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Cloning and Expression of Human Anti-Tumor Necrosis Factor-α Monoclonal Antibodies from Epstein-Barr Virus Transformed Oligoclonal Libraries

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Peripheral blood was obtained from a healthy human volunteer and transformed with Epstein-Barr virus (EBV). This produced an oligoclonal cell library in culture medium that was screened by ELISA for anti-human tumor necrosis factor-α (TNFα) activity. RNA from two positive clones was applied to RT-PCR using antibody-specific primers, and the light (κ and λ) and heavy chain genes (γ and μ) were cloned into the plasmid vector pFab1-His2. The antibodies produced in *Escherichia coli* as Fab fragments were assayed for anti-TNFα activity utilizing ELISA. Two IgG1/κ anti-TNFα antibodies and two IgM/κ anti-TNFα antibodies were isolated. DNA sequence analysis showed that the VL and VH gene families of IgM and IgG were the same. Both the antibodies showed almost the same activity on ELISA-testing. Ten clones randomly selected from light (κ and λ) and heavy (γ and μ) chain genes in the oligoclonal cell library 1D5 were sequenced, and each gene (κ, λ, γ, and μ) was found to be composed of one to three different genes. These data support the conclusion that the cell clone is oligoclonal at the molecular level.

Key words: EBV, Fab, human monoclonal antibody, oligo clone, TNFα.

The cytokine TNFα plays an important role in the pathogenesis of infectious, neoplastic and autoimmune disease as well as in maintaining normal physiological function. Animal models have shown that TNFα is involved in septic shock (*1*), bacterial meningitis (*2*), graft-*versus*-host-disease (GVHD) (*3*), autoimmune lupus (*4*), and multiple sclerosis (*5*). Recently, anti-TNFα monoclonal antibodies (mAbs) have been approved for clinical trials in diseases in human, such as sepsis (*6*, *7*), rheumatoid arthritis (*8*, *9*), GVHD (*10*), Crohn's disease (*11*), and louse-borne relapsing fever (*12*). These studies suggest that anti-TNFα antibodies have therapeutic capacity in a variety of human diseases. The mAbs used in these trials were native mouse mAbs or engineered mouse mAbs such as chimeric (*13*) or humanized (*14–16*) antibodies. In humans, several antibodies against TNFα have been isolated by phage display systems (*17*, *18*) or by Epstein-Barr virus (EBV) transformation (*19*). These antibodies are derived from human materials, are pure human polypeptide, and therefore do not include any rodent polypeptides.

In this study, we developed anti-TNFα antibody-producing cells by EBV transformation of peripheral lymphocytes from a healthy individual. Anti-TNFα antibody genes from these EBV-transformed cells were then cloned in soluble Fab cloning vector (*20*), and finally anti-TNFα antibodies were produced in *Escherichia coli*.

MATERIALS AND METHODS

Establishment of Antibody Producing EBV-Transformed Cells—Peripheral blood was collected from an anti-TNFα antibody-positive healthy human volunteer. Lymphocytes were separated by Ficoll-paque density-gradient centrifugation. EBV was prepared from culture supernatant of the B95-8 cell line and used for transformation (10^5 TD_{50}/ml). The EBV-infected cells were seeded on a 96-well plate at a density of 5×10^3–10^4 with RPMI 1640 culture medium (Gibco BRL, Life Technologies) supplemented with 20% fetal calf serum (Gibco BRL, Life Technologies). The medium was changed every 4 days. After 4 to 6 weeks, the cells were transferred to a 24-well plate and then finally to a 6-well plate. The supernatant was analyzed by ELISA, and anti-TNFα positive oligoclones were selected. The cells and supernatant were kept at −70°C. The cells that were found, on DNA sequencing and immunological analysis, to be an oligoclonal mixture of IgG- and IgM-producing cells were called the oligoclone cell library.

