Results

The phenotype of the Lin$^{-/low}$CD34^{+}TIE2^{+} population

Mononuclear cells were separated by the expression of Lineage, CD34, and TIE2 expression. CD34^{+} cells from umbilical cord blood contained a distinct population of TIE2 expressing cells. Lin$^{-/low}$CD34^{+}TIE2^{+} cells comprised 18.8% of the total Lin$^{-/low}$CD34^{+} population (Fig. 1).

Colony formations by Lin$^{-/low}$CD34^{+}TIE2^{+} cells and Lin$^{-/low}$CD34^{+}TIE2^{-} cells

Each sorting window was established as described in Materials and methods. The presentative colony-forming patterns of five independent experiments are shown in Fig. 2A. Lin$^{-/low}$CD34^{+}TIE2^{+} cells contained BFU-E, CFU-GM, and CFU-Mix. Plating efficiency (PE) of Lin$^{-/low}$CD34^{+}TIE2^{+} cells in the presence of CSFs was approximately 38% (76/200) (Fig. 2A). In contrast, the

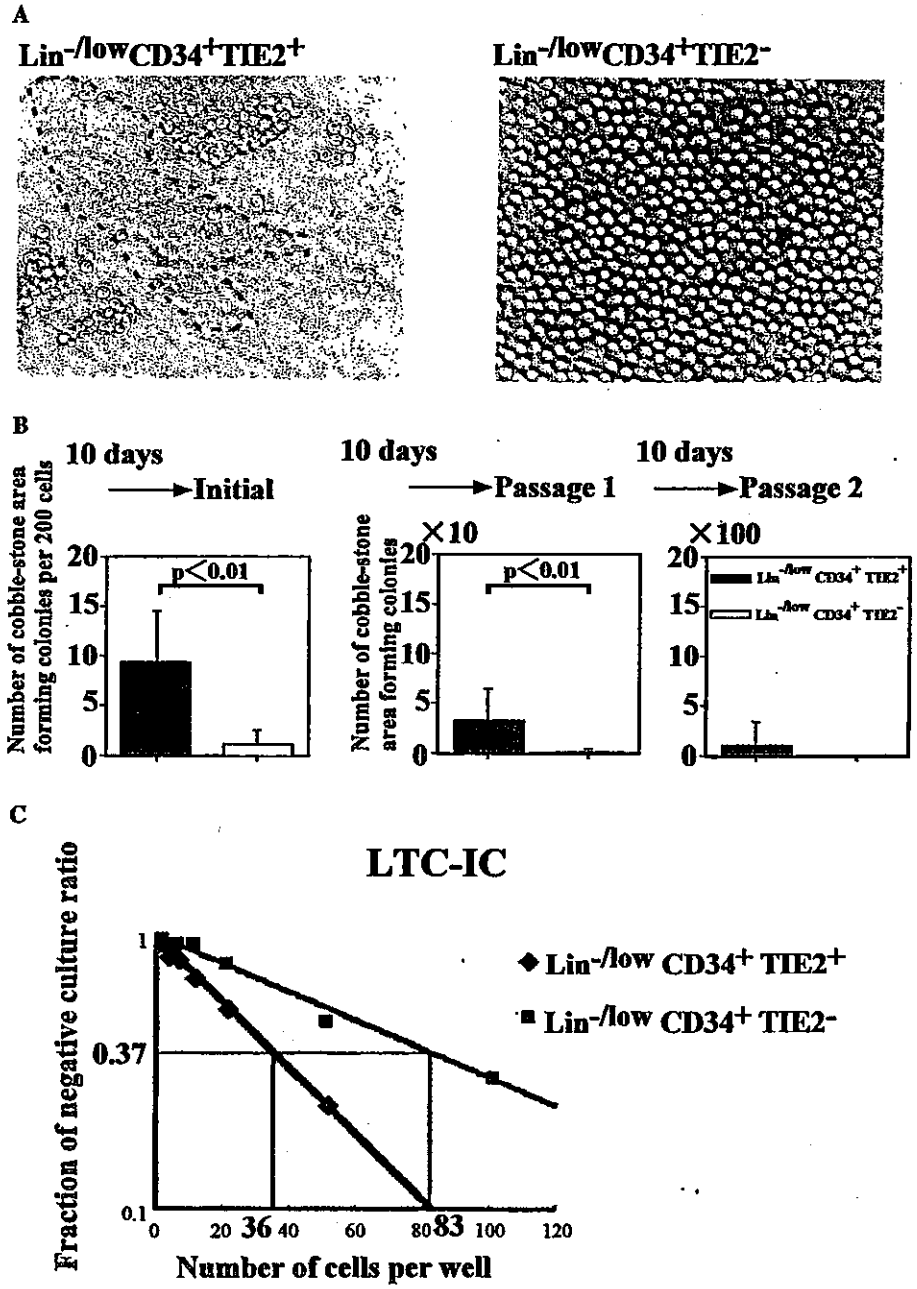


Fig. 3. (A) Cobble-stone appearance of cultured Lin$^{-/low}$CD34^{+}TIE2^{+} cells from cord blood. (B) CD34^{+}TIE2^{+} cells formed cobble-stone area by replating culture. (C) Representative data of limiting dilution analyses are shown. Decreasing numbers of each of Lin$^{-/low}$CD34^{+}TIE2^{+} cells and Lin$^{-/low}$CD34^{+}TIE2^{-} cells were seeded onto MS-5 feeders and the numbers of clonogenic cells detectable after five weeks of cocultures were determined. In these experiments, the frequencies of LTC-IC in the initial cell populations were as follows: 1/36 cells and 1/83 for Lin$^{-/low}$CD34^{+}TIE2^{+} and TIE2^{-}, respectively.

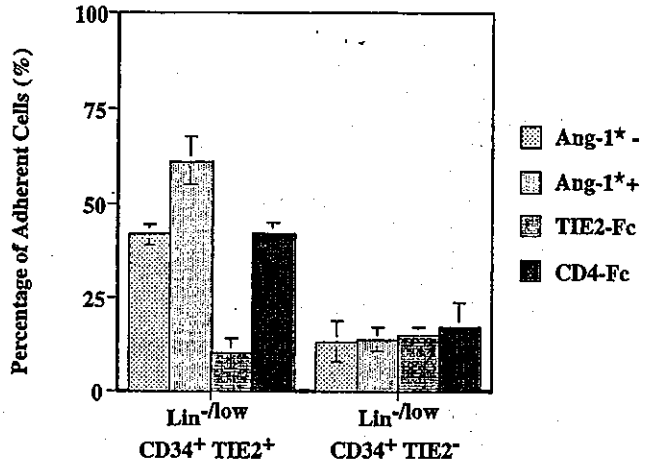


Fig. 4. Angiopoietin-1* (Ang-1*) promotes the adhesion of TIE2$^+$ cells to FN. The adhesion to FN of Lin$^{-/low}$CD34$^+$TIE2$^+$ cells was promoted by Ang-1*. Chimeric protein of extracellular domain of TIE2 and the Fc of human Ig (TIE2-Fc) was added, and CD4-Fc was as a control. Adhesion to FN of Lin$^{-/low}$CD34$^+$TIE2$^+$ cells was promoted by Ang-1* and blocked by saturation of ligand with TIE2-Fc protein.

PE of Lin$^{-/low}$CD34$^+$TIE2$^-$ cells was significantly lower than that of TIE2$^+$ cells (Fig. 2A).

Limiting dilution of cultured Lin$^{-/low}$CD34$^+$TIE2$^+$ cells revealed that 1/3.1 of cells were hematopoietic colony-forming cells (CFC) (Fig. 2B). In Lin$^{-/low}$ CD34$^+$TIE2$^-$ cells, 1/8.5 of cells were CFC (Fig. 2B).

Cobble-stone area formation of cultured Lin$^{-/low}$CD34$^+$ TIE2$^+$ cells and Lin$^{-/low}$CD34$^+$TIE2$^-$ cells

More primitive progenitor cells than CFC are transmigrated under the stromal cells and proliferated in more immature state. Migrated immature cells seem to form cobble-stone features [32,33]. Cobble-stone area formation by Lin$^{-/low}$CD34$^+$TIE2$^+$ cells was significantly more frequent than those of Lin$^{-/low}$CD34$^+$ TIE2$^-$ cells. Even after one or two passages, more cobble-stone areas were formed by Lin$^{-/low}$CD34$^+$ TIE2$^+$ cells than those of Lin$^{-/low}$CD34$^+$TIE2$^-$ cells (Fig. 3B).

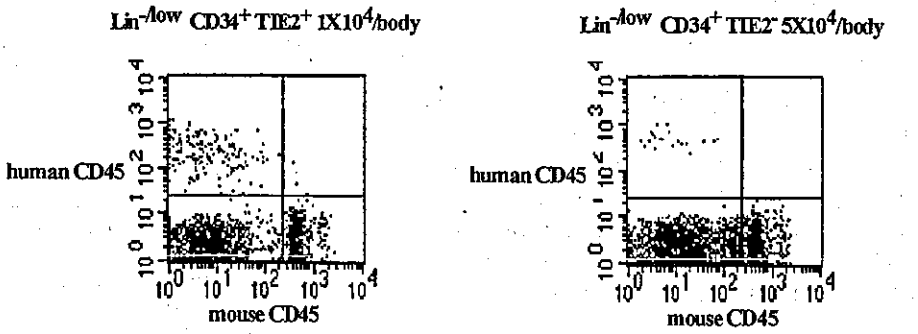
Next, to measure the incidence of LTC-IC in Lin$^{-/low}$CD34$^+$TIE2$^+$ cells and Lin$^{-/low}$CD34$^+$TIE2$^-$ cells, we performed the limiting dilution experiments. The representative data of five different samples that were used are shown in Fig. 3C. The enrichment pattern of LTC-IC was highly consistent. The frequencies of LTC-IC in Lin$^{-/low}$CD34$^+$TIE2$^+$ cells and Lin$^{-/low}$ CD34$^+$TIE2$^-$ cells were 1/36 cells and 1/83 cells, respectively.

