ig gene

The mRNA was extracted from the hybridoma by using a QuickPrep micro mRNA Purification Kit (Amersham Biosciences) and cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The cDNA of the heavy chain was amplified by Pyrobest DNA polymerase (Takara, Otsu, Japan) with several combinations of primer. The primers for the heavy chain consisted of five primers of the 5' terminus end and one primer of the 3' terminus end, and those sequences are:

Heavy 5'-1(s); 5'-CACCATGGACTGGACCTGGAGGTTCCTC-3'
Heavy 5'-2(s); 5'-CACCATGGACTGGACCTGGAGGATCCTT-3'
Heavy 5'-3(s); 5'-CACCATGGGGTCAACCGCCATCCTCGCC-3'
Heavy 5'-4(s); 5'-CACCATGAAACACCTGTGGTTCTTCCTC-3'
Heavy 5'-5(s); 5'-CACCATGGAGTTTGGGCTGAGCTGGCTT-3'
CH3(as); 5'-GCAAGCCTCGAGAATTCTTATTTACCCGGAGACAGGGAGAGGCTCTT-3'.

The CH3(as) primer contained EcoRI and XhoI sites. The cDNA of light chain was amplified with KOD plus DNA polymerase (Toyobo, Osaka, Japan) using the specific primers. The primers for kappa or lambda chain consisted of 2 or 3 primers of the 5' terminus end and one primer of the 3' terminus end, respectively, and those sequences are:

Kappa 5'-1(s); 5'-CACCATGAGGGTCCCCGCTCAGCTCCTGGGGCT-3'
Kappa 5'-2(s); 5'-CACCATGGAAACCCCAGCGCAGCTTCTCTTCCTC-3'
Kappa-CL(as); 5'-TTCCATACTAGTAAGCTTCTAACACTCTCCCCTGTTGAAGCTCTT-3'.
Lambda 5'-1(s); 5'-CACCATGACCTGCTCCCCTCTCCTCCTC-3'
Lambda 5'-2(s); 5'-CACCATGACTTGGACCCCACTCCTCTTC-3'
Lambda 5'-3(s); 5'-CACCATGGCCTGGACCCCTCTCTGGCTCACTC-3'
Lambda-CL(as); 5'-TTCCATACTAGTAAGCTTCTATGAACATTCTGTAGGGGCCACTGT-3'.

The Kappa-CL(as) and Lambda-CL(as) primers contained the restriction sites of HindIII and SpeI. All primers were designed on the basis of the Kabat sequence database and Ig sequences recorded in the Genbank. Full-length cDNAs of heavy and light chain were cloned into a pENTR/D-TOPO vector using a pENTR Directional TOPO Cloning Kit (Invitrogen). Each sequence was determined with an ABI PRISM 3100 genetic analyzer (Applied Biosystems) according to a standard protocol. Variable regions of heavy and light chains, the complementarity-determining regions (CDRs) and framework regions (FRs) were determined with the Kabatman website (http://www.bioinf.org.uk/abs/simkab.html) based on the Kabat sequence database.

2.9. Construction of expression vectors and expression of recombinant mAbs

The cloned Ig gene was subcloned into the expression vector pCADEST2.2 or pCADEST2.3 through the LR-recombination reaction using the Gateway LR Clonase II enzyme mix (Invitrogen) according to the manufacturer's instructions. The blasticidin resistance expression vector pCADEST2.2 was constructed on the basis of pCALNL5 [20], consisting of the CAG promoter, a first loxP site, a neo-resistance gene, a second loxP site, and multi-cloning sites in that order. The pCALNL5, from which a fragment between two loxP sites containing loxP sites was removed by digestion with MluI/XhoI, was filled in with DNA polymerase I, a Large (Klenow) fragment (New England BioLabs) and self-ligated. SacI-KpnI-SmaI sites in multi-cloning sites were converted to a polylinker encoding SacI-NheI-PmeI-NotI-SmaI sites (named pCA7). Concerning the casset of an internal ribosome entry site derived from the Encephalomyocarditis Virus and the blasticidin S deaminase gene (EMCV-IRES-BSD), the blasticidin S deaminase gene taken from pMAM2-BSD (Funakoshi, Tokyo, Japan) by digestion with HindIII/PstI was blunted by Klenow and inserted into the SmaI site of pIRES1neo (Clontech). The EMCV-IRES-BSD casset, which was cut off by digestion with PstI and blunted by T4 DNA polymerase (New England BioLabs), was inserted into the SmaI site of pCA7 (named pCA6eRB). A fragment containing conversion sites of the GATEWAY system was taken from pDEST12.2 (Invitrogen) by digestion with Asp718I/NspI, blunted by a Klenow fragment and inserted into the SwaI/PmeI site of a pCA6eRB vector to make pCADEST2.2. The expression vector pCADEST2.3 was constructed by inserting a puromycin-resistance gene amplified from pPUR vector (Clontech) into pCADEST2.2 after the removal of the blasticidin S deaminase gene with EcoRV.

Constructed expression vectors encoding cDNA of H and L chains of each tetroma were co-transfected to 293T cells using Fugene6 (Roche Applied Science) according to the manufacturer's instructions. Transfected 293T cells were selected in Dulbecco's modified Eagle medium (DMEM, Nissui Pharmaceutical, Tokyo, Japan) containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 μg/ml blastcidin, and 1 μg/ml puromycin. Several colonies were picked up and cloned with the limiting dilution method to establish the cell lines that stably produced recombinant mAbs.

2.10. Detection of the EBV genome by PCR

For the PCR assay, the genomic DNA was extracted from 5×10^6 cells of EBV-transformed B cells and tetromas using a QIAamp DNA Kit (Qiagen), according to the manufacturer's instructions. In the qualitative PCR assay, the BamHI W region of the EBV genome was amplified with specific primers. The upstream and downstream primer sequences were 5'-CAAGAACCCAGACGAGTCCGTAGAA-3' and 5'-AAGAAGCATGTATACTAAGCCTCCC-3', respectively [21]. Ten ng of the extracted DNA was added to the reaction mixture containing 10 mM Tris-HCl (pH8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM dNTP, 0.6 μM each primer, and 0.5 U of Taq polymerase (Roche Applied Science). Following an initial denaturation for 2 min at 95 °C, 28 cycles of 15 sec at 95 °C and 1 min at 60 °C were carried out using the GeneAmp PCR system 9600 (Perkin Elmer). The amplified samples were separated on a 2% agarose gel.

In the real-time quantitative PCR assay, the amplification of the BALF5 gene which encodes the viral DNA polymerase was estimated using an ABI PRISM sequence detector system 7000 (PE Applied Biosystems) [22]. The primers and FAM-labeled fluorogenic probe for BALF5 were synthesized with PE Applied Biosystems as previously described by Kimura et al. [21]. For internal controls, human beta-actin (hACTB) and murine beta-actin (mACTB) were used for EBV-transformed B cells and tetromas, respectively. Regarding hACTB, the sequences of the upstream, downstream primer and VIC-labeled fluorogenic probe were 5'-CCAGTGTGACATGGTGCATCT-3', 5'-ACAGCCTGGATAGCAACGTACAT-3', and 5'-VIC-TGCCTTACAGATCATGTTTGAGACC-3', respectively. Regarding mACTB, the sequences of the upstream, downstream primer and VIC-labeled fluorogenic probe were 5'-GGCCAACCGTGAAAAGATGA-3', 5'-GCTGAGAAGCTGGCCAAAGA-3', and 5'-VIC-AGGTCAGTATCCCGGGTAACCCTT-3', respectively. These primer-probe combinations were designed between intron and exon using Primer Express software (PE Applied Biosystems). The genomic sequence data of hACTB were obtained from the GenBank sequence database (accession number M10277), and those of mACTB were obtained from the Mouse Genome Informatics (http://www.informatics.jax.org/). The PCR reactions were performed in a volume of 25 μl using a TaqMan Universal PCR Master Mix (PE Applied Biosystems). Following an initial denaturation for 2 min at 50 °C and an activation of AmpliTaq Gold DNA polymerase for 10 min at 95 °C, 50 cycles of 15 sec at 95 °C and 1 min at 60 °C were carried out. For positive controls, plasmids that contained the amplified fragment with each combination of the specific primers were constructed from pGEM-T easy vector (Promega) and termed pGEM-BALF5, pGEM-hACTB, and pGEM-mACTB, respectively. Standard curves of the threshold cycle value were obtained from serial dilutions of each vector.

