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Generation of a Human Anti-Tumor Necrosis Factor-α Monoclonal Antibody by *in Vitro* Immunization with a Multiple Antigen Peptide

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We developed the *in vitro* immunization method to induce antigen-specific immune responses in human peripheral blood mononuclear cells (PBMCs). However, when we used a peptide as sensitizing antigen, the antigen-specific immune response was found to be weak, and hence, we could not effectively obtain the antigen-specific antibody gene. In the present study, we attempted to improve the *in vitro* immunization method by augmenting the immune response to the peptide antigen. We used a multiple antigen peptide for sensitization. *In vitro* immunization of the multivalent antigen elicited a strong antigen-specific immune response in the PBMCs, and we succeeded in obtaining antigen-specific antibody genes by the phage-display method. Further, by combining the variable-region genes and constant-region genes of human IgG, we obtained four independent human monoclonal antibodies specific for tumor necrosis factor-α. This might be a good strategy for generating antigen-specific human monoclonal antibodies using a peptide antigen.

Key words: *in vitro* immunization; multiple antigen peptide; human monoclonal antibody

Monoclonal antibodies (mAbs) are considered an epochal breakthrough in medicine. They are suitable reagents for the diagnosis and treatment of cancer, allergy, infection, and other diseases. It is relatively easy to generate antibodies using laboratory animals, but their therapeutic efficacy in humans is limited by their antigenicity.[1)] Hence, human-derived mAbs are desirable for clinical use. Several methods of generating human mAbs have been reported thus far, including (i) the generation of antigen-specific scFv antibodies by the phage display method,[2,3)] (ii) humanization of non-human derived antibodies by genetic engineering, and (iii) the production of antigen-specific human mAbs using transgenic mice with human antibody genes,[4)] but these methods are time-consuming and laborious.[5)] With these specifications in mind, we developed an *in vitro* immunization method to augment the antigen-specific immune response in human PBMCs.[6)] We generated mite-extract-specific human monoclonal antibodies by this method. With a peptide, it is possible to induce an antigen-specific immune response by *in vitro* immunization, but obtaining the antigen-specific antibody gene is difficult because of the weak immune response elicited by the peptide antigen. Hence, in the present study, we attempted to augment the immune response by *in vitro* immunization with a multiple antigen peptide (MAP) instead of a monovalent antigen and to obtain antigen-specific human monoclonal antibodies.

Materials and Methods

Antigens and reagents. On the basis of the 3D-structure of tumor necrosis factor (TNF)-α, deposited in the Protein Data Bank, and epitope structures of previously generated anti-TNF-α monoclonal antibodies, the peptide region (^{1}VRSSSRTPSDKPV^{14}A) of TNF-α was used as a peptide antigen, as reported previously.[7)] The peptide antigen for TNF-α (pTNF-α), and MAP for TNF-α (mTNF-α), have been synthesized by Sigma Genosys (Ishikari, Japan) and Funakoshi (Tokyo) respectively. Recombinant human interleukin-2 (IL-2) and IL-4 were purchased from Genzyme (Cambridge, MA) and Pepro Tech (London, UK) respectively. L-Leucyl-L-leucine methyl ester (LLME) was from Bachem (Torrance, CA). D-Type CpG oligodeoxynucleotide (ODN) (5′-ggTGCATCGATGCAGGGGggG-3′; uppercase and lowercase letters indicate bases with phosphodiester and phosphorothioate-modified backbones respectively) and K-type CpG ODN (5′-tcgagcgttctcC-3′) were from Sigma Genosys.[8)] Fish gelatin (FG) was from BioFX Laboratories (Owings Mills, MD).

In vitro *immunization.* PBMCs were isolated from peripheral blood as described previously.[9)] PBMCs (5×10^6 cells) seeded into a 48-well culture plate were first treated with 0.25 mM LLME for 20 min at room temperature, and then sensitized with peptide antigen (pTNF-α or mTNF-α; 0.1–10 μg) in the presence of IL-2 (50 U/ml), IL-4 (50 ng/ml), D-type CpG ODN (150 μM), and K-type CpG ODN (300 μM), and then were cultured in enriched RDF (eRDF) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2-mercaptethanol (50 μM) for 6–8 d.