Adhesion of Lin$^{-/low}$CD34$^+$TIE2$^+$ cells and Lin$^{-/low}$CD34$^+$ TIE2$^-$ cells to FN

Next we sorted primary Lin$^{-/low}$CD34$^+$TIE2$^+$ cells and TIE2$^-$ cells from CB and determined the adhesion to FN in the presence of Ang-1*. TIE2$^+$ cell adhesion to FN was promoted by Ang-1*. This adhesion was inhibited by TIE2-Fc but not by CD4-Fc, suggesting that cell/matrix adhesion was specific for TIE2 signaling. On the other hand, adhesion of TIE2$^-$ cells was not changed by the addition of Ang-1* (Fig. 4).

NOD/SCID-repopulating cells (SRCs)

In parallel with analysis of cobble-stone formation, the ability of sorted cells to reconstitute hematopoiesis in vivo was examined by transplantation of Lin$^{-/low}$CD34$^+$TIE2$^+$ CB cells or TIE2$^-$ CB cells into sublethally irradiated NOD/SCID mice. After 12 weeks,



	Lin$^{-/low}$ CD34$^+$TIE2$^+$ 1X10^4/body	Lin$^{-/low}$ CD34$^+$TIE2$^-$ 5X10^4/body
human CD45	5.75%	0.55%
human CD34	1.23%	0.02%
human CD13	1.44%	0.08%
human CD20	1.27%	0.36%

Fig. 5. Engraftment of umbilical cord blood cells in NOD/SCID mice. Representative immunophenotyping of NOD/SCID mouse BM 12 weeks after transplantation of Lin$^{-/low}$CD34$^+$TIE2$^+$ or TIE2$^-$ CB cells. Twelve weeks after transplantation, BM was obtained from the femurs and tibiae of NOD/SCID mice and assessed for multilineage human engraftment by 3-color flow cytometry. Cells were initially gated on myeloid and lymphoid fractions. Cells which expressed human specific CD45 but not murine CD45 are considered to be human CB-derived cells. Lin$^{-/low}$CD34$^+$TIE2$^+$ cells show the proliferation and differentiation of CD34 (precursor cells), CD13 (myeloid cells), and CD20 (B cells) in NOD/SCID bone marrow.

the degree of chimerism and the percentage of human $CD45^+$ cells in mouse bone marrow (BM) were determined by flow cytometry (Fig. 5). A cell dose of 1×10^4 of $Lin^{-/low}CD34^+TIE2^+$ cells was necessary for consistent successful engraftment 12 weeks after transplantation. A cell dose of 5×10^4 of $Lin^{-/low}CD34^+TIE2^-$ cells was necessary for consistent successful engraftment (Fig. 5). Transplantation of 1×10^4 of $Lin^{-/low}CD34^+TIE2^+$ cells and 5×10^4 of $Lin^{-/low}CD34^+TIE2^-$ cells resulted in chimerism levels of 5.75% and 0.55%, respectively. Also, immature human $CD34^+$ cells were present in the mouse BM (1.23% for $Lin^{-/low}CD34^+TIE2^+$ cells and 0.02% for $Lin^{-/low}CD34^+TIE2^-$ cells) (Fig. 5). Human myeloid and B cell engraftment was confirmed by flow cytometry; myeloid lineage (CD13), 1.44% for $Lin^{-/low}CD34^+TIE2^+$ cells and 0.08% for $TIE2^-$ cells, and B-lymphoid (CD20), 1.27% for $Lin^{-/low}CD34^+$ $TIE2^+$ cells and 0.36% for $TIE2^-$ cells (Fig. 5). T-lymphoid (CD3) or natural killer (NK) cells (CD56) was not detected (data not shown).

Discussion

HSCs are cells which are able to self-renew and to reconstitute hematopoiesis. Numerous differentiation membrane antigens have been defined to better identify stem cell population. In this study, we introduced a receptor tyrosine kinase, TIE2, which is also expressed on endothelial cells, and tried to purify the CB stem cells, combining CD34 and lineage markers.

$Lin^{-/low}CD34^+TIE2^+$ cells contained committed progenitors including CFU-GM, BFU-E, and CFU-Mix, while $TIE2^-$ cells contained a few CFU-GM. The cobblestone area-forming cell (CAFC) assay and the LTC-IC assay revealed that $Lin^{-/low}CD34^+TIE2^+$ cells contained more primitive cells than $Lin^{-/low}CD34^+$ $TIE2^-$ cells. Moreover, transplantation of sorted NOD/SCID mice confirmed that $Lin^{-/low}CD34^+TIE2^+$ cells contained more hematopoiesis-reconstituting cells than $TIE2^-$ cells.

To clarify the function of TIE2, we show that a ligand of TIE2, Ang-1, promotes adhesion to FN in $Lin^{-/low}CD34^+TIE2^+$ cells. Cell adhesion is mediated by the interaction between integrins on $TIE\text{-}2^+$ HSCs stimulated with Ang-1 and FN beneath endothelial cell layer [25]. This suggests that cell adhesion induced by TIE2 and Ang-1 may be related to cobble-stone area formation. It is suggested that cell adhesion to matrix is critical for the maintenance and proliferation of primitive hematopoietic cells. We will clarify whether Ang-1 is one of the stem cell factors which expand HSC or not.

Acknowledgments

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Isolation of human single chain antibodies (scFv) against human TNF-α from human peripheral blood lymphocyte-SCID mice

H. Nguyen[a,f,**], J. Hay[b], S. Gallinger[c], J. Sandhu[d] and N. Hozumi[b,e,*]
[a]*Department of Laboratory Medicine and Pathology, Samuel Lunenfeld Research Institute at Mount Sinai Hospital, Toronto, Canada*
[b]*Department of Immunology, University of Toronto, Toronto, Canada*
[c]*Department of Surgery, Samuel Lunenfeld Research Institute at Mount Sinai Hospital, Toronto, Canada*
[d]*Sandhu PHD Inc., Burlington, Canada*
[e]*Research Institute for Biological Sciences, Tokyo University of Science, Noda-city, Japan*
[f]*Present address: H. Nguyen, Department of Pathology, University of Massachusetts, Worcester, USA*

Abstract. By developing an appropriate immunization protocol for SCID (hu-PBL-SCID) mice engrafted with human peripheral blood lymphocytes in combination with scFv phage display library, we were able to establish an efficient strategy to obtain human scFv clones against a human self-antigen, TNF-α. The mice pretreated with γ-radiation (3Gy) and anti-asialo GM1 antibody were immunized with a mixture of human TNF-α-keyhole limpet hemocyanin and Freund's adjuvant. Human antibody maturation was suggested to be induced in the mice with the immunization protocol. The scFv clones obtained from the mice were shown to exhibit binding affinities in the range of 10^7–10^8 M^{-1}. Together with our previously published work on the isolation of respiratory syncytial virus neutralizing scFvs, the results of this study have implicated that this combined approach is one of the effective alternatives for the cloning of human monoclonal antibodies specific for a wide range of antigens of interest.

Keywords: scFv, phage display library, hu-PBL-SCID mice, human TNF-α

1. Introduction

TNF-α, a pleiotropic proinflammatory cytokine of 17kDa, was originally identified as an endotoxin-induced factor that caused wasting syndrome and hemorrhagic necrosis of tumors in tumor-bearing mice [1]. TNF-α is produced mainly by activated monocytes and macrophages. It is initially synthesized as a membrane-anchored precursor of 233 amino acid residues. The cleavage of an Ala-Val bond between residues 76–77 of the precursor by a matrix metalloproteinase-like (MMP) TNF convertase, a unique Zn^{2+} dependent endopeptidase, gives rise to a secreted 17kDa mature form [2,3,4]. With an ability to induce the expression of inflammatory and cytotoxic mediators, TNF-α is a potent effector molecule of central importance to the pathogenesis of many disorders such as Crohn's disease, multiple sclerosis, rheumatoid arthritis and the cachexia associated with cancer or human immunodeficiency virus (HIV) infection [5,6]. TNF-α is now known to play pivotal roles in promoting both host defense and pathological processes. It also participates in maintaining the host homeostatic state by influencing cell proliferation and differentiation as well as inducing apoptosis in various cell types.

Clinical trials of cA2, a chimeric mouse/human anti-

*Corresponding author: Dr. N. Hozumi, Research Institute for Biological Sciences, Tokyo University of Science, 2669 Yamazaki, Noda-city, Chiba 278-0022, Japan. Tel.: +81 4 7123 9779; Fax: +81 4 7122 4131; E-mail: nobhozmi@rs.noda.tus.ac.jp.
**Dr. H. Nguyen, E-mail: Hai.Nguyen@umassmed.edu.