2.11. Southern blot analysis of the EBV genome

Ten μg of extracted genomic DNA (as described above) was digested with BamHI and EcoRI, and fractionated by electrophoresis on a 0.8% agarose gel. Then size-fractionated genomic DNA was transferred onto a positive-charged nylon membrane (Roche) for hybridization with the digoxigenin (DIG)-labeled 400-bp PCR fragment, which was amplified with primers 5'-GCAGTAACAGGTAATCTCTG-3' and 5'-ACCAGAAATAGCTGCAGGAC-3' [23] using a PCR DIG Probe Synthesis Kit (Roche). The hybridization was performed overnight at 42 °C in a solution containing 50% formamid, 5× SSC (1× SSC is 0.15 M NaCl, 15 mM sodium citrate), 50 mM sodium phosphate, 2% blocking regent (Roche), 0.1% N-lauroylsarcosine, and 7% sodium dodecyl sulfate (SDS). After hybridization, membranes were washed twice at room temperature in 2× SSC, 0.1% SDS for 5 min, followed by two washes in 0.1 × SSC, 0.1% SDS for 15 min each. Hybridized probes were detected using anti-DIG-ALP (Roche), followed by the detection with CSPD. The light emission was recorded on X-ray film. Semi-quantification of the EBV genome was performed with pGEM-sBamHIW, which was constructed from non-labeled PCR products. The internal control, mACTB, was detected with a DIG-labeled probe, which was amplified with primers 5'-GGCCAACCGTGAAAAGATGA-3' (the same primer as in the quantitative PCR assay) and 5'-GTTGAAGGTCTCAAACATGA-3'.

Table 1
Enrichment of Ab-producing cells in EBV-transformed B cell line

IL-6 [U / mL]	Seeded cells [cells / well]	Ratio of TT-specific Ab positive well [positive well / seeded well]
1	50	1 / 60
	10	0 / 60
	5	0 / 60
4	50	1 / 60
	10	0 / 120
	5	0 / 180

3. Results

3.1. Establishment of stable tetroma cell lines producing human mAbs against TT

B cells isolated from the volunteer immunized with Ttd were transformed by *in vitro* EBV infection, and so the seven EBV-transformed B cell lines were established. As a result of an *in vivo* neutralization assay using doses of TT at 2 LD_{50} per mouse, a satisfactory neutralizing activity was detected in culture supernatants of the 5-11F cell line, which was one of the established EBV-transformed cell lines. The expanded population of the 5-11F cell line was fused with the trioma cell line 6JC5.2; however, none of the hybrid cells producing specific human antibodies against TT was generated. Then, B cells producing specific antibodies against TT in the 5-11F line were enriched by a limiting dilution method using the culture medium containing 1 U/ml or 4 U/ml of IL-6 before the cell fusion. Under each condition, TT-specific antibody reaction was detected with an ELISA in supernatants of only one well which was seeded 50 cells per well (Table 1). The B cell line, which was maintaining the production of specific antibodies against TT after cloning in the medium containing 4 U/ml of IL-6, was designated as the 5-11F D11 line. The 5-11F D11 line was expanded and fused with 6JC5.2 cells. Surprisingly, two different tetroma cell lines, TT1 and TT2, that produce IgG1·λ and IgG4·κ, respectively, were established by two independent cell fusion experiments (Fig. 1) with the 5-11F D11 line. These tetroma cell lines were capable of being continuously cultured after three-time consecutive cell cloning, and maintaining the stable productivity of specific antibodies against TT (Fig. 1). TT1 had a fast growth rate with a relatively low production ability of IgG1·λ (in case of the subclone 2D3 about 11.5 mg Abs were purified from 2700 ml culture supernatant), whereas, TT2 had a slow growth rate with a high production ability of IgG4·κ (in case of the subclone 1B6 about 7.8 mg Abs were purified from 850 ml of culture supernatant).

3.2. Enhancement of the in vivo neutralizing activity of human mAbs

To determine whether the established human mAbs were able to functionally neutralize TT, the neutralizing activities of the representative mAbs TT1 and TT2 were estimated in an *in vivo* neutralization assay using mice. Mice were inoculated with antigen-antibody complexes of TT and titrated human mAb, TT1 or TT2 by itself, and a mixture of equal amounts of each mAb. In this *in vivo* neutralization assay, TT that escapes neutralization by mAbs induces local spastic paralysis, followed by the death; accordingly, the neutralizing ability of mAbs was evaluated by the survival rates of mice. When a total 0.125 mg of mAbs was administered, TT1 and TT2 were incapable of protecting mice from death. All mice inoculated with the mixed mAbs at a ratio of 1:1 of each mAbs survived for the experimental period, although one of them showed mild systemic symptoms, which presented as systemic rigorous paralysis, hyperpnea, and piloerection (Fig. 2A). Although mice inoculated with 0.5 mg of TT1 survived for about two weeks from the inoculation, death followed after about 13 days as a result of the drastic systemic symptoms. TT2 was incapable of protecting mice at this level of administration at all. However, the mixture of both mAbs could completely protect mice from death without any symptoms (Fig. 2B).

TT1 showed partial neutralization activity of TT in a concentration-dependent manner. In contrast, TT2 showed little neutralization activity. Surprisingly, almost complete neutralization of TT was observed when equal amounts of both antibodies were reacted with TT. This result showed that the neutralizing activity of TT1 to TT was strongly enhanced by the addition of non-neutralizing TT2 in an *in vivo* neutralization assay using mice.

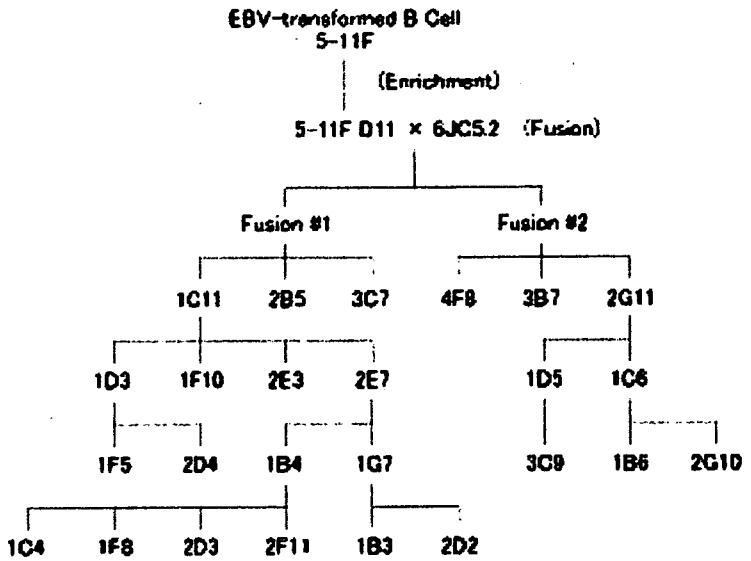

Fig. 1. Establishment of tetroma cell lines stably producing anti-TT antibodies. The enriched EBV-transformed B cells were fused with the fusion partner 6JC5.2 cells. Subclones of each tetromas that maintained TT-specific antibody production were cloned consecutively by the limiting dilution method. Two independent cell fusions yielded two groups of subclones producing the different isotype of IgG. One group consists of subclone 1C4, 1F8, 2D3, 2F11, 1B3, and 2D2 produced TT1 mAbs (IgG1, λ); and the other consists of subclone 3C9, 1B6, and 2G10 produced TT2 mAbs (IgG4, κ).

3.3. Cloning of Ig genes from tetromas

The tetroma cell lines producing TT1 (IgG1·λ) and TT2 (IgG4·κ) mAbs were established from the same EBV-transformed B cell line 5-11F D11, which was the subline of the parental line 5-11F that contained antibody-producing B cells at a probability of 1 in 3,000 (Table 1). Therefore, generation of TT1 and TT2 was speculated to be the result of isotype switching of one tetroma cell after the cell fusion with 6JC5.2. Accordingly, the sequences of Ig genes of each mAb were determined. The Ig genes of the H and L chains of TT1 were amplified with one primer set (H; Heavy 5'-5(s), L; Lambda 5'-3(s)), and those of TT2 were amplified with the other set (H; Heavy 5'-5(s), L; Kappa 5'-1(s)). As a result of the sequencing of the Ig genes of each mAb, the sequences were found to be completely inconsistent with each other (Fig. 3). The complementarity-determining regions (CDRs) and framework regions of the variable region (VH, VL) of each mAb were determined with the Kabat sequence database. It was revealed that TT1 and TT2 were entirely derived from different B cell clones. Moreover, the Ig sequences of six subclones in group TT1 or three subclones in group TT2 were consistent with each other, respectively as expected.