Cloning of Heavy and Light Chain Antibody Genes—Total cellular RNA was isolated from pelleted cells using a commercial kit (RNeasy mini kit, Qiagen). Using random 9-mers, nucleotides and reverse transcriptase (Takara, RNA-PCR kit, Ohtsu), cDNAs were synthesized and were amplified by the polymerase chain reaction (PCR), with heavy

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; Fd, heavy chain portion of Fab, TD50, 50% cell transforming dose; VH, variable region of heavy chain; VL, variable region of light chain

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and light chain primers specific for human immunoglobulins (Ig) (*21*). To amplify the μ chains, the reverse primer (FDM) (5′-CCGCGGCCGCCACTGGAAGAGGCACGTTCT-TTTC-3′) was used. The "touchdown" PCR protocol (*22*) was employed: *i.e.*, three cycles each of denaturation at 95°C for 1 min, annealing for 1 min, and elongation at 72°C for 2 min, for a total of 11 cycles. The annealing temperature was varied from 65 to 55°C in steps of 1°C. The touchdown cycles were followed by 25 cycles using an annealing temperature of 55°C. The resultant PCR product was gel-purified in agarose and extracted using Qiaquick spin-columns (Qiagen). The light chain and heavy chain Fd genes were then cloned into the *NheI*/*Asc*I and the *Sfi*I/*Not*I sites of the expression vector pFab1-His2 (*20*).

E. coli Expression and Extraction of the Soluble Fab Fragment—The ligated pFab1-His2 vectors with the light chain (κ and λ) and Fd heavy chain genes (γ and μ) were introduced into competent *E coli* JM109 cells (Toyobo, Osaka). After transformation, the *E. coli* cells were plated onto Luria-Bertani (LB)/ampicillin (50 μg/ml) plates. Isolated bacterial colonies were incubated at 30°C in 2 ml of Super Broth (SB) with ampicillin (50 μg/ml) and $MgCl_2$ (1.5 mM). Isopropyl-β-D-thiogalactopyranoside (IPTG) was used to induce production of the Fab protein. Cells from the bacterial cultures were pelleted, resuspended in 0.3 ml of B-PER (Pierce) with a protease inhibitor cocktail (Complete, Boehringer Mannheim), and shaken for 5 min at room temperature. Cell lysates were centrifuged at 15,000 ×*g* for 10 min, and the resultant supernatant containing the Fab antibody was collected.

ELISA—ELISA plates (E.I.A/R.I.A, Costar) were coated with recombinant human (rh) TNFα derived from *E. coli* (50 ng/well, Pepro Tech). PBS containing 1% bovine serum albumin (BSA) was added to the plates to block any non-specific antigen-antibody reactions. Fab antitbodies from the bacterial culture were added to the plates and incubated for 1 h at room temperature. Then the second antibody, horseradish peroxidase (HRP)–conjugated goat anti–human IgG Fab–specific antibody (Sigma), was added and the plates were incubated for 1 h at room temperature. To detect IgM/κ or λ, anti–IgG Fab–specific antibody was also used, because it was more reactive toward IgM-derived Fab than anti–IgM whole–specific antibody. Finally, tetramethylbenzidine base (Gibco BRL, Life Technologies) was added and the plates were incubated at room temperature. An optical density (OD) reading at 650 nm was obtained for each well.

Nucleotide Sequence Analysis—The cloned heavy chain Fd and light chain genes in the pFab1-His2 vector showing a positive reaction with rhTNFα were recloned into sequencing vectors CV-1 and CV-2, respectively (*21*). Plasmid DNAs were prepared using a commercial kit (Qiaprep miniprep kit, Qiagen). Cyclic sequencing of these DNAs was performed in both directions using a commercial kit (Thermo Sequence kit, Amersham Pharmacia Biotech) and the M13 forward (5′-CACGACGTTGTAAAACGAC-3′) and reverse (5′-GGATAACAATTTCACACAGG-3′) primers on a DNA sequencer (LI-COR model 4000L, Lincoln).