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TNF-α monoclonal antibody (mAb), showed that a single infusion was effective for short-term treatment in many patients with moderate-to-severe Crohn's disease that was resistant to glucocorticoid treatment [7]. Similar therapeutic effects of cA2 were reported in patients with rheumatoid arthritis [8,9]. However, long-term treatments were ineffective due to the development of anti-cA2 responses that can occur within two weeks of the initiation of the treatment. Fully hu-mAbs are expected to minimize the immunological responses intrinsic to mouse or mouse-derived mAb and thus optimize the efficacy and safety of the administered Ab. Even, fully hu-mAb is speculated to evoke an undesirable anti-idiotypic Ab response. There is a general consensus that a number of distinct mAbs should be available and used to counteract anti-idiotypic Ab responses for long-term passive Ab therapy. However, the enticing goal of producing a large number of fully human mAbs with desired specificities and at reasonable ease to fulfill the promise of Ab therapy in human has remained elusive.

Recent developments in chimeric mouse models, transgenic mice and DNA recombinant technologies have brought large-scale selection and production of hu-mAb closer to reality. Human/mouse radiation chimera, the trimera system, has been used successfully to induce human humoral responses and to generate hu-Abs against various foreign antigens (Ags) [10–12]. Transloci mice bearing large portions of human immunoglobulin (hu-Ig) loci capable of mounting highly diverse hu-Ab responses have been created and used to generate high affinity hu-mAbs to a variety of Ags [13]. However, generation of transgenic mice carrying the entire complete loci of human Ig genes is still the goal for the future. Human chromosomes 2 and 14 (or their fragments) carrying hu-Ig κ and heavy chain gene loci respectively, have been introduced into mouse embryonic stem (ES) cells *via* microcell-mediated chromosome transfer technique to establish trans-chromosomic (Tc) mice [14]. This report demonstrated that hu-Ig transgenes are properly exploited by the mouse machinery for diverse Ab responses, however, hybridomas established from the Tc mice showed instability of a human chromosome fragment carrying the κ gene loci [15]. At the present time, the use of transloci and Tc mice for hu-mAb production is limited due to patent issues.

We previously demonstrated the relative ease of cloning specific hu-scFvs with high binding affinity and neutralizing activity using our combined approach of hu-PBL-SCID mice and scFv phage display library. Specific hu-Ab response to fusion protein F of respiratory syncytial virus (RSV-F) was induced in hu-PBL-SCID mice and RSV-neutralizing hu-scFvs were selected from a scFv phage display library [16]. It is our belief that, due to the lack of the regulatory mechanism for immunological tolerance to human self-Ags in hu-PBL-SCID mice, a hu-Ab response to hu-Ag could be induced in these mice if appropriate immunization protocol was applied. We report the effectiveness of this approach in selection of human scFv clones to human TNF-α. The results of this study have strengthened our evidence that this widely accessible, relatively simple and highly effective approach is an alternative toward the generalization of Ab therapy for human disease.

2. Materials and methods

2.1. SCID mice and materials

Homozygous C.B.-17 scid/scid (SCID) mice were bred and maintained at the Samuel Lunenfeld Research Institute, Toronto, Canada. Anti-asialo GM1 Ab was purchased from Wako Chemicals (Dallas, Texas). Other antibodies were purchased from Serotec Ltd. (Kidlingto, UK). All oligonucleotide primers were purchased from Gibco-BRL (Gaithersburg, MD). Restriction enzymes and Vent^R DNA polymerase were purchased from New England Biolabs (Beverly, MA). Superdex^R 75 and Recombinant Phage Antibody System were obtained from Pharmacia (Piscataway, NJ). All other chemicals were purchased from Sigma Chemicals Co. (St. Louise, MO). Human TNF-α and cA2 mAb were kindly provided by Centocor (Malvern, PA). The Millipore HPLC system was used in this study to isolate scFv monomers. Experimental protocols were approved by the subcommittees of the University of Toronto and Mount Sinai Hospital, Toronto.

2.2. Immunization of hu-PBL-SCID mice with human TNF-α

Human TNF-α was conjugated to keyhole limpet hemocyanin (KLH) at a ratio of 1/1 (w/w) and the resulting TNF-KLH in PBS was stored at −72°C until use. hu-PBL-SCID mice were prepared following our previously described protocol [17,19]. In brief, SCID mice were treated with γ-radiation (3Gy) and injected i.p. with 100 μl of anti-asialo GM1 antiserum/saline (1/9) on day one. Fresh blood from a consenting donor was collected and subjected to Ficoll-Hypaque gradi-

ent. Freshly isolated hu-PBLs (3×10^7) were engrafted into each pretreated SCID mouse ($n = 15$) on day two. Each hu-PBL-SCID mouse of the first group of five was immunized i.p. with 100 μl of complete/incomplete Freund's adjuvant (CFA/IFA) (v/v = 1/10) mixture containing 10 μg of TNF-KLH on day three (TNF-hu-PBL-SCID mouse). Five hu-PBL-SCID mice were immunized with 100 μl of the CFA/IFA emulsion without TNF-KLH. This second group of five mice were referred to as C-hu-PBL-SCID. The last five hu-PBL-SCID mice were immunized with 100 μl of PBS and called P-hu-PBL-SCID. The latter two groups and a group of five TNF-KLH immunized SCID mice were used as negative controls. Mouse sera and spleens were collected and pooled on day sixteen for further analyses. Total RNA fractions were separately prepared from the donor's PBLs and from the splenocytes of TNF-hu-PBL-SCID mice, and were used to construct human scFv libraries of $\gamma\kappa$ and $\gamma\lambda$ classes.

2.3. scFv phage display libraries

The recombinant phage antibody system obtained from Pharmacia Biotech was used to construct scFv libraries following the manufacturer's instructions. Briefly, variable regions of hu-Ig heavy, κ and λ (V_H, V_L) were amplified by RT-PCR using the primer set described by Marks et al. [18]. V_H and V_L repertoires were randomly linked together by oligonucleotide linkers, 5'-TC TCC/T TCA GGT GGC GGC GGT TCG GGC GGA GGA GGC TCT GGC GGT GGC GGA TCG GA-3', that encoded a flexible and hydrophilic peptide (-Gly_4Ser-)$_3$ [13]. The resulting scFv repertoires were then cloned into pCANTAB 5 E phagemid vector.

2.4. Biopanning

Direct panning was conducted as previously described by Marks et al. and Nguyen et al. using recombinant hu-TNF-α [16,18]. Briefly, wells in an Immulon I 12-well strip (Dynatech, VA, USA) were coated overnight at 40C with human TNF-α (100 μl of 1 μg/ml in 0.1M sodium bicarbonate buffer, pH 9.6) and blocked with blotto (3% skim milk in PBS). Wells directly blocked with blotto without TNF-α coating were used as negative controls. A phage library (108 plaque-forming units (pfu)/well in 0.1ml of blotto) was added into the well and left rocking gently at room temperature for 2 hrs. Wells were then washed 60x with PBSTT (PBS + 0.05%Tween 20 + 0.01% Triton X-100), 5x with sodium bicarbonate buffer, pH 9.6, and then 1x with PBS. The bound phages were eluted with 100 μl of 0.1 M triethylamine pH 11.5 and rocked for 5 min before neutralization with 10 μl of 3 M Tris-HCl (pH7.5). Eluted phages were used to infect 2 ml of growing E. coli TG1 (Suppressor F^+ strain) for 30 min at 37°C and plated on 5 of 100 mm LB plus Amp agar plates (150 μg/ml ampicillin). Ninety binding phages were randomly picked and used to infect E. coli HB2151 (Suppressor F^- strain) to produce soluble scFv proteins. Culture supernatants were subjected to electrophoresis on 12% polyacrylamine gel. The presence of soluble scFv proteins was detected by directly staining with basic fucin solution. Test samples containing similar concentrations of soluble scFv proteins were prepared for ELISA.

2.5. ELISA

Immulon I 96-well plates were coated with 100 μl of 1 μg/ml hu-TNF-α in 100 mM sodium bicarbonate buffer, pH 9.6 overnight at 4°C, then blocked with 0.3 ml of blotto (PBS + 3% skim milk). One hundred μl of supernatant containing either phages ($\sim 10^9$ phages) or soluble scFv proteins plus 3% skim milk and 0.05% Tween 20 was added into each well and incubated at room temperature for 2h with shaking. Either HRP-conjugated mouse anti-M13 Ab (0.1 ml) or HRP-conjugated anti-E Tag Ab was added into each well and incubated for 1h with shaking. Binding was detected using an HRP substrate kit (Bio-Rad Lab., CA) following the manufacturer's instructions. Wells directly coated with 10^9 phages were used as positive controls. Both 10^9 phage particles and soluble scFv protein samples of the randomly picked human TNF-α binding clones were subjected to ELISA for selecting strong binding clones. Clones exhibited both phage signals of at least 50% of those of the positive controls and strong soluble scFv signals were arbitrarily called strong binders. DNA samples of 14 strong binders were prepared and digested with BstN1 restriction enzyme. Three clones exhibiting distinct BstN1 digestion patterns were selected and subjected to DNA sequencing analysis.