3.4. Amplification and expression of the Ig gene at an early stage of cloning

Ig-gene cloning from subclones, which were established after multiple consecutive cloning of the tetromas, is laborious and time-consuming. Therefore, we investigated whether Ig genes were able to be cloned or not at an early stage after the cell fusion with 6JC5.2, using clones 1C11 and 2G11, which were the parental clones of groups TT1 and TT2, respectively (Fig. 1). At first, it was examined how many cells were necessary for Ig-gene cloning, and whether the cognate pairing of heavy and light chains was cloned. mRNA was extracted from different numbers of 1C11 and 2G11 cells, and cDNA was synthesized from an equal volume of mRNA for each sample. Each Ig gene was amplified in a cell number-dependent manner with the same primer combination, which was used to amplify those of groups TT1 and TT2, respectively. Except for the light chain of clone 2G11, it was possible to amplify each Ig gene from at least 10 cells (Fig. 4A). Next, it was examined whether the cognate pairing of heavy and light chains was cloned at the first cell-cloning step of parental clones 1C11 and 2G11. Parental clones 1C11 and 2G11 were recloned with the limiting dilution method. After 11 days (1C11) and 18 days (2G11), three subclones which

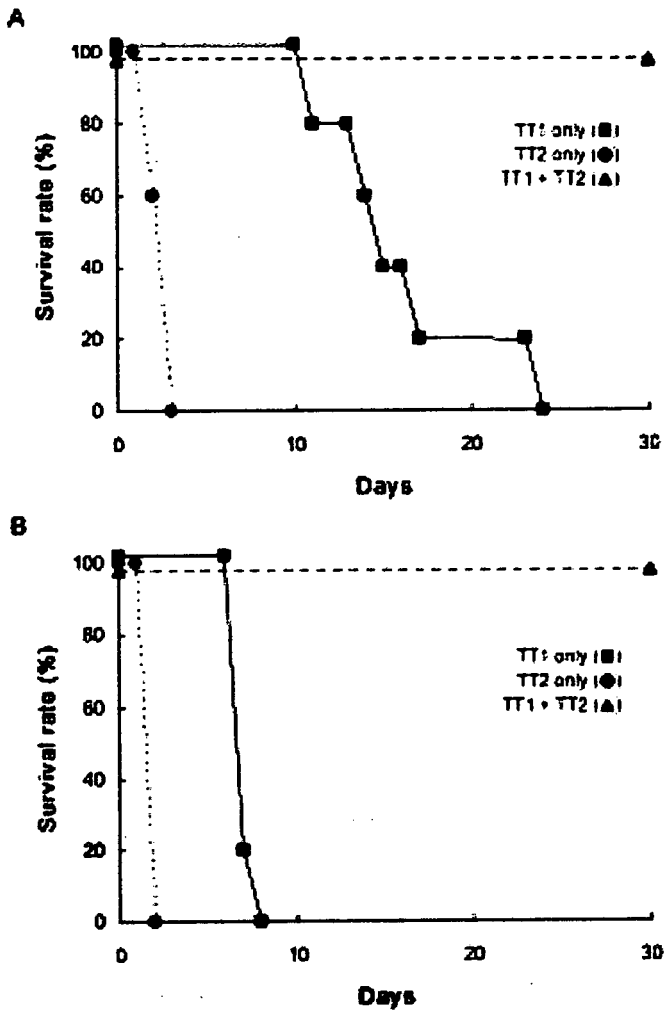

Fig. 2. *In vivo* neutralizing activities of anti-TT mAbs. TT-neutralizing activity of each human monoclonal antibody was determined with *in vivo* neutralizing assay using doses of TT at L+/100 levels. Purified monoclonal antibodies of 500 μg (A) or 250 μg (B) were mixed with 400 LD_{50} of TT. The mixtures (0.4 ml) were injected s.c. to femor of ddY mice. Symptoms were observed everyday, and survival rate of five mice was shown. TT1 mAbs showed partial neutralization activity of TT. In contrast, TT2 mAbs showed little neutralization activity. Surprisingly, almost complete neutralization of TT was observed when equal amounts of both antibodies were reacted with TT.

showed a positive reaction to TT with an ELISA were randomly selected, and cDNA was synthesized from all cells in each well. Each Ig gene was amplified by the use of cDNAs diluted 1/50. From subclone No.2 of clone 1C11, the Ig gene of the light chain was amplified at a low efficiency. Nevertheless, all subclone Ig genes were able to be amplified with the same primers for TT1 and TT2 (Fig. 4B). In addition, the sequences of each Ig gene which was cloned in these processes were completely consistent with those of groups TT1 and TT2 (data not shown).

3.5. Specificity and affinity of anti-TT mAbs

To examine whether cloned Ig genes were derived from the correct pairing of heavy and light chains, the reactivity of recombinantly expressed TT1 and TT2 mAbs was compared with that of native TT1 and TT2 mAbs purified from culture supernatants of tetromas. The binding activity of established human mAbs was first investigated with an ELISA using purified TT and inactivated TT in 0.5% formalin as Ttd. Both human mAbs reacted to the fixed TT

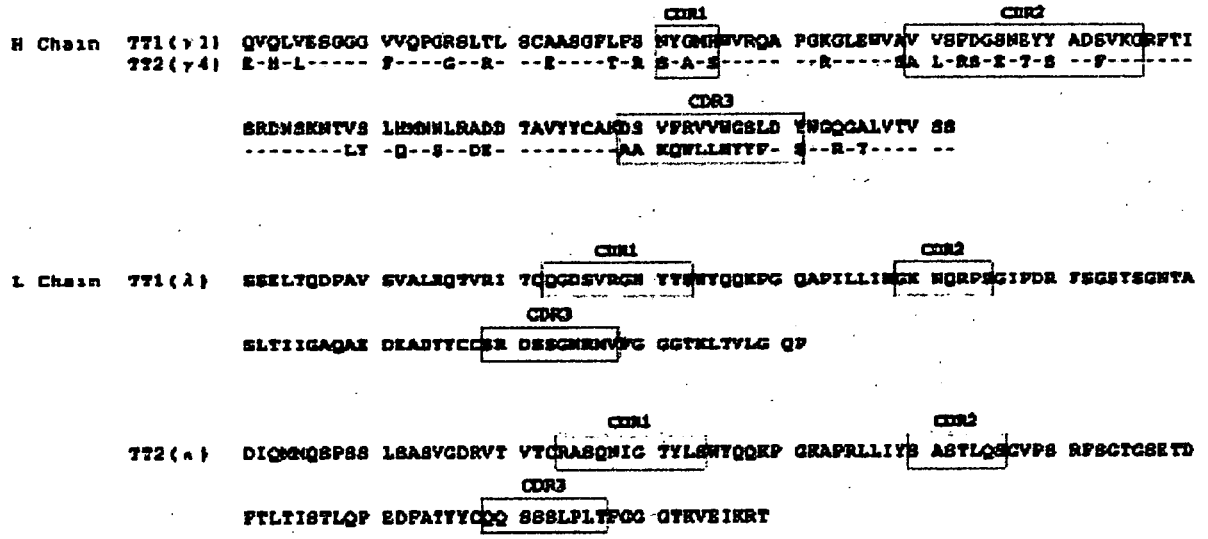

Fig. 3. Amino acid sequences of mAbs specific for TT produced by tetromas. The cDNA of each mAb was amplified with a specific primer combination (TT1; Heavy 5'-5(s) and Lambda 5'-3(s), TT2; Heavy 5'-5(s) and Kappa 5'-1(s)) among a several primer sets. The complementarity-determining regions (CDRs) and framework regions of variable region of each tetroma were determined with Kabatman website (http://www.bioinf.org.uk/abs/simkab.html) based on the Kabat sequence database. The sequence was the same among all subclones producing TT1 or TT2 mAbs.

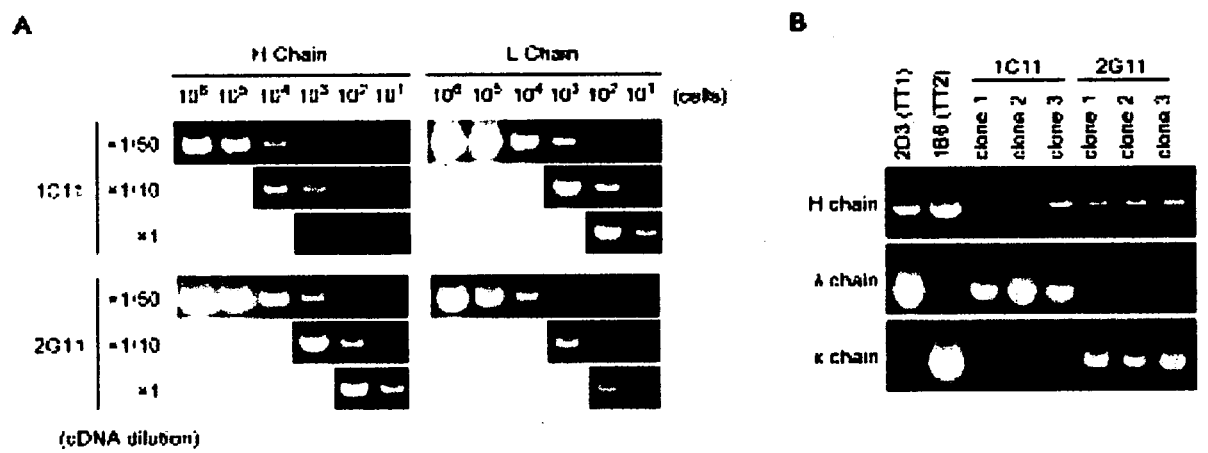

Fig. 4. Efficient amplification of Ig genes at an early stage after the fusion with 6JC5.2. (A) Cell number-dependent amplification of Ig genes from cDNA of tetromas that produced specific antibody against TT after the fusion with 6JC5.2. (B) Amplification of Ig genes from three subclones of each tetromas after the first cloning step. As positive controls, Ig genes were amplified from cDNAs of 2D3 (TT1) and 1B6 (TT2) cells, which were stable cell lines established after three-time cloning steps (Fig. 1). Moreover, each cDNA of 1C11 or 2G11 and those subclones were amplified with the same primer set of TT1 or TT2, and the sequences of them were consistent with those of TT1 or TT2 (data not shown).

