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Anti-Peptide Antibody Production Elicited by *in Vitro* Immunization of Human Peripheral Blood Mononuclear Cells

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Human monoclonal antibodies have great potential for use in the treatment of various diseases. We have established an *in vitro* immunization protocol for inducing antigen-specific antibody production from human peripheral blood mononuclear cells (PBMCs). In the *in vitro* immunization protocol, PBMCs are pretreated with L-leucyl-L-leucine methyl ester (LLME) to remove suppressive cells, and are sensitized and cultured with a soluble antigen in the presence of IL-2, IL-4 and muramyl dipeptide for 8 d, and then an antigen-specific antibody is produced. In this study, we examined the novel possibility of an *in vitro* immunization protocol, specifically, whether LLME-treated PBMCs can be sensitized with a peptide antigen to produce an anti-peptide antibody. The results indicate that antigen-specific immune responses were elicited by a peptide antigen derived from rice allergen, a cholera toxin B subunit, and TNF-α as a sensitizing antigen in *in vitro* immunization. These results suggest that the *in vitro* immunization protocol is applicable in the generation of an anti-peptide antibody against various antigens, including food allergens, foreign antigens, and self-antigens.

Key words: *in vitro* immunization; antibody production; peptide antigen

Human monoclonal antibody (mAb) has a great potential for use in the treatment of cancer, autoimmune disease, allergy, and bacterial infection. Although a number of antigen-specific mouse monoclonal antibodies (mAb) have been generated, mAbs generated in mice are not suitable for direct administration to humans.[1,2] Hence, human mAb is thought to be best suited for clinical use, but techniques to produce human mAb are not yet well established. One method to produce human mAb is the *in vitro* immunization protocol, in which human peripheral blood mononuclear cells (PBMCs) are treated with L-leucyl L-leucine methyl ester (LLME)[3] and sensitized with a soluble antigen. Activated PBMCs are induced to produce antigen-specific mAb.[4] We have improved this technique, and we found that muramyl dipeptide (MDP),[5] IL-2, and IL-4 were effective as additives for inducing production of antigen-specific antibody from human PBMCs *in vitro*.[6]

Here, we tried to evaluate whether peptide antigen can elicit antigen-specific antibody production in *in vitro* immunization using peptides derived from rice allergen, a cholera toxin B subunit and TNF-α were used as sensitizing antigens. Provided that PBMCs are sensitized with peptide antigen by *in vitro* immunization, and that they produce peptide-specific antibody, the usefulness of the *in vitro* immunization technique should be greatly increased.

Material and Methods

Antigens and reagents. Cholera toxin B subunit (CTB) was purchased from List Biological Laboratories (Campbell, CA). Rice allergen (RA) was prepared according to the method described by T. Matsuda *et al.*[7] TNF-α was from Sigma Genosys (Ishikari, Japan). Antigenic peptides of both antigens and the peptides conjugated with keyhole limpet hemocyanin (KLH) were designed and synthesized by Sigma Genosys. Recombinant human interleukin 2 (IL-2) was from Genzyme (Cambridge, MA). Recombinant human IL-4 was from Pepro Tech (London, UK). Muramyl dipeptide (MDP) was from Chemicon (Temecula, CA). L-leucyl-L-leucine methyl ester (LLME) was obtained from

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Bachem (Torrance, CA). ERDF medium was from Invitrogen (Carlsbad, CA).

In vitro *immunization.* In these experiments, all blood samples were obtained from healthy donors. Peripheral blood mononuclear cells (PBMCs) were separated by density-gradient centrifugation using lymphocyte separation medium (LSM; Organon Teknika, Durham, NC), and treated with 0.25 mM LLME for 20 min at room temperature. *In vitro* immunization of LLME-treated human PBMCs was performed as described previously.[8] LLME-treated PBMCs were sensitized with antigen (0.1 to 10 $\mu g \cdot \mu l^{-1}$) in ERDF medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen), MDP (10 $\mu g \cdot \mu l^{-1}$), IL-2 (1–50 units$\cdot ml^{-1}$), IL-4 (1–50 $ng \cdot ml^{-1}$), 2-mercaptoethanol (2-ME; 50 μM), and were further cultured for 8 d. In a previous study, we reported that the sensitivity of PBMCs against antigen sensitization in *in vitro* immunization differed among healthy donors.[9] Hence, we searched for the optimal condition for eliciting an immune response from each PBMC before antigen sensitization. The experiments conducted 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 (ELISA). Culture supernatants were collected from *in vitro* immunized PBMCs and applied to ELISA to determine the amount of immunoglobulins produced by *in vitro* immunization, as described previously.[8] A color development reaction was carried out for 30 min. All experiments were repeated at least 3 times, and reproducibility was confirmed.