2.6. TNF-α binding affinity, BIAcore

scFv protein samples were purified from periplasmic extracts of selected clones ($n = 3$) by affinity chromatography using anti-E taq antibody conjugated, protein G sepharose 4B column. The purified scFv

samples were then subjected to size-exclusion chromatography on Superdex^R 75 HR10/30 to isolate scFv monomers. All buffers and samples had been filtered through 0.22 μm filters before use. Binding affinity of soluble scFv to human TNF-α was measured using BIA-core, Biosensor system (Pharmacia) following our previously described protocol [16]. In brief, a solution of 50 μg of TNF-α in 1 ml of 10mM acetate buffer pH 5.5 was used in the immobilization process. The TNF-α solution was injected onto each activated sensor cell at a continuous flow rate of 2 μl/minute for 10 minutes. This resulted in 1000 to 1500 response units (RU) of immobilized TNF-α. Solutions of scFv (in PBS) were injected onto the chip under continuous flow conditions of 20 μl/minute for 1 minute. The association (k_a) and dissociation (k_d) rates of the scFv on the chip were detected and formulated by BIA Evaluation software version 2.1 provided in a Biosensor system model BIAcore Upgrade (Pharmacia Biosensor AB). The collected data were well fitted to homogeneous single-site interaction between two molecules (homogeneous kinetics). Dissociation model AB = A + B was used to calculate the dissociation rate constant (k_d) by fitting data to the equation: $R = R_0 e^{-k_d(t-t_0)}$ (t: time in seconds; k_d: dissociation rate constant; R_0: response at the start of dissociation; t_0: start time for the dissociation). Association model A + B = AB type 1 was used to calculate the association rate constant (k_a) and the steady state response level (R_{eq}) by fitting data to the equation: $R = R_{eq}[1 - e^{-(k_a C n + k_d)(t-t_0)}]$ (t: time in seconds; k_a: association rate constant; R_{eq}: steady state response level; C: molar concentration of analyte; n: steric interference factor; t_0: start time for the association; k_d: dissociation rate constant).

3. Results

3.1. Increased hu-IgG response to human TNF-α in TNF-hu-PBL-SCID mice

We have previously demonstrated the high degree of hu-PBL engraftment in SCID mice pretreated with low dose of γ-radiation and anti-asialo GM1 rabbit antiserum [16,17,19]. The same results were achieved in this study. FACS analyses of splenocytes from all three groups of hu-PBL-SCID mice confirmed that over 80% of these cells expressed the human CD45 marker. The pooled sera of the two mouse groups, TNF-hu-PBL-SCID and C-hu-PBL-SCID mice, contained similar levels of hu-IgG and -IgM as those of the human donor; about 7 mg/ml and 0.5 mg/ml respectively. Pooled serum of the P-hu-PBL-SCID mice contained only 1 mg/ml of hu-IgG and 0.3 mg/ml of hu-IgM. Splenocytes of TNF-KLH immunized SCID mice did not express the human CD45 marker and hu-Ig were completely absent in their sera. These results confirmed our previous finding that pretreatment with γ-radiation and anti-asialo GM1 is crucial for a high degree of hu-PBL engraftment into SCID mice and that adjuvant is required to activate the engrafted hu-PBLs.

ELISA was used to measure anti-TNF-α hu-Ab titres in mouse sera. In agreement with the general consensus that anti-TNF-α Ab in the donor is mainly IgM isotypes, the anti-TNF-α IgG in the donor serum could only detected by ELISA down to 1/2 dilution (data not shown). Based on unit of hu-Ig, serum hu-IgG and -IgM titres to human TNF-α in the pooled serum of TNF-hu-PBL-SCID mice were 60 fold higher and 238 fold lower than those of the donor serum respectively. Even though the serum hu-Ig levels in C-hu-PBL-SCID mice were similar to those of the donor's serum, both hu-IgG and IgM anti-TNF-α levels were undetectable by this assay. Anti-TNF-α Ab levels were also undetectable in the pooled serum of P-hu-PBL-SCID mice. These data show that adjuvant was necessary for the activation of the engrafted hu-PBL in the pretreated SCID mice and that specific hu-Ab response to human TNF-α was successfully induced in TNF-hu-PBL-SCID.

3.2. Isolation of scFv clones exhibiting human TNF-α binding activity from TNF-hu-PBL-SCID mice

It is generally believed that specific IgG produced during an Ab response usually possess higher binding affinity than IgM. Therefore, we constructed scFv libraries of γκ and γλ classes from the donor PBLs and splenocytes of TNF-hu-PBL-SCID mice. These libraries contained more than 10^8 members each (2.7×10^8 to 3.8×10^8). About 1×10^8 phage particles from each of the four libraries were used to screen for human TNF-α binding by panning. High stringent washing conditions with both strong detergent (Triton-X) and increasing pH (up to pH 9.6) were applied to minimize non-specific binding. Under these washing conditions, less than 10 clones were recovered from each of the negative control wells. Both γλ libraries did not produce any binding clone, while γκ libraries derived from the donor's PBLs and from splenocytes of TNF-hu-PBL-SCID mice resulted in 2 and 4.6×10^3 binding clones respectively (Table1). The results reported here were the averages of three independent panning

assays. At first, the strikingly different numbers of the binding clones of the two γκ libraries seemed not to correlate well with the reported titres since the IgG titre in the serum of TNF-hu-PBL-SCID mice increased by only 60 times. One explanation for this discrepancy is related to the binding affinity/avidity and the stringent washing conditions. Under normal physiological conditions, autoreactive B cells produce IgG with very low binding affinity to self-Ag such as TNF-α, and the γκ library constructed from the donor's PBL contains mainly TNF-α weak binding clones. These weakly bound clones were washed off during the screening procedure. Only clones with binding affinity/avidity higher than one specific value could sustain the stringent washing. These results imply that either the autoreactive B cells to TNF-α were enriched by the TNF-hu-PBL-SCID mice, or human anti-TNF-α Ab maturation actually occurred in these mice. The shifting of anti-TNF-α titre from IgM to IgG and panning results favor the latter.

Randomly picked binding clones were tested by ELISA to look for 'strong binders'. We arbitrarily defined 'strong binders' as clones that exhibited ELISA signals of at least 50% of those of the positive control [16]. Fifteen percent of the randomly picked clones from the mouse derived γκ library were positive (14/90), while none were found in the other γκ library (0/2) (Table 1). It should be noted that only two binding clones were found in the hu-PBL derived γκ library. We selected the strong binders for further analyses.

3.3. Clonality of scFv clones

To find out the diversity of the library, DNA was prepared from the 14 isolated strong binders and subjected to BstN1 restriction enzyme digestion. Three different digestion patterns were found from this group (data not shown). Representative clones were selected and subjected to DNA sequencing analysis to determine their clonality. Clones 2 and 14 contained a similar V_H but different V_L segments, while clone 20 carried a totally different scFv sequence (Fig. 1). These V_H and V_L sequences were derived from different germline IgV families. The results suggest that a relatively large repertoire of anti-TNF-α clones were present in the TNF-hu-PBL-SCID mouse derived γκ library. The redundancy of strong binders may be generated during the amplification process of the mouse derived library.

3.4. Binding affinities of distinct scFv clones

BIAcore assay was then applied to measure binding affinities of these clones. All the three isolated clones exhibited human TNF-α binding affinities in the range of 10^8 M^{-1}; they were 9.42×10^7, 1.50×10^8 and 3.24×10^8 for clones 2, 14 and 20 respectively (Table 2). If complete Ab molecules were constructed from any of these three scFv clones, we expected binding affinities of 10^9 M^{-1} or higher could be achieved since scFv usually exhibited much lower binding affinity than that of the complete Ab molecule from which the scFv was derived [20].

4. Discussion

Human polyclonal Abs are widely used as both prophylactic and microbicidal agents, but the benefits of hu-mAb of desired specificities are obvious [21]. The use of hu-mAb should minimize the anti-globulin responses during therapy by avoiding anti-isotypic Ab responses [22]. A large body of studies has shown the effectiveness of hu-mAb as a therapeutic agent. However, the lack of a simple and efficient system to clone specific hu-mAb has been one of the major setbacks for fulfilling the promise of antibody therapy in human disease.

To continue on our effort to establish a generalized method to clone specific hu-mAbs, we describe here the generation of hu-scFv clones against a human self-Ag, TNF-α, using our previously established procedure of combining hu-PBL-SCID mice and scFv phage display library [16]. The presence of auto-Abs in mice and in humans has been extensively documented and autoreactive Abs against well-defined hu-Ags have been detected in the serum of healthy individuals. Under normal physiological conditions, 10 to 30% of B cells are committed to produce autologous Ab of mainly IgM class with very low binding affinities. It has been suggested that autologous IgM contributes to regulate expression of autoreactive IgG through V region-dependent interactions, resulting in low levels of serum autoreactive IgG [23]. We speculate that this regulatory mechanism may be absent in hu-PBL-SCID mice. The shifting specific Ab titre from IgM to IgG and the presence of a number of strong binders in hu-PBL-SCID mice derived γκ library seems to support our notion that antibody maturation occurs in the mice with the immunization protocol.

Table 1
Screening of human TNF-α binding scFv clones by ELISA

Library	γλ		γκ	
	# of binders[A]	Strong binders[B]	# of binders	Strong binders
Donor's PBLs	0	NA	2	0/2
TNF-hu-PBL-SCID mice	0	NA	4.6×10^3	14/90

Ninety six well plates were coated overnight with 100 μl of 1 μg/ml human TNF-α in 50 mM of sodium bicarbonate buffer, pH 9.6 and then blocked with blotto. Phages (10^8 cfu in blotto) of each library were added into a well and incubated for two hours at room temperature with gentle shaking. Bound phages were eluted, infected into *E. coli* TG1 and plated in 5 LB + Amp plates. The numbers of colonies were reported as # of binders. Uncoated and blotto blocked wells were used as negative controls. No phages bound to these wells and the ELISA signal was at background level. The average of three independent experiments is reported here.