more strongly than to the purified TT (Fig. 5A); that is, these mAbs showed a strong interaction with Ttd used as a vaccine. However, these human mAbs had a sufficient affinity constant to TT. The affinity constant of TT1 (2D3) was 10-fold higher than that of TT2 (1B6) (Table 2). Next, TT-specific binding reactivity of recombinant mAbs was compared with that of native mAbs with an ELISA. Recombinant and native mAbs of TT1 showed similar reactivities to TT in a concentration-dependent manner. The same reactivity was observed concerning recombinant and native mAbs of TT2. TT1 mAb reacted with TT about two-fold higher than TT2 mAbs at a concentration of 1.0 μg/ml (Fig. 5B). In addition, it was investigated whether epitopes recognized by recombinant mAbs were consistent with those of native mAbs or not. Recognized epitopes were identified with a competitive ELISA to TT,

Table 2
Affinites of human mAbs to TT

Human mAb	ka $[M^{-1}s^{-1}]$	kd $[s^{-1}]$	Kd [M]
TT1 (2D3)	$(1.05 \pm 0.01) \times 10^5$	$(1.55 \pm 0.61) \times 10^{-5}$	$(1.48 \pm 0.56) \times 10^{-10}$
TT2 (1B6)	$(2.29 \pm 0.49) \times 10^4$	$(4.11 \pm 0.92) \times 10^{-5}$	$(1.89 \pm 0.81) \times 10^{-9}$

in which non-labeled competitor mAbs including recombinant and native mAbs competed with biotinylated native mAbs of TT1 or TT2, and biotynilated mAbs bound to TT were detected. In a competition assay with biotin-labeled TT1, biotinylated TT1 completely competed with recombinant and native mAbs of TT1, but not with those of TT2 (Fig. 5C). The efficacy of self-competition, that is, biotinylated TT1 versus native TT1, was 97.9% at a concentration of 0.5 μg/ml competitor. The same result was obtained in a competition assay with biotin-labeled TT2, and efficacy of self-competition was 87.4% at a concentration of 0.5 μg/ml competitor (Fig. 5D). These results revealed that it was possible to clone the correct pairing of Ig gene at an early stage in the EBV-hybridoma method using fusion partner 6JC5.2, and that TT1 and TT2 mAbs established in this process recognized the different epitopes.

3.6. Removal of the EBV genome from antibody producing cells

For clinical applications or safety handling, the retention of the EBV genome as the pathogenic agent in stable tetroma cell lines producing human mAb was irrelevant. So, whether the EBV genome was eliminated or not in the established tetroma cell lines was investigated with the PCR method. The BamHI W region, which was repeated about twelve times on the EBV genome and considered as a sensitive region, was amplified by a qualitative PCR assay [21,22]. The amplified BamHI W region was detected in two stable tetromas, clone 1C11 and subclone 2E3, as well as in the EBV-transformed B cell line 5–11F and enriched cell line 5-11F D11 (Fig. 6A). In contrast, none of the EBV genome was detected at all in the final established stable tetromas, that is, group TT1 and group TT2. Thus, as a result of multiple cloning steps, it was possible to select the stable tetromas in which the EBV genome was eliminated to below detectable levels. Regarding the representative subclones including groups TT1 and TT2, a similar result was obtained from semi-qualitative Southern blot analysis, by which the EBV genome was detected at a frequency of 0.04 to 0.4 copies per cell (Fig. 6B).

Next, the frequency of retention of the EBV genome in stable tetromas was estimated with a quantitative real-time PCR assay using primers specific for the BALF5 region encoding the viral DNA polymerase, a single copy gene on the EBV genome [21]. One hundred or more copies of the EBV genome per cell were detected in EBV-transformed B cell lines 5-11F and 5-11F D11. On the other hand, the EBV genome was detected at a frequency of < 10 copies per cell in stable tetroma clone 1C11 and subclone 2E3. In contrast, the EBV genome was eliminated at the level of the detection limit, which was lower than 0.01 copies per cell, in other clones and subclones, TT1 and TT2 (Table 3).

4. Discussion

In this study, we have succeeded in reviving the classical EBV-hybridoma method using a newly established fusion partner 6JC5.2. Our revised EBV-hybridoma system will be superior to the previously reported method in: 1) reproducibility in generating hybridomas that stably produce specific mAbs without producing non-specific Abs of parental cells as well as pathogenic EBV from transformed B cells and 2) efficient cloning of whole sequences of functional and correct pairs of H and L chain genes for production of fully human mAbs for therapeutic use; the genes were cloned from small numbers (less than 10) of the subclone of the first cell-cloning step after selecting the Ab-producing hybridomas after the cell fusion.

Currently, the establishment of fully human antibodies depends on the phage display library technology or the transgenic mice bearing human Ig loci. These antibodies are not selected by the intact human immune system and consequently might be hazardous for the recipient or recognized as xenobiotics and eliminated by the recipient's immune system. If the generation of fully human antibodies from native human B cell sources could become technically easier, the effective development of theoretically safer mAbs for therapeutic use would be possible.

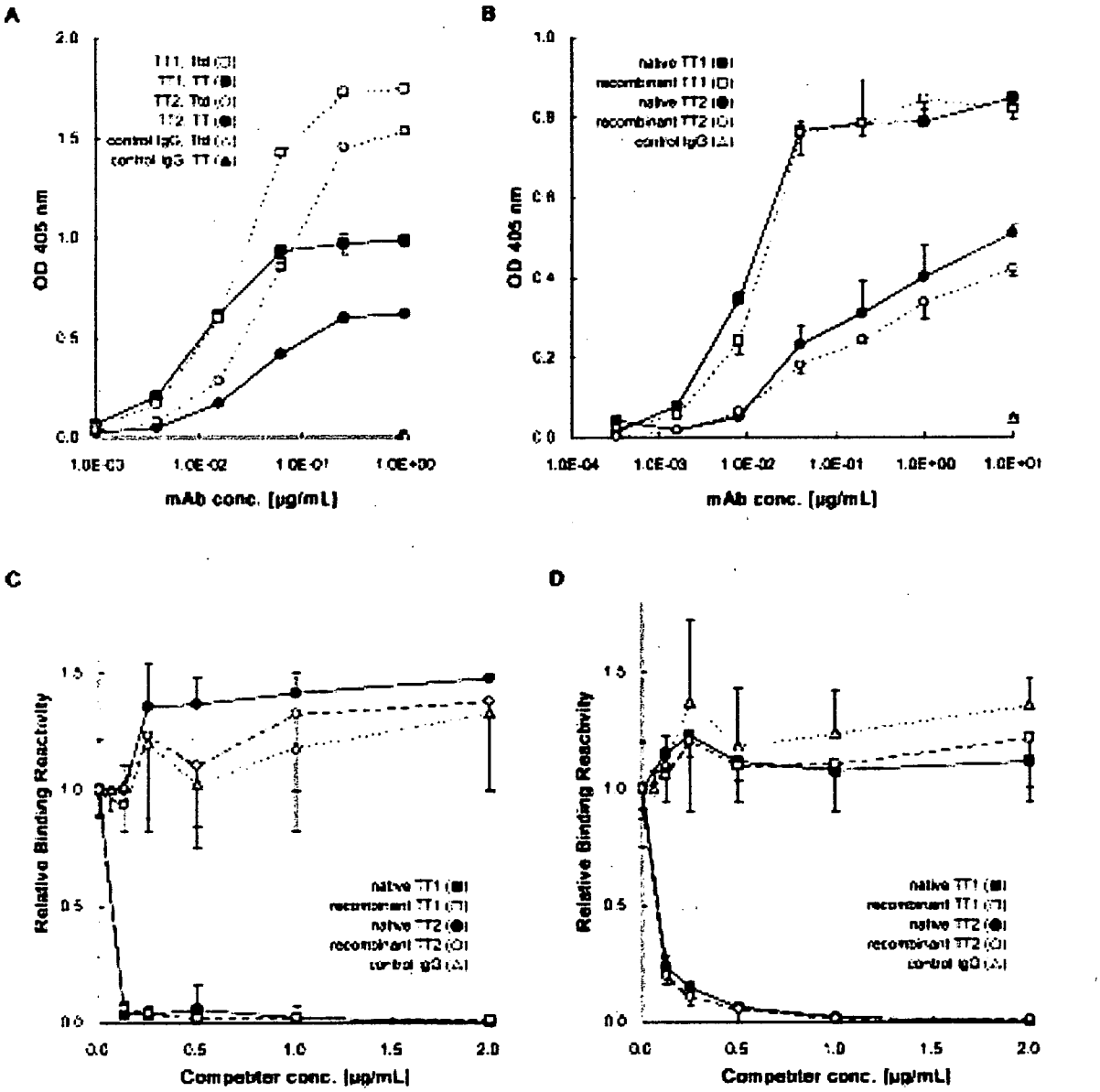

Fig. 5. TT-specific binding of native and recombinant mAbs. (A) Reactivity of TT1 and TT2 mAbs to TT or Tetanus Toxoid (Ttd) was estimated by ELISA. Ttd was prepared by fixation of TT with 0.5% formalin. (B) TT-specific binding activities of serially diluted native antibodies purified from the culture supernatant of TT1 or TT2 tetromas, and that of recombinant antibodies purified from culture supernatants of 293T cells co-transfected with $\gamma 1$ and λ chains of TT1 or $\gamma 4$ and κ chains of TT2 producing tetromas, were determined by ELISA. (C) Competitive binding of the native and recombinant TT1 mAbs to TT was analyzed by competitive ELISA with biotinylated native TT1 and non-labeled native and recombinant TT1 mAbs as the competitors. The reaction without each competitor was regarded as a positive control. The degree of competition was shown as relative binding reactivity to the positive control. (D) Competitive binding of the native and recombinant TT2 mAbs to TT was analyzed by competitive ELISA with biotinylated native TT2 and non-labeled native and recombinant TT2 mAbs as the competitors.