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Abbreviations: ABTS, 2,2′-azino-bis (3-ethylbenzthazoline-6-sulfonic acid); cfu, colony-forming unit; CH, heavy chain constant region; CL, light chain constant region; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunospot assay; FG, fish gelatin; LLME, L-leucyl-L-leucine methyl ester; mAbs, monoclonal antibodies; MAP, multiple antigen peptide; mTNF-α, multiple antigen peptide for TNF-α; PBMCs, peripheral blood mononuclear cells; PBST, phosphate buffered saline (PBS) containing 0.05% Tween 20; pTNF-α, peptide antigen for TNF-α; scFv, single chain antibody variable fragment; VH, heavy-chain variable region; VL, light-chain variable region

All experiments throughout this study were carried out in accordance with the principles of the Declaration of Helsinki and the regulations of the Ethics Committee of the Faculty of Agriculture of Kyushu University.

Enzyme-linked immunosorbent assay. Culture supernatants were collected from *in vitro* immunized PBMCs and were used in the enzyme-linked immunosorbent assay (ELISA) to determine the amount of immunoglobulin produced by *in vitro* immunization, as described previously.[10] All the experiments were repeated 3 times, and the results are expressed as means ± standard error of mean.

Enzyme-linked immunospot assay. B cells producing antigen-specific antibodies were detected by enzyme-linked immunospot (ELISPOT) assay, as described previously.[7] Briefly, multiscreen HA filtration plates (Millipore, Bedford, MA) were coated with 0.5 μg of human recombinant TNF-α (Pepro Tech) or 0.05 μg of streptavidin, and incubated overnight at 4 °C. After three washes with phosphate buffered saline (PBS), 0.05 μg of biotinylated pTNF-α was added to the streptavidin-coated wells, and the plates were incubated for 2 h at 37 °C. After three washes with PBS, blocking was performed with 1% FG in PBS for 2 h at 37 °C. After three washes with PBS, *in vitro* immunized-PBMCs (1×10^5 cells) were added to the wells in triplicate and cultured for 48 h. After the plates were washed, goat anti-human IgM conjugated with horseradish peroxidase (Tago, Burlingame, CA) was added, and the cells were cultured for 2 h at 37 °C. After the plates were washed with PBS containing 0.05% of Tween 20 (PBST), TrueBlue peroxidase substrate (KPL, Gaithersburg, MD) was added, and the plates were incubated for 10 min at 37 °C. The reaction was terminated by washing the plates with water, and the plates were then dried in the dark.

Generation of an antigen-specific phage antibody library by the phage-display method. A phage-antibody library specific for mTNF-α was generated by the phage-display method, as described previously.[11] Finally, antigen-specific phage antibody was selected by several rounds of panning using biotinylated TNF-α and streptavidin magnetic beads (Dynabeads M-280 Streptavidin, Invitrogen, Carsbad, CA). To evaluate the efficiency of the panning, the number of colony-forming units (cfu) of *Escherichia coli* producing the acquired phage antibodies was measured before and after each round of panning by transformation of log-phase *E. coli* TG1 cells with phage, as described previously.[11]