[A] # of binders: Number of scFv clones recovered after panning against RSV-F. Only γκ libraries contained RSV-F binding scFv clones.
[B] Strong binders: Randomly picked TNF-α binding scFv clones (from the previous panning step) were tested by ELISA. Clones that showed both phage signals of at least 50% of positive control signals and strong soluble scFv signals were reported as strong binders. Wells directly coated with 10^9 phage particles were used as a positive control. NA: Not applicable.

V_H amino acid sequence

Clones	FR1	CDR1	FR2	CDR2
2	EVQLVESGGDLVQPGGSLRLSCAASGITVS	SSYMS	WVRQAPGKGLEWVS	VIYSGGSTYYADSVKG
14	EVQLVESGGDLVQPGGSLRLSCAASGITVS	SSYMS	WVRQAPGKGLEWVS	VIYSGGSTYYADSVKG
20	EVQLVESGAEVKKPGESLRITCKGSGYSFT	SYWIT	WVRQMPGKGLEWM	IIYPGDSDTRYSPSFQG

	FR3	CDR3	FR4
2	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR	EGHTGMDV	WGQGTTVTVSS
14	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR	EGHTGMDV	WGQGTTVTVSS
20	QVTISADKSISTAYLQWSSLKASDTAMYYCAR	AGALYYYGSGSYYNDDYYYGMDV	WGQGTTVTVSS

V_L amino acid sequence

Clones	FR1	CDR1	FR2	CDR2
2	DIVMTQSPDSLAVSLGERATINC	KSSQSILYSSDNKNYLA	WYQQKPGQPPKLLIY	CASTRES
14	DIQMTQSPSSLSASVGDRVTITC	RASQGISNALA	WYQQKAGNPPKLLLY	AASRLES
20	DIVMTQSPDSLAVSLGERATINC	KSSQSVLYSSNNKNYLA	WYQQKPGQPPKLLIY	WASTRES

	FR3	CDR3	FR4
2	GVPDRFSGSGSGTDFTLTISSLQAEDVAVYYC	QQSYNLPWT	FGQGTKVEIKR
14	GVPSRFTGSGSGTDYTLTISSLQPEDFATYSC	QQYYSIPLT	FGGGTKVEIKR
20	GVPDRFSGSGSGTDFTLTISSLQAEDVAVYYC	QQYYSTPWT	FGQGTKVDIKR

Fig. 1. Amino acid sequences of the isolated scFv clones (2, 14 and 20). The sequences are shown in single-letter amino acid code.

The induction of specific hu-Ab responses in hu-PBL-SCID mice has had drawbacks and debate especially when the original hu-PBL-SCID mouse model was described without pretreatment [24–28]. Our hu-PBL-SCID mouse model was designed to avoid and overcome those limitations. The pretreatment of SCID mice with γ-radiation and anti-asialo GM1 has been shown to further reduce the residual innate immunity of the mice in order to improve hu-PBL engraftment. By using CFA in immunization and limiting the entire procedure within the first two weeks of hu-PBL engraftment, we speculate that specific Ab response was induced in the mice avoiding the skewing of human immune response toward mouse Ags [16,17,19].

A number of mouse/human chimeric and humanized Abs specific for human TNF-α such as cA2, Inflix-

Table 2
Human TNF-α binding affinities of scFv clones

	2[A]	14	20
k_a $(M^{-1}.s^{-1})^B$	3.73×10^5	3.55×10^5	4.76×10^5
k_d $(s^{-1})^C$	3.91×10^{-3}	2.37×10^{-3}	1.47×10^{-3}
$K_A = k_a/k_d$ $(M^{-1})^D$	9.54×10^7	1.50×10^8	3.24×10^8

BIAcore[R] system was used to measure TNF-α binding affinities of selected scFv clones. Human TNF-α was immobilized on the CM5 sensor chip. A solution of scFv protein was injected into the cell under continuous flow conditions (20 μl/min). Dissociation rate, association rate and binding affinity were measured and automatically calculated.

[A] scFv clones – 2, 14 and 20.
[B] Association rate.
[C] Dissociation rate.
[D] Binding affinity.

imab and CDP 571 have been used in clinical trials for treatments of rheumatoid arthritis, Crohn's disease, septic shock, and multiple sclerosis [7,11,12,29–33]. The beneficial effects of anti-TNF-α mAb therapy, at least in the short term, have clearly been demonstrated by these studies. In one study, up to four cycles of treatment with cA2, an anti-TNF-α chimeric mAb, were well tolerated and beneficial effects extended up to 60 weeks [12]. However, a single mAb was used in each of these trials. Patients developed anti-idiotypic response rendering long-term treatments ineffective. It has been suggested that multiple distinct hu-mAbs with desired specificity are required for long-term Ab therapy. We predict that the mouse-derived γκ library would contain more than a hundred distinct anti-TNF-α scFv clones with high binding affinities since we were able to isolate three clones from the first 90 binders tested (Table 1). Work is in progress to construct complete hu-IgG1 Abs from the three isolated anti-TNF-α scFv clones for further characterization, including their neutralizing activity both *in vitro* and *in vivo*.

The results of this study have demonstrated that the combination of hu-PBL-SCID mice and scFv phage display library can be used to clone multiple distinct scFv exhibiting high binding affinity to a self-Ag, human TNF-α. Together with our previously reported work of isolating neutralizing, anti-RSV-F hu-scFv clones, this study provides for the evidence that this unique procedure is an effective alternative for cloning multiple hu-mAbs against any Ag of interest [16]. This procedure may represent a significant step toward fulfilling the promise of Ab therapy in human disease.

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UPDATE

Various Applications of Direct PCR Using Blood Samples

NAOYUKI NISHIMURA[1], TOMOKO NAKAYAMA[1], HIROSHI TONOIKE[1], KOUICHI KOJIMA[1], YOSHINARI SHIRASAKI[1], KENSUKE KONDOH[2], AND TAKETO YAMADA[2]

[1]*Life Science Laboratory, Analytical and Measuring Instruments Division, Shimadzu Corporation, Ibaraki, Japan*
[2]*Department of Pathology, School of Medicine, Keio University, Tokyo, Japan*

SUMMARY

Samples of blood or other animal fluids contain a variety of substances that inhibit the polymerase chain reaction (PCR), meaning that isolation of DNA, involving multiple labor-intensive steps, is generally necessary prior to PCR. We have developed a novel reagent cocktail that effectively suppresses these inhibitory substances. Using this reagent cocktail, DNA from various targets can be efficiently amplified directly from various forms of blood samples without DNA isolation.

1. **DNA sequences within the β-globin gene could be amplified directly from human blood samples treated with various anticoagulants. Either fresh blood or blood samples stored frozen for up to 4 years could be used for PCR.**
2. **DNA sequences of up to 2056 bp within the β-globin gene could be amplified directly from human blood samples.**
3. **Human chromosomal and mitochondrial DNA from different individuals could be amplified directly from blood samples.**
4. **Low titers of hepatitis B virus could be amplified directly from human blood samples.**
5. **DNA could be amplified directly from various target sequences using dried blood in a PCR tube or on a filter paper.**
6. **Transgenes could be detected directly in blood samples from transgenic mice.**

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KEY WORDS

PCR, blood, inhibitor, viruses, DNA diagnosis

INTRODUCTION

Samples of blood or other animal fluids contain a variety of substances that inhibit the polymerase chain reaction (PCR), meaning that isolation of DNA, involving multiple labor-intensive steps, is generally necessary prior to PCR. Recently, many kinds of DNA isolation kits have become commercially available. While the use of these kits means DNA isolation has become simpler, it is still time-consuming. Furthermore, the yield and/or purity of the final DNA preparation are not always satisfactory. Finally, DNA isolation procedures increase the likelihood of sample contamination with foreign DNA, including DNA from samples processed earlier.

We have developed a novel reagent cocktail (Ampdirect®) that effectively suppresses these inhibitory substances. Using this reagent cocktail, DNA from various targets can be efficiently amplified directly from various forms of blood samples without DNA isolation. This process is hereafter referred to as "Direct PCR".

