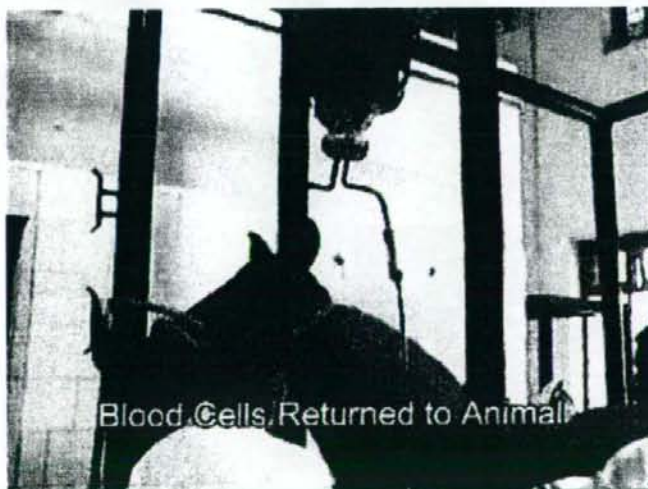
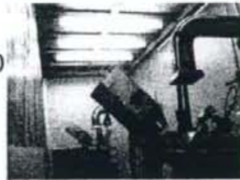
# INDIA: Blood bottle shaker

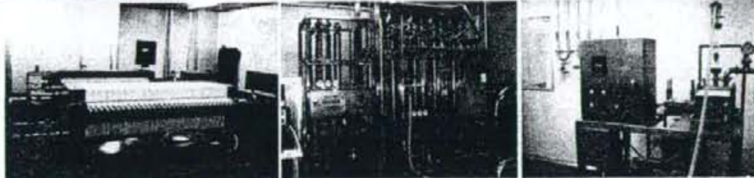
# INDIA: Blood cells returned



## CHINA: Production Capability

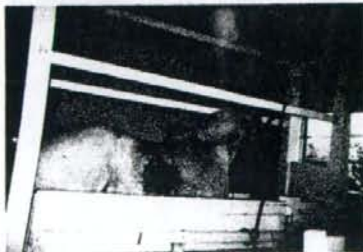
- TAT 5,000,000 vials
- ARS 500,000vials (100,000 doses)
- Antivenom 60,000vials

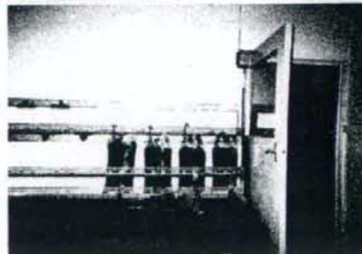




35

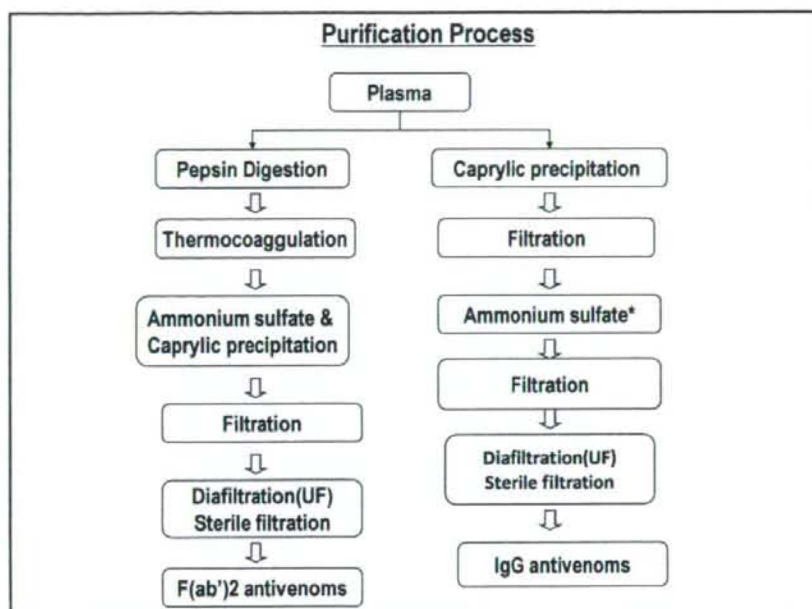
## Australia : Farm & bleeding





## Australia : Production area

7 March 2009




## *CONTROL OF INFECTIOUS RISKS*

Factors contributing to the viral safety of biological products

- The potential viral load in the starting material should be kept to a minimal:出発原料中の潜在的ウイルス混入は最小限にするべきである:
  - Epidemiological control of "donor" population 地域管理
  - Follow-up/health control of each "donor" 個体管理
  - Quality control assays of source material prior to downstream processing 原材料の品質管理