Affinity Purification of Fab Antibodies—One liter of bacterial cell culture producing the recombinant Fab antibody was pelleted by centrifugation, resuspended in 50 ml B-PER with a protease inhibitor cocktail (Complete), and shaken for 15 min at room temperature. To collect the Fab protein fraction, cell lysates were centrifuged at 15,000 ×*g* for 15 min, and the resulting supernatant, containing the Fab antibody, was transferred to an anti-Fab antibody affinity column, as described by Harlow and Lane (*23*). The eluted Fab protein was dialyzed against PBS and concentrated by centrifugation (Centriplus 30, Amicon).

Cell Culture—TNFα-sensitive L929, mouse connective tissue–derived cells were obtained from the Riken Cell Bank (RCB1422; Tsukuba). The cell line was maintained in Eagle MEM culture medium (Nissui, Tokyo) supplemented with 10% horse serum (Gibco BRL, Life Technologies).

RESULTS

cDNA Cloning and Expression of the Fab Fragments—The anti-TNFα activity of the supernatant from the oligoclone libraries was analyzed by ELISA. The supernatant of oligoclones 1D5 and 1F8 showed anti-TNFα activity. Both oligoclone cell libraries were used to obtain total cellular RNA. Both the light chain and Fd region genes were amplified by RT-PCR. Light chain (κ and λ) and heavy chain genes (γ and μ) were cloned into pFab1-His2 vector. Eight libraries were generated: 1D5 heavy chains (γ or μ) with 1D5 light chains (κ or λ), and 1F8 heavy chains (γ or μ) with 1F8 light chains (κ or λ).

To identify specific bacterial clones expressing the Fab antibody of interest, we transformed the library DNA to JM109 and screened each colony's Fab activity against rhTNFα by ELISA. Four positive clones isolated from the oligoclone cell libraries, 1D5(IgM/κ), 1F8(IgM/κ) and two 1F8-(IgG/κ) were selected for further analysis and comparison.

DNA Sequence Analysis—The amino acid sequences of the heavy and light chain variable regions, which were deduced from the base pair sequences of the positive clones DNA, are shown in Fig. 1. Light and heavy chain Fd regions were compared with those in non-redundant DNA databases (DDBJ, GenBank, EMBL) by using the MPsrch program (http://disc.dna.affrc.go.jp/) and the Kabat database (http://www.ncbi.nlm.nih.gov/igblast/) (*24*). The Ig gene family of each gene and the most homologous germ-line are indicated in Table I. H-chain of clone 1D5-mk29 was highly homologous with the anti-TNFα antibody genes reported by Griffiths *et al.* (*17*) and Jespers *et al.* (*18*). The alignment of the H-chain variable region of 1D5-mk29 and the homologous genes shows that the framework regions (FR) and complementarity determining regions 1 and 2 (CDR1 and CDR2) are essentially identical and that there is amino acid variation among the clones in the CDR3 domain (Fig. 2). The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB027433–AB-027440.

These cell clones were not monoclonal, but oligoclonal, because light chains κ and λ and heavy chains γ and μ were detected by immunological analysis of the culture medium. In addition, molecular cloning and DNA sequencing analysis revealed that both 1D5 and 1F8 cells were oligoclonal. In these analyses, 10-randomly selected clones of both the light (κ and λ) and heavy (γ and μ) chain genes derived from 1D5 were DNA-sequenced (Table II). It was found that in the amplified heavy chains, only one γ and one μ sequence were detected, but in the light chains, three κ sequences and two λ sequences were detected. These data

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Fig 1 **The deduced protein sequences of the heavy and light chain V-genes of the anti-TNFα antibody fragments 1D5-mk29, 1F8-mk26, 1F8-gk10 and 1F8-gk20.** Abbreviations CDR, complementarity-determining region, FR, framework region Dashes and dots indicate identical residue and deletions, respectively