MATERIALS AND METHODS

Human Samples

Human blood was collected from healthy volunteers into hypobaric tubes containing one of three different

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Table 1: Primer sets designed for specific target sequences

Target DNA	bp	Oligo Sequence
Human β-globin gene*	437	5'-TGAAGTCCAACTCCTAAGCC-3'
	641	5'-ATGGTAGACAAAGCTCTTCC-3'
	742	5'-CTTAGAACTGAGGTAGAGTT-3'
	1.127	5'-ACACTCTTGCAGATTAGTCC-3'
	1.238	5'-AAACGCAGTATTCTTAGTGG-3'
	1.497	5'-CCTACGCTGACCTCATAAATG-3'
	1.745	5'-TCTGATAACTAGAAATAGAGG-3'
	2.056	5'-CACGTGTGCCTAGATCCTCAT-3'
Human mitochondrial DNA	293	5'-GTACCATAAATACTTGACCACCTGTAGTAC-3'
		5'-TGATTTCACGGAGGATGGTGGTCAAGGGAC- 3'
Transgene in transgenic mouse	521	5'-TCCAGTAAGGTCCATGGTGATT-3'
		5'-CAGCAAGTGGGTAACTAGACTA -3'
HBV C region	137	5'-AGACCACCAAATGCCCCTAT-3'
		5'-GATTGAGATCTTCTGCGACG-3'

* GH21 was used for common antisense primer.

anticoagulants (sodium citrate, dipotassium EDTA or sodium heparinate: Terumo Corp., Tokyo, Japan). Informed consent was obtained from all volunteers. The collected blood samples were stored at 4 °C or –20 °C for 4 years. The frozen samples were freeze-thawed once a month during the first year of storage. Dried blood samples were prepared by drying 1 µl of EDTA-treated human blood in a PCR tube or onto a 4 mm disc of 3MM paper (Whatman International Ltd, Maidstone, England). DNA from EDTA-treated healthy human blood was purified using a SepaGene® DNA purification kit (Sanko Junyaku Co., LTD. Tokyo, Japan).
EDTA-treated human blood containing hepatitis B virus (HBV) was collected from an HBV-infected individual. The HBV titer in the collected blood was estimated at 5,000 copies/µl based on the results of nested PCR of diluted blood in TE (10 mmol/l Tris-HCl, 1 mmol/l EDTA, pH 8.0), using a primer set which had independently been shown to detect a single copy of purified HBV DNA (data not shown). The HBV-containing blood was subjected to serial ten-fold dilutions in HBV-free human blood, and each blood dilution was used for PCR either directly or following purification of DNA using a QIAamp DNA Blood kit (QIAGEN GmbH, Hilden, Germany).

Mouse Samples

C57BL/6J mice transformed with the *lck* promoter-human D4-GDI transgene were used for studies of PCR efficiency in differently treated samples. Following surgical removal of the tail, blood was collected from the tail stump with a micropipette. Blood samples were immediately mixed with sodium citrate, dipotassium EDTA or sodium heparinate. DNA was purified from the amputated tail using a DNA purification kit (DNeasy tissue kit: QIAGEN GmbH, Hilden, Germany).

Primers

Primer sets for detection of the human β-globin gene (GH20, GH21) (1), human protein S gene (PS exon2s, PS exon2as) (2), HLA DPB1 gene (5'DPB1, 3'DPB1) (3), and human p53 gene (p53 Primer Exon 6 and Exon 11: Nippon Gene Corp., Tokyo, Japan) were used for PCR. Other primer sets designed for specific target sequences are shown in Table 1.

Reaction Mixture for Direct PCR

The reaction mixture for Direct PCR ("Ampdirect mixture") contained 1x Ampdirect® (Shimadzu Corp., Kyoto, Japan), 200 µmol/l each of dATP, dGTP, dCTP and dTTP, 500 nmol/l each of the specific primers, and 25,000 U/l of *Taq* DNA polymerase (TaKaRa Taq™; Takara Shuzo Corp., Kyoto, Japan). To improve the detection sensitivity, *Taq* DNA polymerase for hot start PCR (Platinum® *Taq* DNA polymerase: Invitrogen Corp. San Diego, CA, USA) was used for Direct PCR detection of HBV DNA in blood.

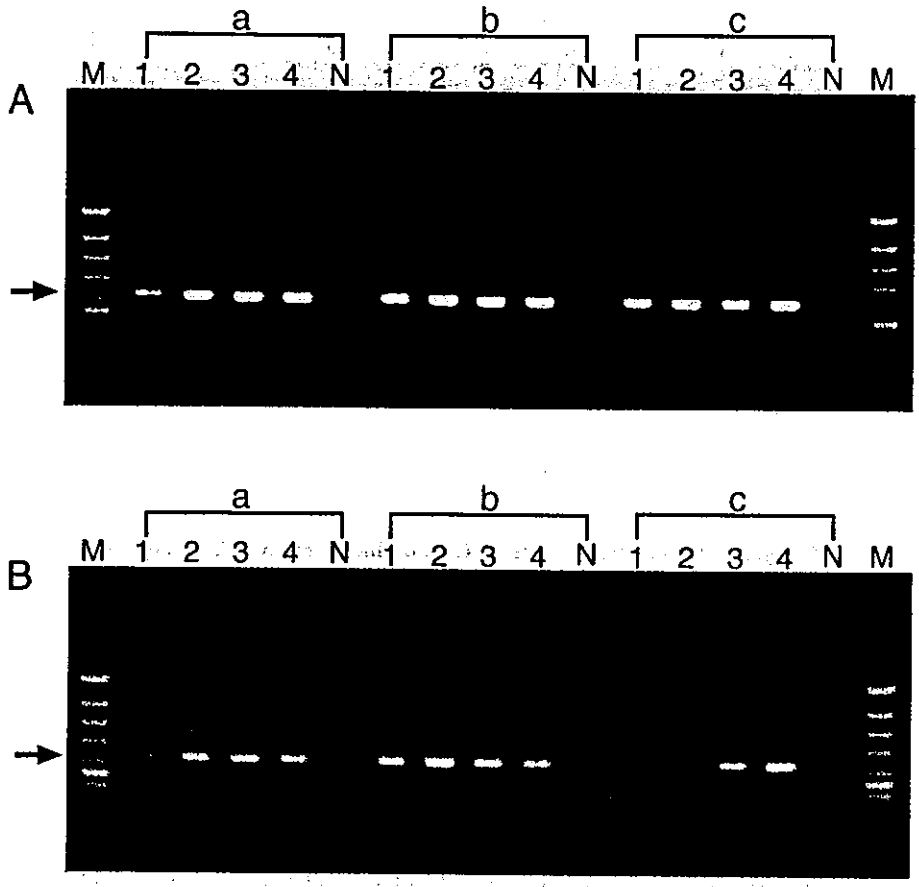

Figure 1: Direct PCR from whole human blood. Direct PCR using four different volumes of human blood treated with three types of anticoagulants (a: sodium citrate, b: dipotassium EDTA, c: sodium heparinate) was carried out to detect a fragment of the β-globin gene. Fresh blood (A) or blood samples stored at -20 °C for four years (B) were added directly to 50 µl of Ampdirect mixture. Sample volumes used were: 5.00 µl (lane 1), 2.50 µl (lane 2), 1.25 µl (lane 3), or 0.63 µl (lane 4). Lane N is a negative control. Lane M contains a molecular size marker (*Hinc* II-digested ϕX174 RF DNA). The specific PCR product for the human β-globin gene is indicated by an arrow at 408 bp.

Reaction Mixture for Conventional PCR Using Purified DNA

The reaction mixture for PCR of purified DNA ("Standard mixture") contained 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 200 µmol/l each of dATP, dGTP, dCTP and dTTP, 500 nmol/l each of the specific primers, and 25,000 U/l of *Taq* DNA polymerase. Platinum® *Taq* DNA polymerase was used for detection of HBV DNA.

PCR Conditions

PCR was performed in a DNA Thermal Cycler PJ2000 (Applied Biosystems, Foster, CA, USA) at 94 °C for 4.5 min (pre-heating), followed by 40 cycles of 94 °C for 0.5 min (denaturation), 51-60 °C for 1 min (annealing), and 72 °C for 1 min (extension). After these cycles, a final extension was carried out at 72 °C for 7 min. Annealing temperatures were varied for each primer set (51 °C for protein S, 55 °C for β-globin and transgene in transgenic mouse, 58 °C for HLA DPB1, 60 °C for p53 exon 6, p53 exon 11, mitochondrial sequence and HBV C region). The number of PCR cycles was changed from 40 to 45 for detection of HBV DNA to improve the detection sensitivity.

Electrophoresis

After PCR, a 5 µl aliquot of each reaction mixture was subjected to electrophoresis at 5 V/cm for 50 min in 2.5% agarose gel (Agarose- ME, Nacalai Tesque Inc.,

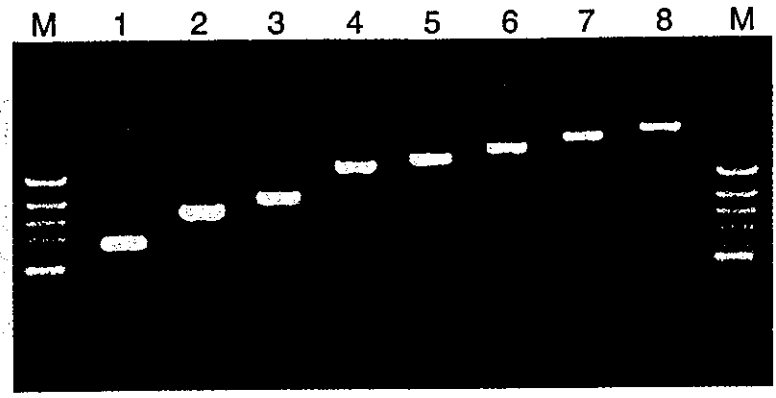

Figure 2: Dynamic range of target amplification by Direct PCR on blood. Direct PCR using 1 µl of citrated blood in 50 µl of Ampdirect mixture was carried out using eight primer sets amplifying fragments of various sizes from the human β-globin gene. The sizes of specific PCR products were: 437 bp (lane 1), 641 bp (lane 2), 742 bp (lane 3), 1127 bp (lane 4), 1238 bp (lane 5), 1497 bp (lane 6), 1745 bp (lane 7) and 2056 bp (lane 8). Lane M contains a molecular size marker.