Enzyme-linked immunospot assay (ELISPOT). B cells producing antigen-specific antibodies were detected by ELISPOT assay, as described previously.[10] Briefly, after *in vitro* immunized PBMCs were cultured for 8 d, cells (1×10^5) were transferred to Multiscreen HA plates (Millipore, Bedford, MA) coated with fish gelatin (FG) or protein antigen, and cultured for an additional 2 d in ERDF medium supplemented with 10% FBS, IL-2 (1 unit$\cdot ml^{-1}$), and IL-4 (1 $\mu g \cdot ml^{-1}$). After the plates were washed with 2.24×10^{-2} M phosphate buffer containing 1.37×10^{-1} M NaCl (PBS), goat anti-human IgM antibody conjugated with horseradish peroxidase was added and the plates were incubated for 2 h at 37 °C. After the plates were washed, a substrate solution (0.1 M Tris–HCl buffer, pH 7.6, containing 0.03% H_2O_2 and 0.3 $mg \cdot ml^{-1}$ 3,3′-diaminobenzidine tetrahydrocholoride) was added and the plates were incubated for 20 min at 37 °C. After the plates were washed again, the number of spots was counted using ImageJ software.

Immunoblotting. RA-specific antibodies in the supernatant was detected by Immunoblotting, as previously described.[10] RAs were resolved on 17.5% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Hybond-P membrane (Amersham Biosciences, Piscataway, NJ). The membrane was incubated with culture supernatant of PBMCs immunized *in vitro* with RA protein or RA peptide. The bound antibody was captured with anti-human IgM antibody conjugated with horseradish peroxidase, and visualized by the ECL-plus Western blotting detection system (Amersham Biosciences) and Image Analyzer LAS-1000 (Fuji Film, Tokyo).

Statistical analysis. Statistical significance was tested by Student's t-test. The differences were considered to be significant at $P < 0.05$ (*, $P < 0.05$; **, $P < 0.01$).

Results

pRA-specific immune response elicited by in vitro *immunization*

We have established B cell hybridomas producing human monoclonal antibody specific for 14–16 kDa rice allergen (RA) by immunizing human PBMCs *in vitro* with RA protein and subsequent immortalization with Epstein Barr virus and human-mouse hetero myeloma.[6] Here, we determined whether peptide derived from RA, known to be a food allergen,[11] can induce an antigen-specific immune response by *in vitro* immunization. A hydrophilic peptide corresponding to p35-45 (^{35}VGRGASAADE^{45}Q; pRA) was synthesized and used as a sensitizing antigen in *in vitro* immunization. After treatment of PBMCs isolated from peripheral blood of healthy donors with LLME, the PBMCs were sensitized either with RA protein (RA), RA peptide (pRA), or pRA conjugated with KLH (pRA-KLH) in the presence of IL-2 and IL-4. After 8 d of culture, the amounts of IgM and IgG secreted into the supernatants were measured by ELISA (Fig. 1A). The results showed that antibody production was augmented in PBMCs immunized *in vitro* with RA or pRA-KLH, although pRA did not induce efficient antibody production (Fig. 1A). Further, we investigated the production of RA-specific antibody from PBMCs immunized with these antigens by ELISPOT assay (Fig. 1B) and Western blotting (Fig. 1C). The results indicated that peptide-antigen as well as protein-antigen can induce the production of antigen-specific antibody in *in vitro* immunization regardless of conjugation with KLH. Similar results were obtained using PBMCs from different donors (data not shown).

pCTB-specific immune response elicited by in vitro *immunization*

Next we used cholera toxin B subunit (CTB), which is known to be highly antigenic, but a foreign antigen, and its derivative peptide as sensitizing antigens in *in vitro* immunization. The region p60-73 of the CTB molecule (^{60}SQKKAIERMKDTLR^{73}; pCTB) forms an α-helix and is exposed to the surface area. Further, anti-CTB