§ 13.1

WHO Workshop, Jakarta, 2008

## Proven complementary measures

- 1 = Minimizing the risks of viral contamination of the raw material
- 2: Inactivating and removing residual viruses during the manufacturing process (2 robust steps of viral reduction considered to provide a satisfactory level of viral safety)

§ 13.1

WHO Workshop, Jakarta, 2008

## Proven complementary measures

- 3: Adhering to GMP at all steps of the production
  - Consistency in processing
  - Monitoring
  - Recording of important safety parameters
- 4: Responding appropriately to new events/information arising from post-marketing surveillance
  - From clinical use of the product
    - Evidence of transmission of an infectious agent
  - From epidemiological surveillance
    - Occurrence of a new transmissible disease

§ 13.1

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## GMP

- Considering already-existing general GMP documents from WHO, regulatory authorities and other organizations, these Guidelines should ideally provide recommendations that are specific to animal derived antivenoms

既存GMPに免疫動物を上乗せ

- Additional inputs are therefore welcome from manufacturers and regulators to improve the Guidelines

新ガイドラインの製造所と規制当局の受け入れ

WHO Workshop, Jakarta, 2008

## GMP Aspects of Venom Production

- Snakes selected from range of relevant geographic locations
- Procedures established, training conducted & records maintained for:
  - Snake collection, identification, healthcare, handling, feeding
  - Venom collection (frequency, technique), freeze drying & storage
  - Facility, snake housing and equipment cleaning
  - Personnel gowning, health & safety considerations
    - Eg. Respirator masks, biohazard/cytotoxic cabinets, face shields
- 蛇の管理？

7 March 2009

## GMP Aspects of Venom Production

- Preventative measures to minimise of bioburden/cross-contamination of snakes and venom, eg nitrile gloves, fresh section of diaphragm per snake
- Traceability, snake identification, venom identification
  - Eg. Colour coding of venom type
- Maintenance/Validation/Calibration/Monitoring of critical equipment
  - Eg. Freeze drier, balances, freezers, fridges
- Establishment of quality control parameters & ongoing monitoring
  - Eg. Trending of venom potency results, snake venom volumes

7 March 2009

## GMP Aspects of Plasma Production

- Horses obtained from approved suppliers, quarantine
- Procedures established, training conducted & records maintained for:
  - Horse healthcare (screening & vaccination), handling, feeding
  - Venom dose preparation, storage & transport
  - Plasma collection (frequency, quantity, technique), storage & transport
  - Facility & equipment cleaning/sterilisation, animal hygiene
  - Personnel gowning, health & safety considerations
    - Eg. Respirator masks, biohazard/cytotoxic cabinets, face shields

7 March 2009

## GMP Aspects of Plasma Production

- Preventative measures to minimise cross-contamination/bioburden
  - Eg. Injection/collection site disinfection, nitrile gloves
- Traceability, venom & plasma identification, quantities, dates
- Maintenance/Validation/Calibration/Monitoring of critical equipment
  - Eg. balances, freezers, fridges, dataloggers, separation equipment
- Establishment of quality control parameters & ongoing monitoring

7 March 2009

## Background formation of personnel

- Relevant scientific discipline where background for the various manufacturing steps of antivenoms/venoms is needed e.g:

bacteriology, biology, chemistry, medicine, pharmacy, pharmacology, virology, immunology and veterinary medicine

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## Routine Assays (1)

§ 14.1

- Solubility (freeze-dried preparations)
- Extractable volume
- Osmolality
- Identity test
- Protein concentration
- Purity
- Molecular-size distribution
- Pyrogen test (rabbit and limulus test)
- Sterility test

WHO Workshop, Jakarta, 2008 49

## Routine Assays (2)

§ 14.2

- Concentration of sodium chloride and other excipients
- Determination of pH
- Concentration of preservatives
- Determination of agents used in plasma fractionation
- Visual inspection
- Residual moisture

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## The WHO Preclinical Tests of Venom Toxicity and Antivenom Efficacy (Section 17)