Evaluation of the antigen specificity of the phage antibody. The culture supernatants containing phage antibodies were analyzed by ELISA to determine their binding affinity for TNF-α. Microtiter plates (Nunc, Roskilde, Denmark) were coated with 1 μg/ml of TNF-α or 0.5 μg/ml of pTNF-α in 50 mM carbonate buffer and incubated overnight at 4 °C; they were then blocked with 1% FG in PBS for 2 h at 37 °C. After the plates were washed, the culture supernatants were added to the wells and the plates were incubated for 2 h at 37 °C. After the plates were washed, horseradish peroxidase-conjugated mouse anti-M13 mAb (GE Healthcare, Amersham Place, UK) was added, and the plates were incubated for 2 h at 37 °C. After the plates were washed, a substrate solution containing 0.3 mg/ml of 2,2′-azino-bis(3-ethylbenzthazoline-6-sulfonic acid) (ABTS; Sigma, St. Louis, MO, USA) and 0.06% H_2O_2 in 0.1 Mcitrate buffer (pH 4.0) was added. Absorbance was measured at 405 nm using a microtiter plate reader (Wako, Osaka, Japan).

Production of recombinant human monoclonal IgG in Chinese hamster ovary cells. We amplified the variable region genes of the heavy and light chains (VH and VL) of the antibody using vectors expressing scFv specific for TNF-α as templates and appropriate primers, as described previously.[11] The VH and VL genes were cloned into the pSecTag2A/IgGc-bearing heavy chain constant region (CH) gene and the pSecTag2A/IgLc-bearing light chain constant region (CL) gene vectors respectively. Approximately 5×10^6 Chinese hamster ovary (CHO) cells were transfected with these expression vectors (5 μg each) using Lipofectamine (Invitrogen), following the manufacturer's instructions. Cell lines stably expressing antibody genes were established, and the supernatants were used for further analysis.

Evaluation of the TNF-α peptide-specific binding of recombinant human IgG. Microtiter plates were coated with 1 μg/ml of TNF-α, 1 μg/ml of streptavidin, 1% ovalbumin, or 1% FG and incubated overnight at 4 °C. After the plates were blocked with 1% FG, equal amounts of recombinant human IgG were added to the plates, and they were further incubated with goat anti-human IgG conjugated with horseradish peroxidase (Biosource, Carlsbad, CA). After a substrate solution containing ABTS was added, the absorbance was measured at 405 nm using a microtiter plate reader, as described above.

Results and Discussion

mTNF-α elicited a TNF-α-specific immune response in the PBMCs upon in vitro *immunization*

We have succeeded in eliciting an antigen-specific immune response in PBMCs by *in vitro* immunization with antigenic peptides as sensitizers,[7] but the antigen-specific immune responses elicited were weak, and the frequency of B-cell production of antigen-specific antibodies was low. Hence, we did not obtain the antigen-specific VH and VL genes. To overcome this problem, we augmented the antigen-specific immune response by *in vitro* immunization with MAP as a sensitizing antigen (Fig. 1). The use of the MAP system is a novel approach to anti-peptide antibody elicitation. The system comprises a small immunologically inert core molecule of radically branching lysine dendrites onto which a number of peptide antigens are anchored. In the present study, we used a MAP system, mTNF-α, which is composed of a highly hydrophobic region of the TNF-α protein, as a sensitizing antigen for *in vitro* immunization of PBMCs.[12,13] The results indicated that although the amounts of IgM, IgG, and IgE were similarly augmented in PBMCs by *in vitro* immunization with pTNF-α and mTNF-α (Fig. 2A), the antigen-specific antibody response was greatly enhanced by the mTNF-α (Fig. 2B). Further, mTNF-α elicited an antibody response specific for the TNF-α protein as well as the TNF-α peptide. These results indicate that the MAP system is useful in eliciting the antigen-specific antibody response in *in vitro* immunization of PBMCs as well as *in vivo* immunization of mice. As compared to coupled antigenic peptides in parallel, the high molar ratio and dense packing of multiple copies of the antigenic peptides in a MAP produced a strong antigen-specific antibody response.

Thus when proteins, such as toxic antigens, tolerized antigens and self antigens cannot be used as sensitizing antigens, this MAP system can be adopted as it is well suited to induce an antigen-specific immune response in PBMCs by *in vitro* immunization.