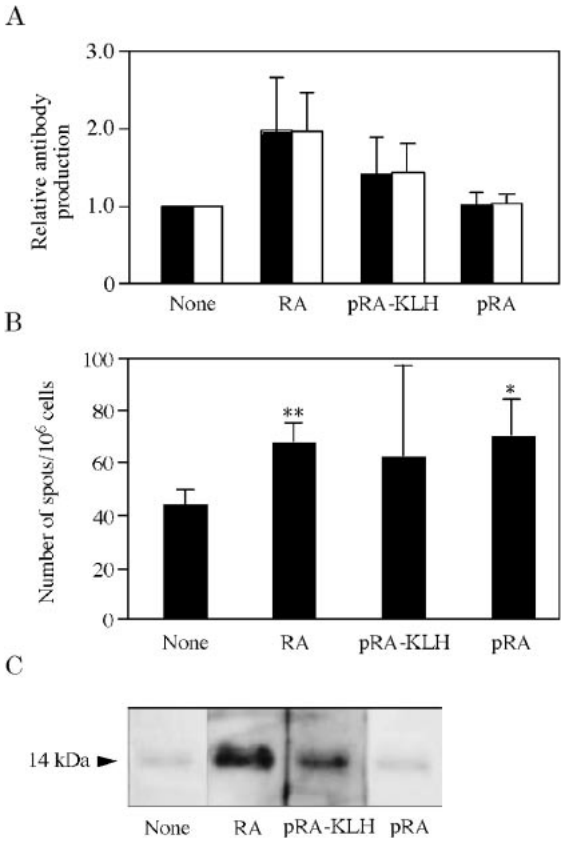

Fig. 1. pRA-Specific Immune Responses Elicited by *in Vitro* Immunization.

PBMCs were *in vitro* immunized with three types of RA antigens (RA, pRA-KLH, and pRA; 10 μg·μl^{-1}) in the presence of IL-2 (10 units·ml^{-1}), IL-4 (1 ng·ml^{-1}), and MDP (10 μg·μl^{-1}). After 8 d of culture, the amounts of IgM (filled bars) and IgG (open bars) in the supernatants were measured by ELISA (A). After 6 d of culture, B cells secreting RA protein-specific IgM in *in vitro* immunized PBMCs were evaluated by ELISPOT assay (B). Immuno-reactivity of antibodies secreted from *in vitro* immunized PBMCs against RA protein was evaluated by Western blot analysis (C).

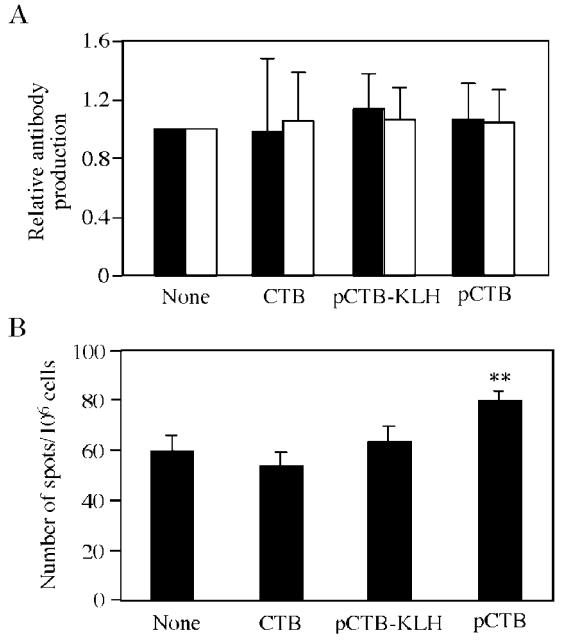

Fig. 2. pCTB-Specific Immune Responses Elicited by *in Vitro* Immunization.

PBMCs were *in vitro* immunized with three types of CTB antigens (CTB, pCTB-KLH, and pCTB; 10 μg·μl^{-1}) in the presence of IL-2 (10 units·ml^{-1}), IL-4 (1 ng·ml^{-1}), and MDP (10 μg·μl^{-1}). After 8 d of culture, the amounts of IgM (filled bars) and IgG (open bars) in the supernatants were measured by ELISA (A). After 6 d of culture, B cells secreting CTB protein-specific IgM in *in vitro* immunized PBMCs were evaluated by ELISPOT assay (B).