Thus, we focused on the classical method to generate fully human mAbs, that is, the EBV-hybridoma method [10]. Since no reliable method for *in vitro* immunization of human B cells has been well-established yet, the EBV-hybridoma method seems to be the best conveniently to produce fully human mAbs for therapeutic use. The wide application of this method, however, has been hampered by the lack of suitable parental fusion partners for

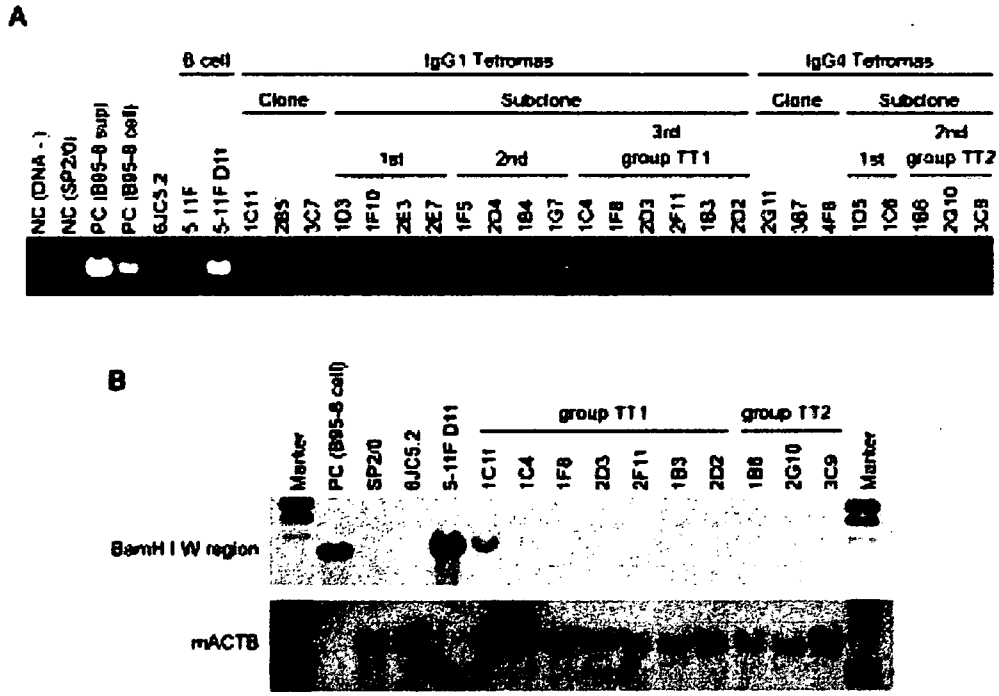

Fig. 6. Detection of the EBV genome at each cloning step of tetromas. (A) Qualitative PCR for BamHI W region. BamHI W region, which has been obtained by BamHI digestion of EBV genome and repeated about 12 times, was amplified with the specific primers. The genomic DNA extracted from mouse myeloma cell line SP2/0 was used as a negative control, and that from the EBV-positive cell line B95-8 was used as a positive control. (B) Elimination of EBV genome in the stable tetroma TT1 and TT2 was confirmed by southern blot experiments using BamHI W region probe, as described in *Materials and Methods*. In this analysis, EBV genome was detectable at the level of 0.04 ~ 0.4 copies per cell at least.

stable production of specific mAbs without production of non-specific Abs of the parental cells. Therefore, we have established the unique fusion partner 6JC5.2, which was a trioma cell line that has the ability to produce human Abs stably after fusion with the EBV-transformed B cell line producing human Abs. Using this revised EBV-hybridoma method, human IgG mAbs specific for HBs [16], TT (this paper) and Hepatitis virus type A (manuscript in preparation) were produced and cDNA clones encoding functional and correct pairs of Ig genes have been isolated and their recombinant mAbs were successfully expressed by 293T cells as described in Figs 3 and 4. Reproducibility of this hybridoma technology was demonstrated by the fact that two different kinds of scientifically interesting and possibly pharmacologically valuable human mAbs specific for TT were mined from the single well of the EBV-transformed B cell line; in fact fusion experiment No.1 and No.2 (Fig. 1) were carried out about 2 years apart with frozen stock of the cells. An *in vivo* neutralization assay to TT showed that TT1 (2D3) could concentration-dependently neutralize TT, in contrast, TT2 (1B6) was incapable of neutralizing the toxin by itself at all. It was noteworthy that almost complete neutralization of TT was achieved when equal amounts of both antibodies were reacted with TT and injected *in vivo* (Fig. 2). Although the detailed mechanism involved in the enhancement of the neutralizing activity remains to be elucidated, the underlying mechanism in this phenomenon will shed light on the effectiveness of oligoclonal or polyclonal Abs for the development of pharmacological Ab agents [24]. The synergistic effects of combined antibodies, which can to some extent neutralize toxin by themselves, have been reported until now [19,25]. If many more human mAbs specific for TT were to be obtained, it would be possible to reveal this enhancement mechanism in detail by the analysis of the neutralizing epitopes on TT. We are establishing other human mAbs using this method as well as KM mouse bearing human Ig loci (collaborative work with Kirin Co.). The analysis of epitopes recognition and the enhanced neutralizing properties of these antibodies will be published elsewhere.

It is noteworthy that the enrichment of Ab-producing B cells in the primary EBV-transformed B cell line using IL-6 was always necessary before cell fusion even in this revised method. The probability of the existence of an

Table 3
The elimination of the EBV genome from antibody-producing tetromas

		BALF5 count (copies)	mACTB count (copies)	hACTB count (copies)	EBV existent frequency** (EBV copies / cell)
	B95-8	3.27E + 08	N.D.*	N.D.	N.D.
	SP2/0	320.66	3.72E + 05	N.D.	0.00
Fusion partner	6JC5.2	498.04	6.18E + 05	3.16E + 04	0.00
EBV transformed	5-11F	3.60E + 07	N.D.	3.84E + 05	93.75
B cell	5-11F D11	3.25E + 08	N.D.	1.36E + 06	238.97
Tetroma (IgG1)	1C11	4.94E + 06	1.34E + 06	N.D.	3.69
	2B5	3.43E + 04	3.68E + 05	N.D.	0.09
	3C7	84.01	1.74E + 05	N.D.	0.00
	1D3	312.76	5.02E + 05	N.D.	0.00
	1F10	222.42	8.77E + 05	N.D.	0.00
	2E3	1.88E + 07	1.95E + 06	N.D.	9.64
	2E7	85.78	8.12E + 05	N.D.	0.00
	1F5	630.88	5.17E + 05	N.D.	0.00
	2D4	916.30	1.05E + 06	N.D.	0.00
	1B4	2883.34	6.77E + 05	N.D.	0.00
	1G7	787.31	1.88E + 06	N.D.	0.00
	1C4	3689.00	3.80E + 05	N.D.	0.01
	1F8	2218.83	1.74E + 06	N.D.	0.00
	2D3	1839.65	1.23E + 06	N.D.	0.00
	2F11	1919.69	4.46E + 05	N.D.	0.00
	1B3	487.33	3.70E + 05	N.D.	0.00
	2D2	4609.42	6.63E + 05	N.D.	0.01
Tetroma (IgG4)	2G11	1.09E + 06	1.64E + 06	N.D.	0.66
	3B7	1.02E + 06	1.23E + 06	N.D.	0.83
	4F8	480.07	9.55E + 05	N.D.	0.00
	1D5	24.92	5.59E + 05	N.D.	0.00
	1C6	3.54	4.37E + 05	N.D.	0.00
	1B6	6184.62	5.35E + 05	N.D.	0.01
	2G10	180.34	3.37E + 05	N.D.	0.00
	3C9	2449.74	1.06E + 06	N.D.	0.00

*N.D.: not determined.
**The copy number of mACTB or hACTB was considered as the substantial cell count, and the EBV existent frequency per cell was calculated.