Essential Tests

- Toxicity determination ($LD_{50}$)
- Neutralisation efficiency ($ED_{50}$)

Recommended Tests

- Haemorrhagic activity (MHD)
- Necrotising activity (MND)
- Procoagulant activity (MCD-P, MCD-F)
- Defibrinogenating activity (MDD)

- Neutralising MHD, MND, MCD-P, -F, MDD

# II. 研究成果の刊行に関する一覧表

## II. 研究成果の刊行に関する一覧表

雑誌

| 発表者氏名 | 論文タイトル名 | 発表誌名 | 巻名 | ページ | 出版年 |
|---|---|---|---|---|---|
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特許出願
抗E型ボツリヌス神経毒素抗体
公開番号 特開2007-274924
公開日 平成19年10月25日
発明者：前田浩明、向本雅郁、幸田知子、小崎俊司、高橋元秀

組換え抗ボツリヌス神経毒素抗体
公開番号 特開2006-311857
公開日 平成18年11月16日
発明者：前田浩明、向本雅郁、幸田知子、小崎俊司、高橋元秀

A型ボツリヌス毒素中和組成物及びヒト抗A型ボツリヌス毒素抗体
出願番号 特願2008－017152
出願日2008.1.29
国際出願番号 PCT/JP2009/000250
出願日2009.1.23
発明者：東 成見、高橋元秀、小崎俊司、幸田知子、黒澤良和、黒澤仁

# III. 研究成果の刊行物・別刷り

INFECTION AND IMMUNITY, June 2006, p. 3682–3683 Vol. 74, No. 6
0019-9567/06/$08.00+0 doi:10.1128/IAI.01731-05
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# Isolation of a Human Monoclonal Antibody with Strong Neutralizing Activity against Diphtheria Toxin

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Received 21 October 2005/Returned for modification 1 November 2005/Accepted 29 March 2006

**We isolated a human monoclonal antibody against diphtheria toxin (DT). It bound to fragment B with a binding activity ($K_d$) of 3.01 nM. The neutralizing activity assayed by the rabbit skin test was estimated to be 73,600 IU/g. This could be used as a therapeutic drug against DT in place of the traditional equine sera.**

Antisera prepared from hyperimmune horse blood are still used as drugs against diphtheria toxin (DT) in emergency situations. Since equine antisera could induce serious side effects such as serum sickness, there is a strong need to develop a human monoclonal antibody (Ab) against DT. DT excreted by *Corynebacterium diphtheriae* has been well characterized (12). It is a single polypeptide chain ($M_r$, 58,000) composed of two structurally distinct regions with three functional domains and contains a protease-sensitive site. The nicked toxin produced upon limited proteolysis consists of two polypeptides that are held together by a disulfide bond. The $NH_2$-terminal region, fragment A, catalyzes the transfer of the ADP-ribose moiety from NAD to elongation factor 2 and thus blocks protein synthesis (4). The COOH-terminal region, fragment B, binds to a specific receptor on the cell surface and mediates transfer of fragment A to the cytoplasm (6, 11, 14). DT is lethal for susceptible animals, including humans, in doses of 100 ng/kg or less (12). Mass immunization of children has been performed on a worldwide scale since the 1940s. The degree of immunity to DT in the serum of each person should be critical for determination of susceptibility to diphtheria. There is a good correlation between clinical protection and the presence of serum antitoxin, whether this results from disease or immunization. According to internationally accepted definitions, an antitoxin concentration of less than 0.01 IU/ml indicates susceptibility, 0.01 to 0.09 IU/ml indicates basic protection, and >0.1 IU/ml indicates full protection (2). Once the symptoms of this disease start to appear, the antiserum should be given to the patient as soon as possible. The amount of Abs required for curing is much larger than that required to prevent infection. It ranges from 5,000 to 50,000 IU, depending on the degree of disease progress (2).