antibody specific for this region has neutralizing activity.[12] Hence, we used this peptide region of CTB (pCTB), KLH-conjugated pCTB (pCTB-KLH), and CTB protein (CTB) as sensitizing antigens in *in vitro* immunization. We did not observe significant changes in antibody production in PBMC sensitized with these antigens (Fig. 2A), but the number of cells producing antigen-specific antibodies was significantly augmented in PBMCs sensitized with pCTB, as evidenced by ELISPOT assay (Fig. 2B). Similar results were obtained using PBMCs from different donors (data not shown). These results indicate that *in vitro* immunization with peptide antigen does not always augment antibody production in PBMCs, while it elicits antigen-specific immune responses. Further, they indicate that peptides derived from a foreign antigen as well as protein can induce antigen-specific immune response in PBMCs by *in vitro* immunization, and further suggest that peptide fragments might induce antigen-specific immune responses in PBMCs by *in vitro* immunization, although its originating whole protein does not induce them.

pTNF-α specific immune response elicited by in vitro *immunization*

Since TNF-α is an inflammatory cytokine with varied functions,[13,14] and is thought to perturb immune responses elicited in PBMCs by *in vitro* immunization, TNF-α protein is not suitable as a sensitizing antigen *in vitro* immunization. Indeed, TNF-α protein neither augments antibody production, nor increases the number of cells producing TNF-α-specific antibodies by *in vitro* immunization (Fig. 3A–D). Hence, we tried to immunize PBMCs with TNF-α-derived peptides. We synthesized two types of TNF-α-derived peptides: p1-14 (^{1}VRSSSRTPSDKPVA^{14}, pTNF-α1), which is a relatively hydrophilic peptide,[15] and p84-95 (^{84}AVSYQTKVNLLS^{95}, pTNF-α2), which is a relatively hydrophobic peptide. The region p84-95 has been reported to be involved in binding to TNF-α receptors on the cell surface.[16]

As shown in Fig. 3B, pTNF-α1 significantly increased the number of cells producing TNF-α-specific antibody, suggesting that it is more effective as a sensitizing antigen than its conjugated form or TNF-α protein (Fig. 3A and B). Similar results were obtained using PBMCs from different donors, although the cytokine

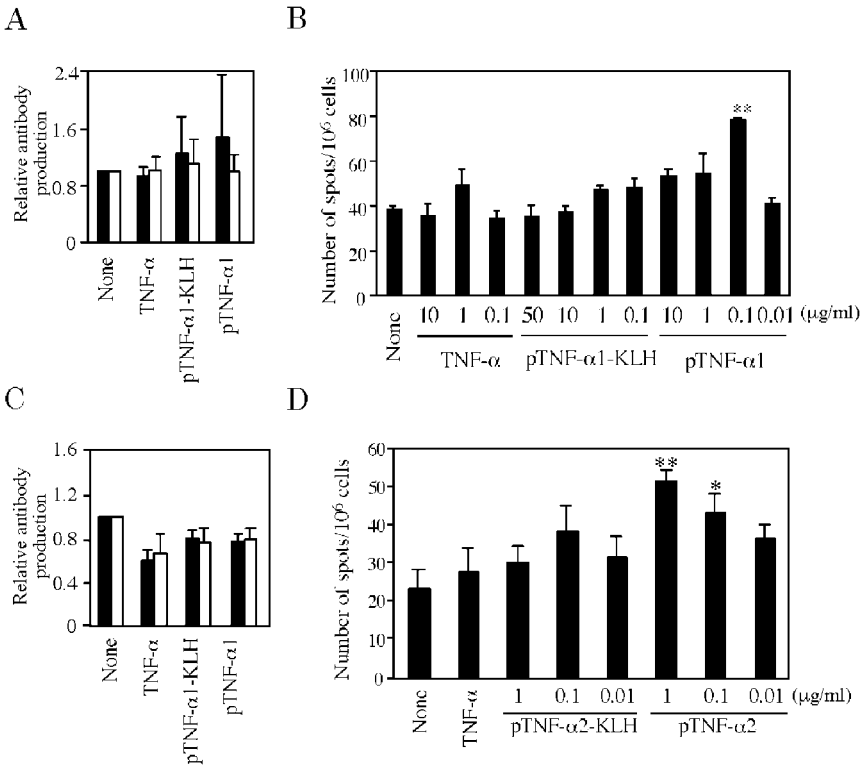

Fig. 3. pTNF-α-Specific Immune Responses Elicited by *in Vitro* Immunization.