Ab-producing B cell in the primary EBV-transformed B cell line was usually very low, 1 in 3000 in the 5-11F line in Table 1, but it was possible to enrich them by 10 to 100-fold using the limiting dilution method with IL-6 (Table 1). It was, however, difficult to enrich further or isolate Ab-producing clones by this method (data not shown) and immnortalization of transiently enriched Ab-producing B cells by immediate cell fusion with the 6JC5.2 cells was essential for stable production of specific mAbs.

Although stable production of anti-TT was possible after three-time consecutive cell cloning of the tetromas, such repeated cell cloning steps are laborious and time-consuming. In addition, although the quantities of the mAbs produced by the tetromas were sufficient for the experiment, the level will be no doubt insufficient for large-scale production of mAbs as pharmaceutical agents. From these points of view, the most important advantage of our revised method would be that efficient cloning is possible of functional and correct pairs of the H and L chain genes from tetromas after establishment of a stable cell line with repeated cloning (Fig. 3) or even after simply selecting Ab-producing cells after the cell fusion (Fig. 4). Cloned cDNA encoding H and L chain genes derived from tetromas were full length and easily transferable to any desired expression vectors for any scale of expression of recombinant mAbs. Furthermore, little but any amplification of cDNA encoding non-specific Igs from the tetromas was the best characteristic of 6JC5.2 cells because it was very convenient for efficient cloning of the functional and correct pairs of H and L chain genes (Fig. 4). Accurate cloning of correct pairs of Ig genes was confirmed by the fact that Ig genes isolated at an early stage were entirely consistent with those isolated from monoclonal tetromas, and that the reactivity of recombinantly expressed mAbs from isolated Ig genes was equal to that of native mAbs (Figs 5B, 5C, 5D). Isolation of the correct-pairing of Ig genes employing our revised method will become one effective technology, together with the single-cell RT-PCR technology that has been recently reported [26,27].

Another minor but important advantage of the revised method will be the removal of the EBV genome from Ab-producing tetromas. Although the detailed mechanism of the deletion of the EBV genome remains to be determined, the EBV genome derived from the EBV-transformed B cells was shed from the cell by the fusion with 6JC5.2 or gradually by continuous cultivation. Quantitative real-time PCR using the BALF5 region revealed that the EBV genome had decreased to below the level of 0.01 copies per cell in the final stable tetromas (Fig. 6 and Table 3). This will be advantageous for safety control in laboratory experiments and the production of pharmaceutical mAbs.

In the latest studies, human memory B cells have been activated and more effectively immortalized by *in vitro* EBV infection than the traditional methods, in the presence of polyclonal activator CpG 2006 or with co-ligation of CD19 and BCR [28–30]. If these procedures were combined with our method, it would be possible to complete a system to develop efficient production of specific mAbs in appropriate volumes for therapeutic use.

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References

[1] O.H. Brekke and I. Sandlie, Therapeutic antibodies for human diseases at the dawn of the twenty-first century, *Nat Rev Drug Discov* **2** (2003), 52–62.

[2] J.M. Reichert, C.J. Rosensweig, L.B. Faden and M.C. Dewitz, Monoclonal antibody successes in the clinic, *Nat Biotechnol* **23** (2005), 1073–1078.

[3] L.L. Green, Antibody engineering via genetic engineering of the mouse: XenoMouse strains are a vehicle for the facile generation of therapeutic human monoclonal antibodies, *J Immunol Methods* **231** (1999), 11–23.

[4] K. Tomizuka, T. Shinohara, H. Yoshida, H. Uejima, A. Ohguma, S. Tanaka, K. Sato, M. Oshimura and I. Ishida, Double trans-chromosomic mice: maintenance of two individual human chromosome fragments containing Ig heavy and kappa loci and expression of fully human antibodies, *Proc Natl Acad Sci USA* **97** (2000), 722–727.

[5] D. Kozbor and J.C. Roder, Requirements for the establishment of high-titered human monoclonal antibodies against tetanus toxoid using the Epstein-Barr virus technique, *J Immunol* **127** (1981), 1275–1280.

[6] M. Steinitz, G. Klein, S. Koskimies and O. Makel, EB virus-induced B lymphocyte cell lines producing specific antibody, *Nature* **269** (1977), 420–422.

[7] M. Sugimoto, Y. Furuichi, T. Ide and M. Goto, Incorrect use of"immortalization" for B-lymphoblastoid cell lines transformed by Epstein-Barr virus, *J Virol* **73** (1999), 9690–9691.

[8] T. Toda and M. Sugimoto, Proteome analysis of Epstein-Barr virus-transformed B-lymphoblasts and the proteome database, *J Chromatogr B Analyt Technol Biomed Life Sci* **787** (2003), 197–206.

[9] G. Kohler and C. Milstein, Continuous cultures of fused cells secreting antibody of predefined specificity, *Nature* **256** (1975), 495–497.

[10] J.C. Roder, S.P. Cole and D. Kozbor, The EBV-hybridoma technique, *Methods Enzymol* **121** (1986), 140–167.

[11] G.F. Kalantarov, S.A. Rudchenko, L. Lobel and I. Trakht, Development of a fusion partner cell line for efficient production of human monoclonal antibodies from peripheral blood lymphocytes, *Hum Antibodies* **11** (2002), 85–96.

[12] A. Karpas, A. Dremucheva and B.H. Czepulkowski, A human myeloma cell line suitable for the generation of human monoclonal antibodies, *Proc Natl Acad Sci USA* **98** (2001), 1799–1804.

[13] N.N.H. Teng, G.R. Reyes, M. Bieber, K.E. Fry, K.S. Lam and J.M. Herbert, Strategies for stable human monoclonal antibody production: construction of heteromyelomas, *in vitro* sensitization and molecular cloning of human immunoglobulin genes, in: *Human Hybridomas and Monoclonal Antibodies*, E.G. Engleman, S.K.H. Foung, J. Larrick and A. Raubitschek, eds, Plenum Press, New York, 1985, pp. 71–91.

[14] K. Ohnishi, J. Chiba, Y. Goto and T. Tokunaga, Improvement in the basic technology of electrofusion for generation of antibody-producing hybridomas, *J Immunol Methods* **100** (1987), 181–189.

[15] K. James and G.T. Bell, Human monoclonal antibody production. Current status and future prospects, *J Immunol Methods* **100** (1987), 5–40.

[16] H. Ohba, K. Kashima, T. Tomozawa and J. Chiba, *Molecular expression of human antibody and prediction of antigen-binding structure*, The 24th Annual Meeting of the Japanese Society for Immunology, 1994, Kyoto, 639.

[17] V.T. Oi and L.A. Herzenberg, Immunoglobulin-producing hybrid cell line., in: *Selected Methods in Cellular Immunology*, B.B. Mishelland and S.W. Shiigi, eds, W. H. Freeman, San Francisco, 1980, pp. 351–371.

[18] A.S. Sheoran, S. Chapman-Bonofiglio, B.R. Harvey, J. Mukherjee, G. Georgiou, A. Donohue-Rolfe and S. Tzipori, Human antibody against shiga toxin 2 administered to piglets after the onset of diarrhea due to *Escherichia coli* O157:H7 prevents fatal systemic complications, *Infect Immun* **73** (2005), 4607–4613.
[19] A.B. Lang, S.J. Cryz, Jr., U. Schurch, M.T. Ganss and U. Bruderer, Immunotherapy with human monoclonal antibodies. Fragment A specificity of polyclonal and monoclonal antibodies is crucial for full protection against tetanus toxin, *J Immunol* **151** (1993), 466–472.
[20] Y. Kanegae, G. Lee, Y. Sato, M. Tanaka, M. Nakai, T. Sakaki, S. Sugano and I. Saito, Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase, *Nucleic Acids Res* **23** (1995), 3816–3821.
[21] H. Kimura, M. Morita, Y. Yabuta, K. Kuzushima, K. Kato, S. Kojima, T. Matsuyama and T. Morishima, Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay, *J Clin Microbiol* **37** (1999), 132–136.
[22] R. Baer, A.T. Bankier, M.D. Biggin, P.L. Deininger, P.J. Farrell, T.J. Gibson, G. Hatfull, G.S. Hudson, S.C. Satchwell, C. Seguin, P.S. Tuffnell and B.G. Barrell, DNA sequence and expression of the B95-8 Epstein-Barr virus genome, *Nature* **310** (1984), 207–211.
[23] M. Savard, C. Belanger, M. Tardif, P. Gourde, L. Flamand and J. Gosselin, Infection of primary human monocytes by Epstein-Barr virus, *J Virol* **74** (2000), 2612–2619.
[24] M.C. Glassy and M.E. McKnight, Requirements for human antibody cocktails for oncology, *Expert Opin Biol Ther* **5** (2005), 1333–1338.
[25] J. Chin, Y. Sohn, S.H. Lee, Y.I. Park and M.J. Choi, Production of neutralizing human monoclonal antibody directed to tetanus toxin in CHO cell, *Biologicals* **31** (2003), 45–53.
[26] M. Ali, K. Hitomi and H. Nakano, Generation of monoclonal antibodies using simplified single-cell reverse transcription-polymerase chain reaction and cell-free.
[27] P.J. Meijer, P.S. Andersen, M. Haahr Hansen, L. Steinaa, A. Jensen, J. Lantto, M.B. Oleksiewicz, K. Tengbjerg, T.R. Poulsen, V.W. Coljee, S. Bregenholt, J.S. Haurum and L.S. Nielsen, Isolation of human antibody repertoires with preservation of the natural heavy and light chain pairing, *J Mol Biol* **358** (2006), 764–772.
[28] G. Hartmann and A.M. Krieg, Mechanism and function of a newly identified CpG DNA motif in human primary B cells, *J Immunol* **164** (2000), 944–953.
[29] D.Y. Hur, M.H. Lee, J.W. Kim, J.H. Kim, Y.K. Shin, J.K. Rho, K.B. Kwack, W.J. Lee and B.G. Han, CD19 signalling improves the Epstein-Barr virus-induced immortalization of human B cell, *Cell Prolif* **38** (2005), 35–45.
[30] E. Traggiai, S. Becker, K. Subbarao, L. Kolesnikova, Y. Uematsu, M.R. Gismondo, B.R. Murphy, R. Rappuoli and A. Lanzavecchia, An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus, *Nat Med* **10** (2004), 871–875.
[31] L.J. Reed and H. Muench, A simple method of estimating fifty per cent endpoint, *Am J Hyg* **27** (1938), 493–497.