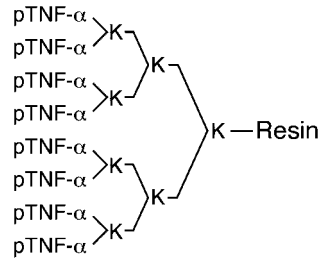

Fig. 1. Multiple Peptide Antigen of Tumor Necrosis Factor-α.

A highly hydrophilic region (^{1}VRSSSRTPSDKPV^{14}A) of tumor necrosis factor (TNF)-α was used as a monovalent antigen (pTNF-α). A multiple antigen peptide (MAP) of TNF-α (mTNF-α) was generated by coupling pTNF-α *via* lysine residues.

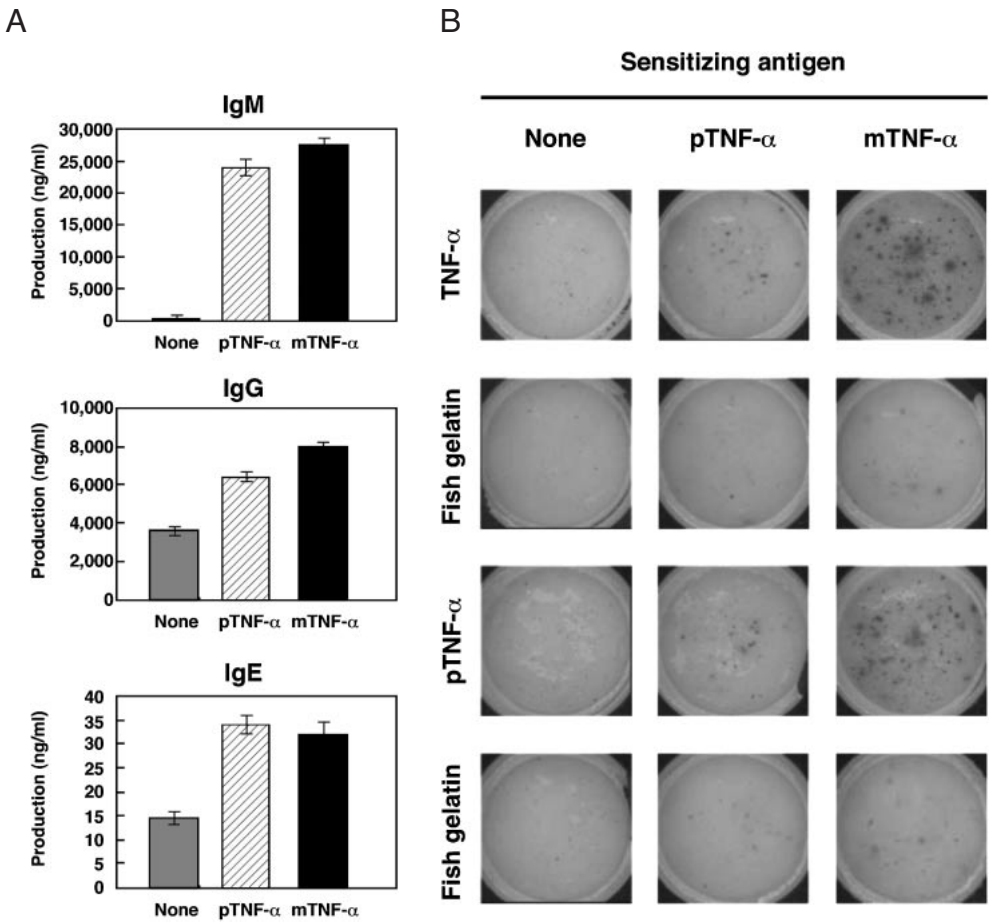

Fig. 2. TNF-α-Derived Peptide Elicited an Immune Response in Peripheral Blood Mononuclear Cells in *in Vitro* Immunization.