PBMCs were *in vitro* immunized with three types of TNF-α antigens (TNF-α, pTNF-α1, and pTNF-α1-KLH) in the presence of IL-2 (10 units·ml^{-1}), IL-4 (10 ng·ml^{-1}), and MDP (10 μg·μl^{-1}). After 8 d of culture, the amounts of IgM (filled bars) and IgG (open bars) in the supernatants were measured by ELISA (A). After 6 d of culture, B cells secreting pTNF-α protein-specific IgM in *in vitro* immunized PBMCs stimulated with 0.1 to 10 μg·μl^{-1} of each antigen were evaluated by ELISPOT assay (B). PBMCs were *in vitro* immunized with three types of TNF-α antigens (TNF-α, pTNF-α2, and pTNF-α2-KLH) in the presence of IL-2 (10 units·ml^{-1}), IL-4 (10 ng·ml^{-1}), and MDP (10 μg·μl^{-1}). After 8 d of culture, the amounts of IgM (filled bars) and IgG (open bars) in the supernatants were measured by ELISA (C). After 6 d of culture, B cells secreting pTNF-α protein-specific IgM in *in vitro* immunized PBMCs stimulated with 0.1 to 10 μg·μl^{-1} of each antigen were evaluated by ELISPOT assay (D).

conditions feasible for inducing antigen-specific antibody production varies between them (data not shown). These results suggest that the antigen-specific immune response can be elicited by peptide forms of antigens that might perturb the in *in vitro* immunization condition. Next we tested for ability to induce antigen-specific immune responses of another hydrophobic TNF-α peptide, corresponding to the region involved in binding to the TNF-α receptor. As shown in Fig. 3D, TNF-α2 peptide rather than its conjugated form elicited antigen-specific antibody production by *in vitro* immunization. These results indicate that peptide forms of antigens can be used as sensitizing antigens in *in vitro* immunization to induce antigen-specific antibody production.

Discussion

We have reported that the B cell epitopes for RA-specific human mAb generated by *in vitro* immunization and subsequent immortalization were p45-55 (^{45}QVWQDCCRQ^{54}L), p56-67 (^{56}AVDDGWCRCGA^{67}L), and p91-98 (^{91}FPGCRRG^{98}D). These regions are thought to be highly antigenic, but are hard to use in sensitizing antigens in *in vitro* immunization because of their cysteine rich structure.[17] Hence, we designed and used an another peptide region (p35-45; ^{35}VGRGASAADEQ^{45}) as a sensitizing antigen, one that contains hydrophilic amino acids. As shown by the results, pRA elicited antigen-specific antibody production from PBMCs by *in vitro* immunization. PBMCs from a healthy donor were thought to be tolerized against RA antigens, but produced antigen-specific antibodies upon sensitization with RA peptide, suggesting that tolerized B cells or naïve B cells were sensitized with RA antigen by *in vitro* immunization. These results suggest that varied antigens, including food antigens and self-antigens, can be used as sensitizing antigens in *in vitro* immunization. Further, pCTB elicited antigen-specific antibody production by *in vitro* immunization, indicating that naïve B cells as well as memory B cells were sensitized by antigens and expanded by *in vitro* immunization. These results suggest that peptides derived from foreign antigens can also elicit antigen-specific antibody production in *in vitro* immunization. In addition, peptides derived from TNF-α, but not TNF-α protein, induced antigen-specific antibody production by *in vitro* immunization, suggesting that TNF-α protein

perturbed the cellular milieu of *in vitro* immunization, and that its peptide form functioned as sensitizing antigen without perturbing the milieu. Further, both TNF-α-derived peptides (pTNF-α1 and pTNF-α2) induced antigen-specific antibody production irrespective of their primary structure and their presumed secondary structure, and their hydrophobicity. These results suggest that anti-cytokine antibodies can be obtained by using their derivative peptides in *in vitro* immunization, which opens up the possibility of *in vitro* immunization.

Although *in vitro* immunization does not always augment antibody production in PBMCs, it reproducibly elicits antigen-specific immune responses, which are thought to be important for generating antigen-specific antibodies. We succeeded in obtaining high affinity human monoclonal antibodies specific for mite allergens by *in vitro* immunization ($K_d > 10^8$ M, paper in preparation). Furthermore, the present study suggests that B cells producing antibodies that recognize specific regions of a protein, that are specific for toxic antigens, and that work against cytokines which might perturb cellular milieu of *in vitro* culture, can be increased by *in vitro* immunization using peptide forms of the antigens. All these results increase the usefulness of the *in vitro* immunization protocol in generating high affinity antibodies against a desired antigen.

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