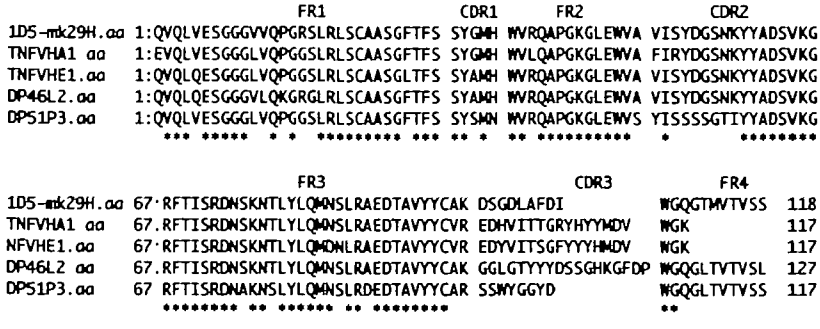

Fig 2 **The deduced protein sequences of the heavy chain V-genes of several anti-TNFα IgM antibody fragments isolated from human samples.** TNFVHA1 and TNFVHE1 were reported by Griffiths *et al* (*17*) and DP46L2 and DP51P3 were reported by Jespers *et al* (*18*) Amino acids residues conserved among all the sequences are indicated by asterisks

TABLE I **Ig class, V-gene family, closest germ line, and percentage identity of closest germ line of Fab fragments from the EBV-transformed oligoclones.**

Clone	Ig class	V_H family	Closest germline	Percentage identity	V_L family	Closest germline	Percentage identity
1D5-mk29	IgM	V_H3	VH3-30	99%	V_k3	L6	96%
1F8-mk26	IgM	V_H3	VH3-23	96%	V_k3	L6	98%
1F8-gk10	IgG1	V_H1	VH1-69	92%	V_k1	L12	97%
1F8-gk20	IgG1	V_H1	VH1-69	99%	V_k1	L12	95%

TABLE II **V-gene family, numbers of the same gene in 10 sequenced genes, closest germ line, and percentage identity of closest germ line of Fab fragments from the EBV-transformed oligoclone 1D5.**

	Chain	Gene family	[Numbers]	Closest germline	Percentage identity
H-chain	γ	VH5	[10/10]	VH5-51	95%
	μ	VH3(=1D5-mk29)	[10/10]	VH3-30	99%
L-chain	κ	Vκ1	[2/10]	L12	97%
		Vκ3	[2/10]	L6	97%
		Vκ3(=1D5-mk29)	[6/10]	L6	96%
	λ	Vλ1	[5/10]	V2-7	99%
		Vλ3	[5/10]	V1-17	99%

support the premise that clone 1D5 is oligoclonal.

Purification and Characterization of the Recombinant Fab Antibody—To characterize further the recombinant Fab antibodies, bacterial cultures containing each recombinant plasmid were induced to produce Fab antibodies by the addition of 50 μg/ml IPTG. The cells were then lysed and the Fab antibodies were purified by affinity chromatography. The purity of the Fab antibodies as determined by SDS-PAGE analysis was greater than 95% (data not shown). The recovery of purified Fab antibodies was from

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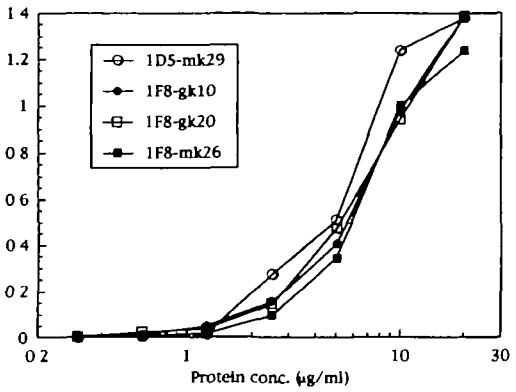

Fig 3 **Analysis of the avidity of anti-TNFα antibodies by ELISA.** Serial dilutions of 1D5-mk29 (○), 1F8-gk10 (●), 1F8-gk20 (□), and 1F8-mk26 (■) were incubated in microtiter plates precoated with 50 ng/well of rhTNFα

58 to 125 μg per liter of culture.

Quantitative Analysis of the Recombinant Fab—The antigen-binding avidity of the purified Fab proteins was measured by ELISA (Fig. 3). The amount of the antibodies estimated from the absorbance of about 0.65–0.70 at OD_{650}, the value at which 50% maximal binding was observed, was from 5 to 7 μg There was no significant difference in avidity between the clones.