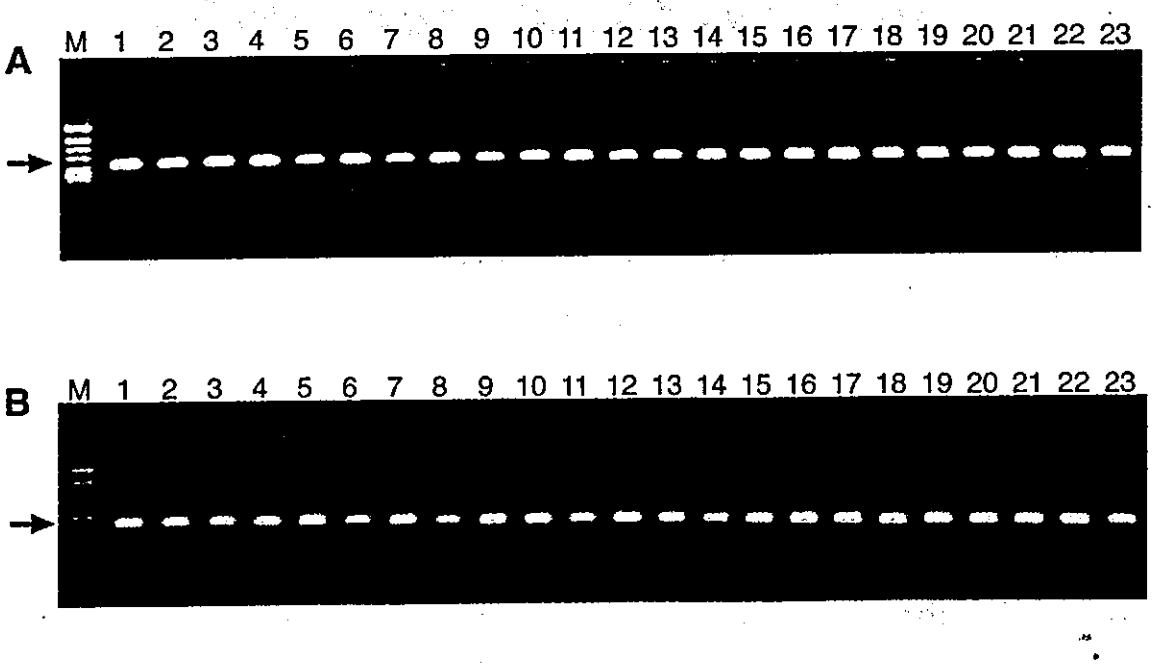

Figure 3: Direct PCR from a population of blood samples. Direct PCR was carried out using 1 µl of 23 different human blood samples (EDTA-treated) and primers for human β-globin gene (A) and mitochondrial DNA (B) in 50 µl of Ampdirect mixture. Lane M shows a molecular size marker. The specific products of PCR for the above genes are indicated by arrows at 408 and 293 bp, respectively.

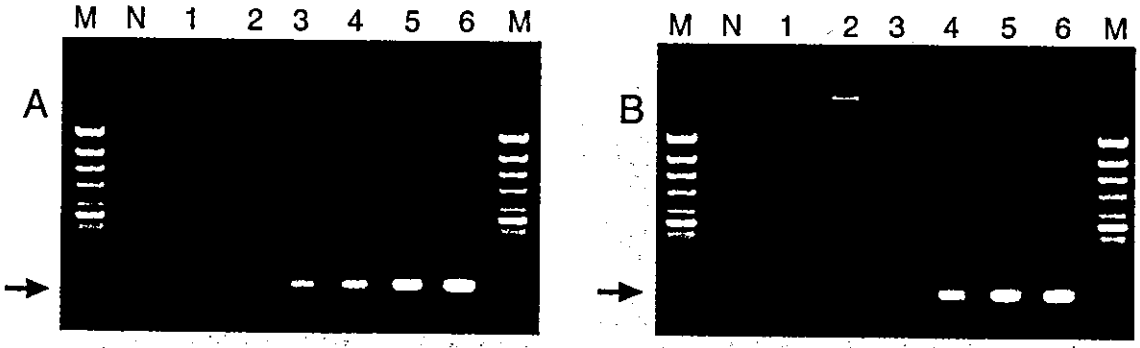

Figure 4: Comparison of the detection sensitivity for HBV in blood using Direct PCR with that observed using conventional PCR for DNA purified from blood containing HBV. Blood containing about 5,000 copies/µl of HBV DNA was subjected to serial ten-fold dilutions with blood from a healthy volunteer. For Direct PCR, aliquots (1 µl) of each diluted blood sample were added to 50 µl of Ampdirect mixture, and PCR was carried out using the primer set for the HBV C region (A). For conventional PCR, DNA was extracted from each diluted blood sample using a QIAamp DNA Blood kit and re-suspended in the same volume of TE. Aliquots (1 µl) of each DNA sample were then added to 50 µl of Standard mixture, and PCR was carried out with the same primer set as above (B). The dilutions used were: 10^5 (lane 1), 10^4 (lane 2), 10^3 (lane 3), 10^2 (lane 4), 10 (lane 5), 1 (lane 6). Lane N is a negative control using blood from a healthy volunteer. Lane M contains a molecular size marker (*Hinc* II-digested ϕX174 RF DNA). The specific PCR product for the HBV C region is indicated by an arrow at 137 bp.

Kyoto, Japan) containing 0.5 mg/l ethidium bromide in TAE buffer (40 mmol/l Tris-acetate, 1 mmol/l EDTA, pH 8.0). DNA bands were visualized using ultraviolet irradiation.

RESULTS

Direct PCR using four different sample volumes (0.63-5.00 µl) of fresh human blood pretreated with three different anticoagulants (sodium citrate, dipotassium EDTA or sodium heparinate) in 50 µl of Ampdirect mixture was first carried out to demonstrate detection of a human β-globin gene fragment. The predicted PCR products were detected for all volumes of each anticoagulant-treated blood sample (Figure 1A). Subsequently, the PCR efficiency for the same blood samples was examined following storage at 4 °C or -20 °C, using the same protocol as described above. To mimic actual sample handling, the frozen samples were freeze-thawed once a month during the first year of storage. Blood samples stored at 4 °C or -20 °C for one year gave results similar to fresh blood regardless of the type of anticoagulant used (4). After four years of storage, blood samples stored at 4 °C had coagulated, while heparinized blood samples stored at –20 °C showed a weaker signal than fresh blood (Figure 1B, lanes c.1-c.4). However, blood samples pretreated with citrate or EDTA and then stored at –20 °C gave results similar to fresh blood (Figure 1B, lanes a.1-a.4 and b.1- b.4). Thus, for this method it is recommended that citrated or EDTA-treated blood be used for Direct PCR, and that samples be stored frozen.

To evaluate the dynamic range of target amplification, Direct PCR was performed using 8 primer sets that produced fragments ranging in length from 437 to 2056 bp from the human β-globin gene (Figure 2). DNA fragments up to 2056 bp in length were successfully amplified. This result shows that Direct PCR can amplify target DNA with a minimum length of 2 kb, in the same range as conventional PCR using *Taq* DNA polymerase with purified DNA.

To confirm the reproducibility and versatility of this method, 23 blood samples from healthy individuals were examined by Direct PCR using primer sets for the human β-globin gene and a mitochondrial DNA sequence (Figure 3). All samples gave the expected PCR products for each primer set.

To study whether low numbers of target sequences in blood could be detected directly, the detection sensitivity of Direct PCR using Ampdirect mixture was compared with that of conventional PCR using Standard mixture. Using Direct PCR, HBV in blood obtained from a single infected individual could be detected following 10^3-fold dilution with blood from a healthy volunteer (representing detection of about 5 copies of HBV DNA) (Figure 4A, lane 3). Using conventional PCR with purified DNA obtained from diluted blood samples, the detection limit for HBV was reached after 10^2-fold dilution (representing detection of about 50 copies of HBV DNA) (Figure 4B, lane 4). The detection sensitivity for HBV DNA was thus ten-fold higher