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Research paper

Toxoid flocculation assay by laser light-scattering

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Abstract

Internationally accepted designations of antigen content for toxoid vaccines are provided by the WHO in Lf (limes flocculationis) units, based on the formation of antigen–antibody complexes. The current assay method for Lf determination involves observation of the complexes by eye, making the development of a more objective system highly desirable. Here we report a novel detection system using a laser light-scattering platelet aggregometer. The system was highly reproducible and more objective than the current method. Only three sets of duplicate data were sufficient for statistically significant determination of toxoid Lf by parabolic regression.

Keywords: Vaccine; Diphtheria; Tetanus; Quality control; Antigen; Antitoxin

1. Introduction

Toxoid vaccines are chemically or physically inactivated bacterial toxins, retaining sufficient immunogenicity but with diminished toxicity (WHO Expert Committee on Biological Standardization, 1990). Quantification of toxoids can be carried out by physicochemical methods such as protein content determination, but such tests do not necessarily reflect antigenicity. This is because toxoids are not always produced from pure toxin preparations and the degree of protein denaturation markedly affects the antigenic integrity of toxoids. As antigenicity is a crucial factor for vaccine quality control, a reliable method for its determination is necessary. Such a method was established soon after the development of toxoid vaccines, by utilizing antigen–antibody reactions. This method is called flocculation (Glenny and Okell, 1924; Ramon, 1922b), based on the observation that the time needed for forming visible antigen–antibody complexes (flocculation) is shortest for an optimal ratio of antibody (antitoxin) and antigen (toxoid) in aqueous solution. Thus, through determining the ratio giving the most rapid flocculation, antigen content of the toxoid preparation can be calculated. The index defined by the above method was termed Lf (limes flocculationis or limit of flocculation) and has been approved widely for use in vaccine quality control, as well as for the quantification of toxins. Lf is the current official index of antigen content for diphtheria and tetanus toxoid vaccines according to the WHO minimum requirements and is used for vaccine production (WHO Expert Committee on Biological Standardization, 1990;

Abbreviations: Dtd, diphtheria toxoid; Ttd, tetanus toxoid.

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World Health Organization, 1977b,a). In addition, flocculation can be used for antitoxin titration (Murata et al., 1953).

Methodology for the quantitative flocculation assay involves the observation of macroscopic flocculation complexes visible to the naked eye, which is influenced by subjectivity of the observer. More objective methods for detection of flocculation complex are required, but have not been described so far. Optical detection of antigen–antibody complexes is one of the most promising approaches to this problem.

In the middle of 1990s, a laser light-scattering platelet aggregometer was described (Ozaki et al., 1994; Yamamoto et al., 1995). This device was originally developed in order to overcome the inability of conventional turbidimetry-type platelet aggregometers to provide information on particle sizes of platelet aggregates, often critical for clinical diagnosis. The novel laser light-scattering-type device described here was designed to quantify not only numbers of particles but also their sizes, including the sizes of platelet aggregates. Particle suspensions are subjected to aggregometry in a cylindrical glass cuvette (7-mm diameter) containing a small stirrer bar. A laser beam is passed through the suspension near the surface of the cuvette, and scattered light intensity and signal counts recorded. These values reflect size and number of detected particles, respectively, and can be presented using special PC software (Harrison, 2000; Ozaki et al., 1994; Yamamoto et al., 1995). As the principle of the flocculation assay is to detect a certain size of flocculation complexes as particles, the laser light-scattering platelet aggregometer could potentially be applied to this assay as well. Therefore, we have attempted to establish a novel flocculation assay system using this new device, and report here that it could be suitable for use in routine vaccine quality control.

2. Materials and methods

2.1. Toxoids and antitoxins

Two different batches of diphtheria toxoid (Dtd) and one batch of tetanus toxoid (Ttd) (approximately 200 Lf/ml for each) were kind gifts of two Japanese manufacturers, A and B. Diphtheria antitoxin (lot 3, 1100 units/vial) and tetanus antitoxin (lot 1, 1100 units/ampoule) were Japanese national reference products for use in flocculation. The toxoid content necessary to compound the most appropriate mixture for 1 unit of antitoxin is defined as 1 Lf (World Health Organization, 1977b,a). Toxoids and antitoxins were appropriately diluted with saline (0.15 M sodium chloride) before use.

2.2. Conventional flocculation assays

Conventional flocculation assays were performed essentially according to WHO recommendations (World Health Organization, 1977b,a). Briefly, 207, 227, 250, 275 or 303 μl of antitoxin solution (100 units/ml) were distributed into 13 mm-diameter glass tubes and the volume adjusted to 500 μl with saline. Five hundred microliters of two-fold or 1.85-fold dilutions of Dtd solution from manufacturers A (Dtd A) or B (Dtd B), respectively, was then added to each tube at time zero. The tubes were immediately transferred to a 50 °C water bath, so as to cover approximately 1/3 of the tube content with water. Flocculation was monitored by occasional observations by eye. The first tube which showed visible flocculation was identified and recorded, together with the time required (Kf). The antitoxin content of the first tube to flocculate was used for calculating the Lf content of the toxoid solution. For example, when the first tube contained 250 μl of 100 units/ml antitoxin (25 units), a corresponding amount (i.e. 25 Lf) of toxoid was defined as being contained in the 500 μl of toxoid solution added. In this example, the concentration of toxoid in the toxoid solution was then 50 Lf/ml. Calculated Lf values of the toxoid solution, expressed in Lf per ml, were multiplied by a factor of 2.0 or 1.85 for Dtd A or B, respectively, to yield Lf/ml values of the original toxoid solutions. Assays were repeated 6 times for Dtd A (3 times by one investigator and 3 times by another) and 7 times for Dtd B (3 and 4 times for the two investigators, respectively). Mean values and standard errors of the results were calculated for each toxoid.

2.3. Flocculation assays using the platelet aggregometer

A laser light-scattering platelet aggregometer model PA-20 (Kowa Co., Nagoya, Japan) was used for these experiments, which were performed in aggregometry cuvettes (7 mm-diameter) containing a metal stirrer bar. The composition of the reaction mixture was the same as for the conventional assay except that the total volume was 400 μl (for assays at the 25 Lf/ml level). For assays at the 50 Lf/ml level, concentrations of toxoid and antitoxin were twice those of the 25 Lf/ml level assays. All the contents were mixed at time zero and the cuvettes were immediately mounted on the aggregometer. The mixture was kept at 37 °C with continuous stirring at 1000 rpm and particle count per unit flow was recorded every second by a personal computer with data logger software PA20 version 3 (Kowa Co., Nagoya, Japan). The detection limit of the device was 9 nm and

preliminary experiments showed that particles larger than 35 μm in diameter were scarcely seen (probably because of blocking of further aggregation by constant stirring at 1000 rpm). Thus, particles of 9 to 35 μm in diameter were detectable by the system.

2.4. Statistical analyses

Time (seconds) to acquire particle counts of 10,000, 20,000, 50,000, 100,000 and 150,000, respectively, were extracted from the recorded data and analyzed. A parabolic regression curve was fitted to the relationship of log-transformed antitoxin units (log[antitoxin]) and time (log-time) according to quadratic regression analysis. Homogeneity of variance was tested for repeated log-time measurements among antitoxin doses in each assay by Bartlett's method (Finney, 1978). Goodness of fit of the regression curve was evaluated by analysis of variance. Lf values were estimated as the local minimum value of the regression curve, which, therefore, was not always a value actually observed but a theoretical estimation based on the fitted equation.