A, The TNF-α-derived peptide elicited an immune response in *in vitro* immunization. Peripheral blood mononuclear cells (PBMCs) were *in vitro* immunized using two TNF-α-derived peptides (pTNF-α and mTNF-α) as sensitizing antigens. After 8 d of culture, the amounts of IgM, IgG, and IgE in the supernatants were measured by ELISA. B, *In vitro* immunization with mTNF-α augmented the antigen-specific immune response. LLME-treated PBMCs were cultured in enriched RDF (eRDF) medium (no stimuli), or sensitized with pTNF-α or mTNF-α by *in vitro* immunization. After 6 d of culture, the PBMCs were seeded into multiscreen HA plates coated with FG or pTNF-α. B-cells secreting antigen-specific antibody were detected as blue spots by TrueBlue staining.

Acquisition of the antigen-specific scFv phage

cDNA was obtained from PBMCs immunized *in vitro* with mTNF-α, and was used in the amplification of the VH and VL genes, as described previously.[11] Then we constructed an scFv phage library by combining the VH, linker, and VL genes by polymerase chain reaction (PCR), cloning them into the pCANTAB5E vector, introducing the vector into the bacterial host, and then infecting the cells with a M13KO7 helper phage. Thus we obtained an scFv phage library containing 2.5×10^5 independent clones. Then we selected antigen-specific phage antibodies by panning, as described in "Materials and Methods."[14] After three-rounds of panning, *E. coli* TG1 cells were infected with the enriched phage and induced to produce phage antibodies. Supernatants of 60 clones containing phage antibodies were screened by ELISA to select antigen-specific phage antibodies. Some phage antibodies showed strong antigen specificity for the TNF-α protein and others for the TNF-α peptide, suggesting that we had obtained several phage antibody clones with different antigen-specificities (Fig. 3). We selected 12 clones (9, 13, 25, 31, 35, 40, 42, 43, 46, 47, 53, and 59) that displayed high antigen specificity for further analysis. We investigated the genetic diversity of the 12 selected clones by sequence analysis, and found that they consisted of 11 different CDR family sets (data not shown). Then we selected five independent clones (13, 25, 35, 40, and 59) displaying high antigen specificity for further analysis.

Acquisition of recombinant human anti-TNF-α IgG antibodies

The VH and VL genes of these 5 scFv antibodies were amplified and inserted into the respective expression vectors, as described in "Materials and Methods." CHO cells were transfected with these vectors and cultured for several weeks in the presence of a selection drugs. After determination of the amount of recombinant human IgG in each of the recombinant CHO cell supernatants, the supernatant containing approximately 6.68 ng of recombinant human IgG was used in direct ELISA to evaluate the antigen-specificity of the antibodies produced (Fig. 4). As shown in Fig. 4, four clones (13, 35, 40, and 59) bound more specifically to the TNF-α protein than to FG, streptavidin, or ovalbumin. Among these antibodies, binding specificities were found to be maintained after conversion of the scFv phage antibody to human IgG antibody.

In this study, we found that peptide antigen-specific human monoclonal antibody can be generated by