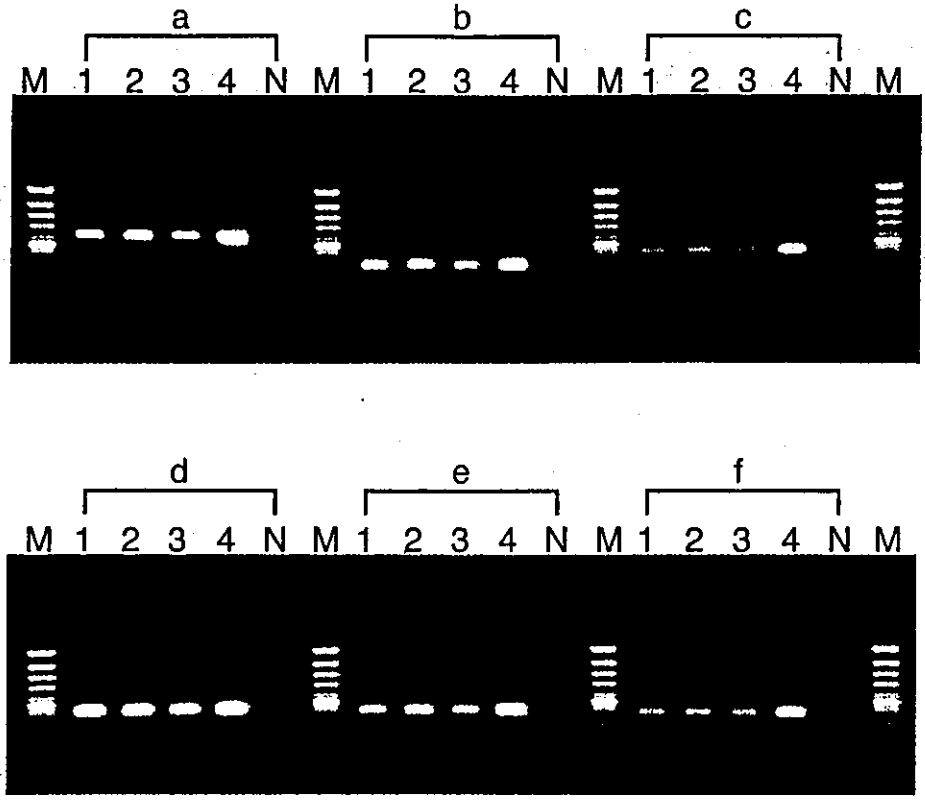

Figure 5: Direct PCR from various forms of blood samples. Direct PCR using 6 different primers was carried out using 1 µl of fresh blood (EDTA-treated) (lane 1), dried blood in a PCR tube (lane 2), dried blood on a filter paper (lane 3), or purified DNA equivalent to 1 µl of blood (lane 4) in 50 µl of Ampdirect mixture. Primers used in this experiment were for human β-globin gene (a), protein S gene (b), HLA DPB1 gene (c), mitochondrial DNA (d), p53 exon 6 gene (e) and p53 exon 11 gene (f). Lane N is a negative control. Lane M contains a molecular size marker. The sizes of specific PCR products were: 408, 213, 280, 293, 276 and 266 bp, respectively.

when Direct PCR was used than when conventional PCR was used with purified DNA, meaning that 1 µl of blood analyzed using Direct PCR corresponds to 10 µl of blood required for conventional PCR.

For archiving or transport of blood samples, it is frequently convenient to use dried blood. The efficiency of Direct PCR using 1 µl samples of blood dried in a PCR tube or on a filter paper was compared with results using 1 µl fresh blood or an amount of purified DNA equivalent to 1 µl blood in 50 µl of Ampdirect mixture (Figure 5). Primer sets for six different targets (human β-globin, protein S, HLA DPB1, mitochondrial sequence, p53 exon 6, and p53 exon 11) were used. All samples gave the predicted PCR products for each target. These results show that Direct PCR can be employed with various forms of blood samples for various target sequences.

Transgenic animals are important models in genetic studies of the causes and therapy of human diseases. Direct PCR detection of the *lck* promoter-human D4-GDI transgene in transgenic C57BL/6J mice, using heparinized blood, was compared with the results of conventional PCR using purified DNA obtained from tissue samples of mouse tails (Figure 6). Direct PCR from blood samples gave the same detection sensitivity as conventional PCR. Using citrated, EDTA-treated or heparinized blood from 325 transgenic C57BL/6J mice, the transgene could be also detected successfully, regardless of the anticoagulant used (data not shown). These results show that Direct PCR can be used for the detection of introduced genes in transgenic mice.

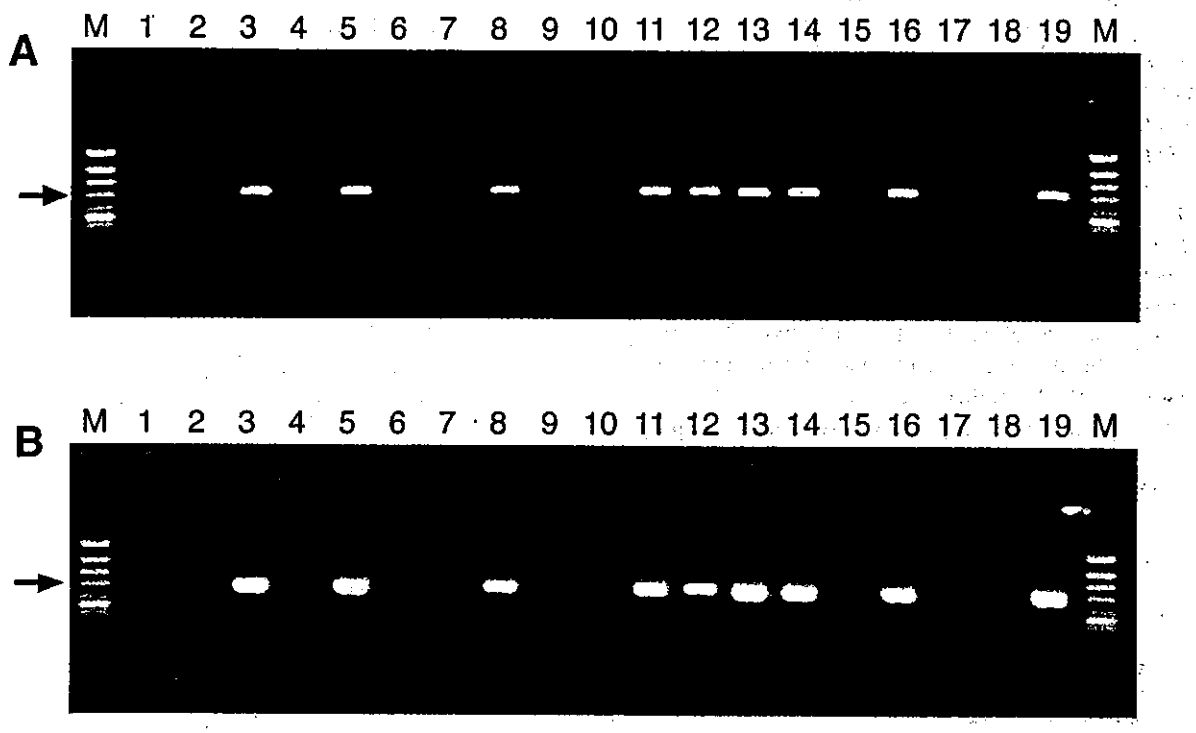

Figure 6: Detection of transgene from transgenic mice. Direct PCR was carried out using 1 µl of transgenic mouse blood (heparinized) and primers for *lck* promoter-human D4-GDI transgene in 50 µl of Ampdirect mixture (A). For comparison, conventional PCR with Standard mixture was carried out using purified DNA from mouse tails (B). Lane M contains a molecular size marker. The specific product of PCR is indicated by an arrow at 521 bp.

DISCUSSION

Samples of blood or other animal fluids contain a variety of substances that inhibit PCR, such as heme (5), immunoglobulin G (6), and anticoagulants including EDTA (7) and heparin (8). Various reports for PCR methods that do not require prior isolation of DNA have been published (8-16). However, almost all the methods reported so far for Direct PCR from blood have used empirical approaches (e.g. pretreatment by heating or freeze-thawing, or modification of the salt composition or addition of detergents to the reaction mixture). Therefore, these methods cannot overcome quantitative and qualitative differences in the inhibitors present in different samples, nor are they applicable for many kinds of primer sets for wider application.

Based on these considerations, we developed a reagent cocktail intended to neutralize the various charge-bearing inhibitory substances that might otherwise bind to DNA polymerase and template DNA and thus inhibit PCR.

Using this reagent cocktail, we showed that various targets in chromosomal or mitochondrial DNA could be efficiently amplified directly, either from fresh human blood or from blood samples stored frozen for long periods. Further, low titers of hepatitis B virus could be amplified directly from human blood samples. The latter experiments also showed the detection sensitivity for HBV DNA using Direct PCR to be ten-fold higher than the sensitivity observed using conventional PCR with purified DNA from the same samples. This result may be rationalized as being due to poor DNA recovery during sample purification prior to conventional PCR. Our results show that Direct PCR is useful for the detection of even low titers of virus in blood, in addition to representing a simpler method. It is anticipated that the method will be similarly effective for the detection of low numbers of fungi, bacteria and mutated cells or similar targets in blood.

We also showed that DNA from dried blood in a PCR tube or on a filter paper could be amplified directly in the same way as whole blood. This shows that Direct PCR was able to amplify even partially degraded DNA

fragments which could not be efficiently recovered using conventional DNA purification methods. The Direct PCR method may therefore also be useful in forensic medicine, archaeological research and epidemiological analysis, which frequently deal with older and lower quality DNA samples from disrupted cells.

In addition, it has been shown that Ampdirect could be adapted for use with the blood of other animals, for example mice.

As this reagent cocktail can eliminate DNA preparation with the associated likelihood of sample mishandling or contamination with foreign DNA, PCR analysis could become easier, faster and more accurate in a wide range of basic and practical fields (4, 17, 18). The concepts used in the development of this reagent may also be applied to PCR amplification from various other types of biological materials (19). We are thus developing similar cocktails applicable to a broader range of biological samples.

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Correspondence: Naoyuki Nishimura, Ph. D.
Life Science Laboratory,
Analytical and Measuring Instruments Division,
Shimadzu Corporation
3-17-1 Azuma, Tsukuba, Ibaraki 305-0031, Japan
Tel: +81-298-51-6641, Fax: +81-298-51-6682
E-mail: nisimura@shimadzu.co.jp