TNFα neutralizing activity of each purified 10 μg/ml Fab protein was assayed by using L929 cells, but no neutralizing activity was detected (data not shown).

DISCUSSION

To our knowledge, this is the first report on genetic analysis of EBV-transformed oligoclone libraries generated from a healthy individual. In previous attempts to obtain monoclonal anti-TNFα human antibodies, one method that has been used to get IgM-derived scFv antibodies is the phage display method (*17*) This method has also been modified to make human antibody against TNFα (*18*). Human antibody fragments directed against TNFα were selected from phage display libraries using mouse monoclonal IgM antibody as a template. The isolated human antibodies were IgM Fab. At the same time, several monoclonal human IgM antibodies were prepared by using EBV-transformation followed by the hetero-hybridoma method (*25*) EBV-transformation has also been used to produce monoclonal IgM antibodies from the plasma of autoimmune individuals, although this has not been possible using the plasma of normal individuals (*19*). Anti-TNFα neutralizing antibody has been developed for the treatment of some chronic diseases. Initially chimeric antibody (cA2) was developed (*13*) and has been used for clinical trials in both rheumatoid arthritis (*8*) and Crohn's Disease (*11*). In these studies, cA2 treatment was highly effective and had a low incidence of adverse events. A humanized anti-TNFα monoclonal antibody has been developed (*14–16*). Human antibody is expected to produce less immunogenicity than chimeric antibody or humanized antibody.

In this study, we first established anti-TNFα antibody–producing oligoclonal cells by EBV-transformation of peripheral blood obtained from a healthy individual, and then constructed a Fab library. From this library, four anti-TNFα antibody clones were isolated. It is difficult to clone single anti-TNFα antibody producing cell lines from an oligoclonal cell library, because of the instability of a single cell line. It is, however, relatively easy to isolate clones showing anti-TNFα antibody activity from a Fab library.

Comparison of the DNA sequences between 1D5-mk29, TNFVHA1, and TNFVHE1 reported by Griffiths *et al.* (*17*) revealed that all FRs and CDRs except CDR3 were identical (Fig. 2). Light chains from 1D5-mk29 and 1F8-mk26 are almost identical to each other, as are those of 1F8-gk10 and 1F8-gk20 (Fig. 1). Heavy chains from 1D5-mk29 are almost identical at the FR to 1F8-mk26, and the same is true of 1F8-gk10 and 1F8-gk20. Preliminary genetic analysis of oligoclone 1D5 showed that the clone is oligoclonal at the molecular level (Table II). The heavy chain is composed of one γ chain and one μ chain Comparison of the heavy chain with germ lines revealed that the γ chain is 95% homologous with the germ line VH5-51 (*26*) and that the μ chain is 99% homologous with the germ line VH3-30 (*26*). The light chain is composed of three κ chains and two λ chains. Comparison of the κ chain with the germ lines revealed that one κ chain is 97% homologous with the germ line L12 (*27*) and that the other two κ chains are 97 and 96% homologous with the germ line L6 (*28*), respectively. Comparison of the λ chain with the germ lines revealed that one λ chain is 99% homologous to the germ line V2-7 (*29*) and the other λ chain is 99% homologous to the germ line V1-17 (*29*). Light chain homology to the germ line is higher than that of heavy chains, because heavy chain CDR3 diversity is greater than that of light chains (*30*).

In this study, bioassay using L929 cells did not show any TNFα neutralizing activity in the Fab clones (data not shown). Epitope mapping studies on TNFα have revealed that there were at least six different epitopes on TNFα and three of them reacted with neutralizing antibodies (*31*, *32*). The Fab clones may have reacted with non-neutralizing epitopes. The aim of our research is to develop human anti-TNFα antibody with neutralizing activity for clinical use. Further work is in progress to develop another oligoclonal EBV-transformed library for TNFα neutralizing antibody.

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