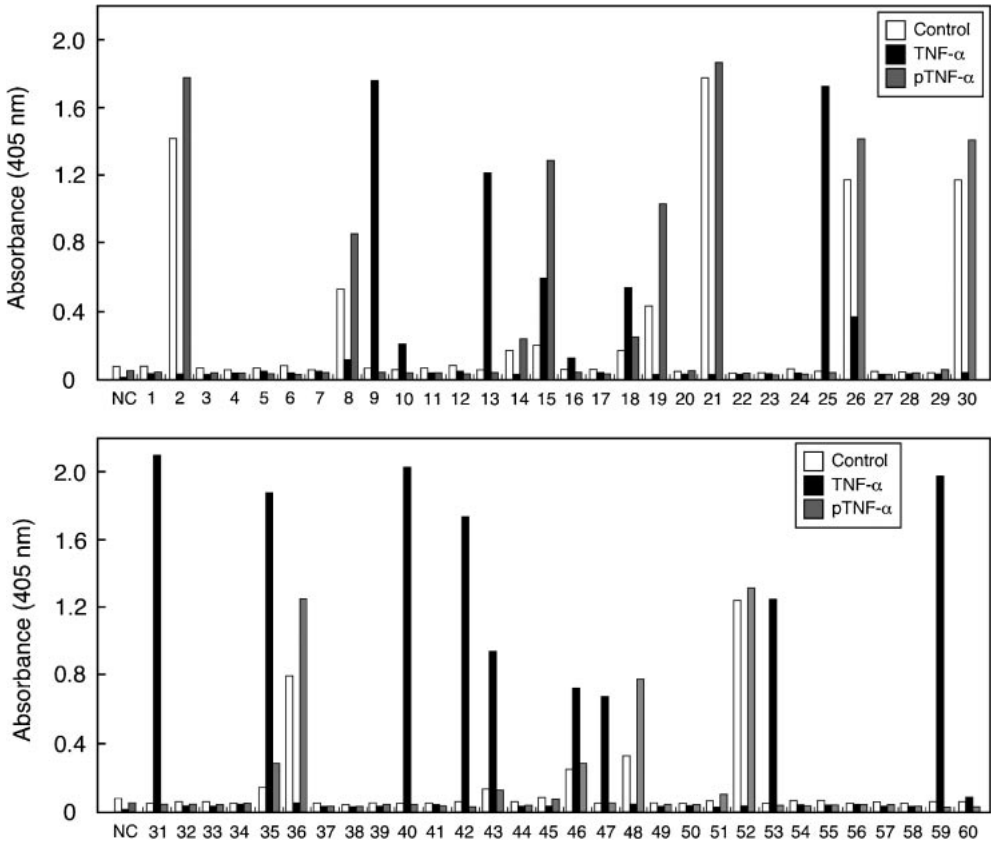

Fig. 3. Antigen-Specificities of Phage Antibodies.

After three rounds of panning, 60 individual colonies were selected and infected with M13KO7 helper phage to produce phage antibody. The antigen specificities of the phage antibodies were tested by enzyme-linked immunosorbent assay (ELISA). This experiment was repeated 3 times, and similar results were obtained.

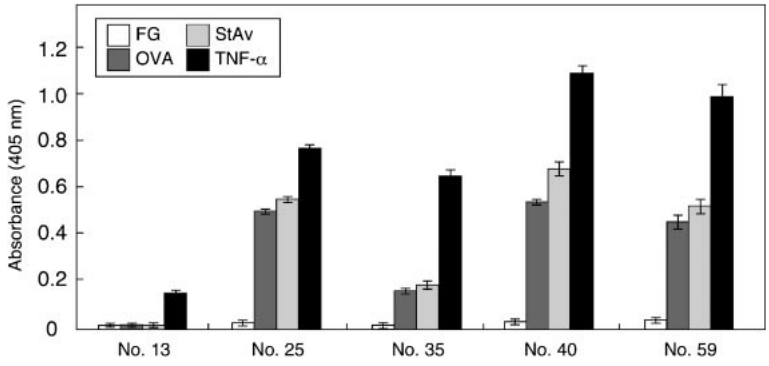

Fig. 4. Antigen-Specificities of Recombinant Human IgG.

Antigen-specific binding of the recombinant human-IgG produced in this study was evaluated by direct ELISA. Microtiter plates were coated with FG, ovalbumin, streptavidin, or TNF-α protein and incubated overnight at 4 °C. After blocking of the plates with 1% FG, equal amounts of recombinant human IgG was added, and they were further incubated with goat anti-human IgG conjugated with horseradish peroxidase. After a substrate solution containing ABTS was added, the absorbance was measured at 405 nm using a microtiter plate reader.

augmenting the immune response by *in vitro* immunization with MAPs and subsequent selection of antigen-specific combinations of VH and VL by the phage display method. This methodology can be applied for generation of human monoclonal antibodies against toxic antigens, self-antigens, and tolerized antigens.

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