A human Ab library was screened with DT and diphtheria toxoid (DTD) as the antigen (Ag) by the panning method (3, 5). DT and DTD were kindly given to us by Kunio Ohkuma (Kaketsuken, Kumamoto, Japan). DTD is inactivated toxin that is used for vaccination. It has been prepared by treatment with formaldehyde (13). The Abs were initially prepared in the form of an Ab fused with truncated cp3 (Fab-cp3) and converted to immunoglobulin G1 (IgG1) (3). In this paper, we report data obtained with IgG. Fifty-five different clones were isolated. Four of them, DTD4, DTD8, DTD10, and DTD76, distinctively showed neutralizing activities. The amino acid sequences of these four clones are shown in Fig. 1. Western blotting with separated fragments A and B indicated that DTD4 and DTD76 bound to fragment B and DTD8 and DTD10 bound to fragment A. The rate constants, and thus the binding constants, of these four clones against DTD and DT were measured with the BIAcore instrument (5) (Table 1). Abs were coupled to the sensor chip, and Ags were injected to avoid the influence of divalency. Clones DTD4, DTD8, and DTD10 bound to DT more strongly than to DTD, whereas DTD76 bound to DTD more strongly than to DT.

In vitro DT-neutralizing activities were estimated by the pH color change method (9, 10). When the cells were metabolically active, the color of the medium changed to yellow. When cellular metabolism was stopped by toxin action, it remained red. Thus, the titration endpoint for anti-DT neutralizing activity was taken at the highest dilution of anti-DT Ab to be tested in the well in which the color of the medium was orange. The results are indicated in the left column of values in Table 2. The antitoxin titers are expressed in international units by comparison with the result obtained with equine sera. The in vivo neutralizing activities of Abs against DT were determined by the rabbit skin test as described previously (1, 7). In brief, DT

TABLE 1. Rate constants ($k_a$, $k_d$) and dissociation constant ($K_d$) of IgG form of Abs with DTD and DT assayed by the BIAcore instrument

| Clone | Anti-DTD | | | Anti-DT | | |
|---|---|---|---|---|---|---|
| | $k_a$ ($10^4$ $M^{-1}$ $s^{-1}$) | $k_d$ ($10^{-4}$ $s^{-1}$) | $K_d$ ($10^{-9}$ M) | $k_a$ ($10^4$ $M^{-1}$ $s^{-1}$) | $k_d$ ($10^{-4}$ $s^{-1}$) | $K_d$ ($10^{-9}$ M) |
| DTD4 | 4.14 | 8.70 | 21.2 | 10.6 | 3.19 | 3.01 |
| DTD8 | 4.99 | 3.82 | 7.66 | 16.2 | 0.831 | 0.513 |
| DTD10 | 8.89 | 3.76 | 4.22 | 8.52 | 0.243 | 0.285 |
| DTD76 | 11.9 | 2.29 | 1.93 | 7.8 | 4.83 | 6.19 |

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Heavy chain

```
                FR1                           CDR1     FR2                CDR2                 FR3                                CDR3                   FR4
         1        10        20        30      ab35  40          50 ab      60     64  70        80 abc       90  94                                103    110
DTDH4    QVQLQQSGPGPAKPSQTLSLTCAISGDSVS SDSAAWN WIRQSPSRGLEWLG RTYYRSTWYRDYAPSVKS RITINPDTSKNQFSLQLNSVTPEDTAVYYCAR DKDSFESSGSLYTAKKMGFDP WGQGTLVTVS
DTDH8    QVQLQESGPGLVKPSETLSLTCTVSGGSIS SYYWS.. WIRQPPGKGLEWIG YIY..YNGSTNYNPSLKS RVTISVDTSKNQFSLKLSSVTAADTAVYYCAG QPFLQRSLYPGAVWH       WGQGTLVTVS
DTDH10   QVQLQQSGPGLVKPSETLSLTCTVSGGSIS SYYWS.. WIRQPPGKGLEWIG YIY..YNGSTNYNPSLKS RVTISVDTSKNQFSLKLSSVTAADTAVYYCAG QPFLQRSLYPGAVWH       WGQGTLVTVS
DTDH76   QVQLQESGPGLVKPSETLSLTCSVSGGAVS SSDHAWG WIRQSPGKGLEWIG SIN..YSGGTYYNPSLKS RATLSLDTSKNQFSLNLRSVTAADTAVYYCAR RGRSLTVTFDH           WGQGTLVTVS
```