3. Results

3.1. Conventional flocculation assays at 50 °C

Lf values for Dtds from manufacturers A (Dtd A) and B (Dtd B) were 196.9±3.1 and 172.9±3.1 Lf/ml (mean± standard error for 6 and 7 assays), respectively, (Table 1). Kf values for these toxoids were 8.8±0.4 min and 9.7± 0.4 min, respectively. As the concentration of antitoxin in the reaction mixtures was around 25 units/ml, the results of these assays can be compared to those at the 25 Lf/ml level of light scattering assays (below). Similar assays were carried out for tetanus toxoid, to compare the duration of the assay with that of Dtd. In this case, a longer time was needed for flocculation (Kf=approximately 19 min at 25 Lf/ml).

3.2. Light scattering flocculation assays using the platelet aggregometer

Flocculation assays on Dtd A and the Japanese reference diphtheria antitoxin for flocculation were performed at the 25 Lf/ml level by a laser light-scattering platelet aggregometer as described in Materials and Methods.

The diameter of the toxoid–antitoxin complexes to be detected as particles was set to 9–35 μm. The increase of particle number reflected the progression of complex formation. Fig. 1A shows the particle counts measured in nine independent flocculation assays at the 25 Lf/ml level, for a mixture containing 200 μl of saline, 100 μl of twofold-diluted Dtd A solution and 100 μl of 100 units/ml antitoxin solution (25 units/ml of final antitoxin concentration). In each assay, particle counts per unit flow were stable at <5000 during the first ca. 240 s and then quickly increased linearly. The linear increase of particle counts continued until they reached approximately 150,000 in around 360 s, and then the rate of increase slowed

Table 1
Conventional flocculation assays of diphtheria toxoids from manufacturers A and B

Toxoid manufacturer	Dilution of toxoid	Assay no.	Observer	Flocculation of mixture composed of						Kf (min)	Calculated Lf
				100 units/ml antitoxin solution (ml)	0.207	0.227	0.250	0.275	0.303		
				Saline (ml)	0.293	0.273	0.250	0.225	0.197		
				Toxoid solution (ml)	0.500	0.500	0.500	0.500	0.500		
A	4×	1	1		+	++	+++	++	+	8.0	200.0
		2	1		+	++	+++	+		8.0	200.0
		3	1		+	++	+++	++		7.8	200.0
		4	2			++	+++	+		10.0	200.0
		5	2			++	+++	+		10.0	200.0
		6	2		+	+++	++			9.0	181.6
		Mean±SE								8.8±0.4	196.9±3.1
B	3.7×	1	1		+	++	+++	+	+	9.1	185.0
		2	1		+	+++	++	+		9.1	168.0
		3	1		++	+++	++	+		9.0	168.0
		4	1		+	+++	++	+		9.0	168.0
		5	2			++	+++	+		10.0	185.0
		6	2		+	+++	++			11.0	168.0
		7	2		+	+++	++			11.0	168.0
		Mean±SE								9.7±0.4	172.9±3.1

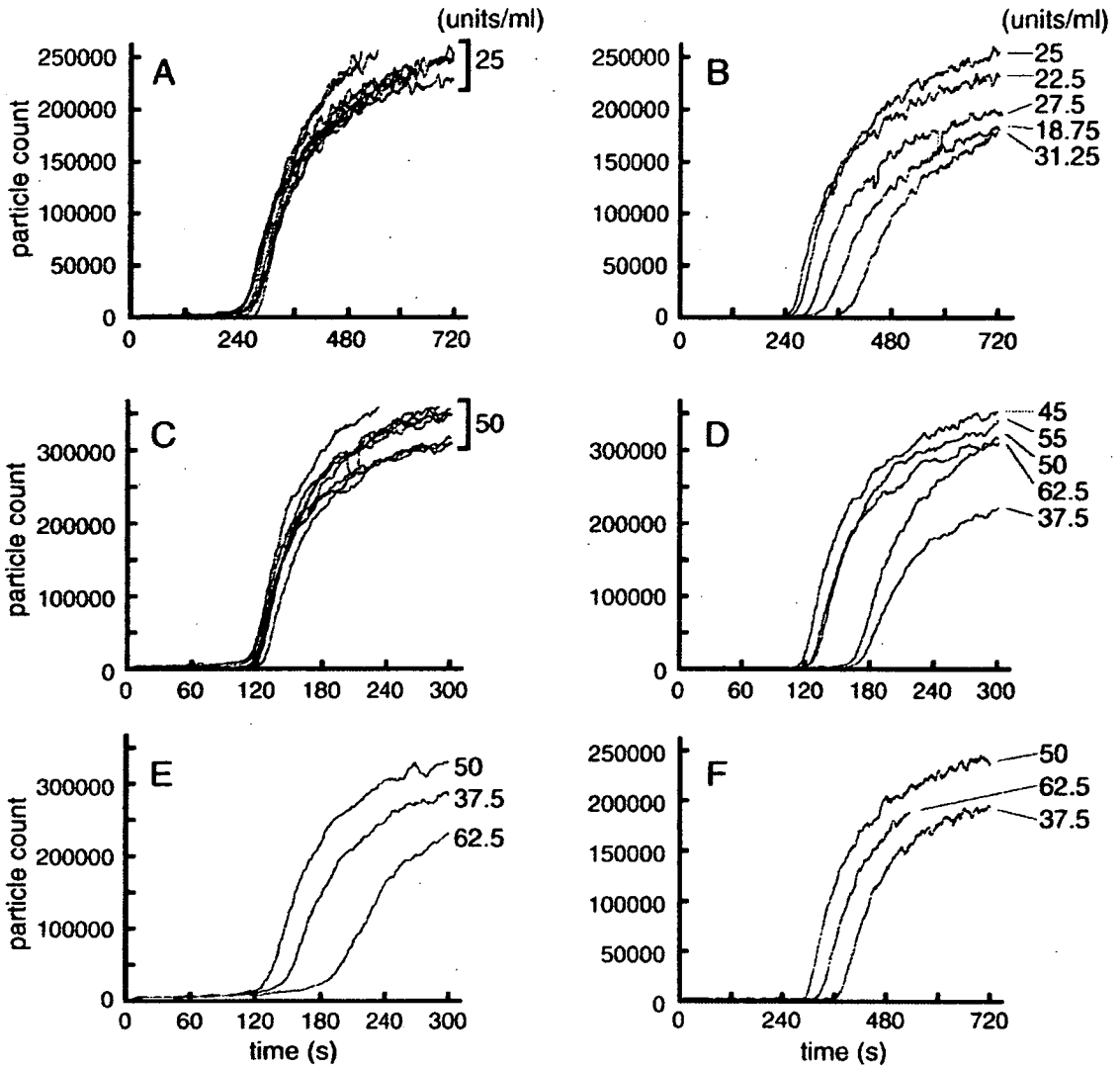

Fig. 1. Detection of particle formation by laser light-scattering platelet aggregometer. Toxoid and antitoxin were mixed to observe flocculation as described in Materials and Methods. A, Diphtheria antitoxin; 25 units/ml, Dtd A, approximately 25 Lf/ml. Results of nine independent assays are shown. B, Diphtheria antitoxin; 18.75–31.25 units/ml as indicated in the figure, Dtd A, approximately 25 Lf/ml. C, Diphtheria antitoxin; 50 units/ml, Dtd A, approximately 50 Lf/ml. Results of eight independent assays are shown. D, Diphtheria antitoxin; 37.5–62.5 units/ml as indicated in the figure, Dtd A, approximately 50 Lf/ml. E, Diphtheria antitoxin; 37.5–62.5 units/ml as indicated in the figure, Dtd B, approximately 50 Lf/ml. F, Tetanus antitoxin; 37.5–62.5 units/ml as indicated in the figure, Ttd A, approximately 50 Lf/ml.

gradually. The patterns seen in the linear portions of all nine curves (Fig. 1A) were indistinguishable, confirming the high degree of reproducibility of the assay.

When the antitoxin content in the mixture was altered, a notable difference was observed between the curves. Fig. 1B shows examples of curves obtained in assays with antitoxin content ranging from 18.75 to 31.25 units/ml, with a constant toxoid content of approximately 25 Lf/ml. The curve obtained from mixtures containing 18.25 or 31.25 units/ml of antitoxin (25% alteration) was clearly distinguishable from that obtained from the mixture containing 25 units/ml antitoxin. However, patterns at 22.5 and 27.5 units/ml of antitoxin (10% alteration) were not distinguishable from the pattern at 25 units/ml.

Doubling the toxoid and antitoxin concentration to the 50 Lf/ml level resulted in a more rapid increase of particle number, apparently without affecting reproducibility or quantitative properties of the assay. For the mixture containing 50 units/ml of antitoxin and approximately 50 Lf/ml of Dtd A, the increase started at about 120 s and continued in linear fashion up to ca. 150,000 particles per unit flow at 150–180 s (Fig. 1C). As shown in Fig. 1D, a 25% alteration in antitoxin content (37.5 and 62.5 units/ml) resulted in clearly different curves, while 10% (45 or 55 units/ml) did not.