Light chain

```
                FR1                    CDR1          FR2             CDR2           FR3                          CDR3         FR4
         1        10         20 21     ab      35   40         50      57 60        70        80      88               100
DTDL4    ETTLTQSPGTLSLSPGERATLSC RASQS.VRSSYLA WYQQKPGQAPRLLIY GASSRAT GIPERFSGSGSGTDFTLTISRLEPEDFAVYYC QQYGSSPIT... FGQGTRLEIKRTVAAPS  Kappa
DTDL76   DIQMTQSPSTLAASVGDRVTITC RASQ..SISSWLA WYQQKPGKAPKLLIY KASSLES GVPSRFSGSGSGTEFTLTISSLQPDDFATYYC QQYNSYST.... FGQGTKLEIKRTVAAPS

DTDL8    QSVLTQPPS.ASGTPGQRVTISC SGSSSNIGSNTVN WYQQLPGTAPKLLIY SNNQRPS GVPDRFSGSKSGTSASLAISGLQSEDEADYYC AAWDDSLNGYV. FGTGTKVTVLGQPKANP  Lambda
DTDL10   QSVLTQPPS.ASGTPGQRVTISC SGSSSNIGSNTVN WYQQLPGTAPKLLIY SNNQRPS GVPDRFSGSKSGTSASLAISGLQSEDEADYYC AAWDDSLNGYVV FGGGTKLTVLGQPKAAP
```

FIG. 1. Amino acid sequences of variable regions of the heavy and light chains of Abs that exhibited neutralizing activities against DT. The numbering of amino acid positions is according to the definition of Kabat et al. (8).

mixed with serially diluted Abs was injected into rabbit back skin. The diameter of local erythema was measured at the site of injection at 48 h postinjection. The results are shown in the rightmost column of Table 2. The antitoxin titers are expressed in international units as relative potency with respect to the result obtained with the standard antitoxin. The neutralizing activities of Abs assayed by the pH color change method and by the rabbit skin test were similar to each other in four cases, indicating a good correlation between the two systems (10).

In the case of DTD4, which showed the strongest neutralizing activity of the four clones, it was estimated to be 73,600 IU/g by the in vivo assay. The binding activity ($K_d$) with DT was 3.01 nM. On the other hand, while DTD76 bound to DTD with strong affinity, it showed very weak neutralizing activity. Although both clones bound to fragment B, they should recognize completely different epitopes. It is possible that clone DTD4 corresponded to the Ab that had matured in vivo by immunization with vaccine against DT. We propose that DTD4 be clinically tested for therapeutic use.

**Nucleotide sequence accession numbers.** The nucleotide sequences of the eight genes described in Fig. 1 have been registered in GenBank under accession numbers AB063724 (DTDH4), AB063723 (DTDH8), AB063729 (DTDH10), AB063743 (DTDH76), AB063937 (DTDL4), AB064049 (DTDL76), AB063977 (DTDL8), and AB064205 (DTDL10).

TABLE 2. Neutralizing activity against DT shown by IgG form of Abs

| Clone | Neutralizing activity[a] | |
|---|---|---|
| | In vitro assay[b] | In vivo assay[c] |
| DTD4 | 52,000 | 73,600 |
| DTD8 | 4,800 | 3,360 |
| DTD10 | 2,300 | 1,612 |
| DTD76 | 215 | 372 |

[a] In international units per gram.
[b] Measured by the pH color change method.
[c] Measured by the rabbit skin test.

*Editor:* J. B. Bliska

We thank Kunio Ohkuma and Eisuke Mekada for providing materials. We also thank Atsuko Suzuoki for preparation of the manuscript.

This study was supported in part by a grant for Research on Pharmaceutical and Medical Safety from the Ministry of Health, Labor, and Welfare of Japan.

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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 K öhler 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 $\mu$g/ml gentamicin (Sigma), 50 $\mu$M 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 $\mu$g/ml gentamicin, 50 $\mu$M 2-mercaptoethanol, 5 $\mu$g/ml bovine insulin (Sigma), and 8 $\mu$g/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. $4.0 \times 10^7$ cells of the enriched EBV-transformed B cell line were fused with $1.0 \times 10^7$ 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 $2.5 \times 10^5$ 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 $\mu$g/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 $\mu$g/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 $\pm$ 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 $\mu$g/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 $\mu$g of each purified mAbs were biotinylated with 100 $\mu$g 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 $\mu$l 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 $\mu$l of 0.5 $\mu$g/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 $\mu$g/ml of each human mAb was applied at a flow rate of 10 $\mu$l/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 $\mu$l/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 $\mu$g/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 $\pm$ 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.