***In vitro* immunization of human peripheral blood lymphocytes: establishment of B cell lines secreting IgM specific for cholera toxin B subunit from lymphocytes stimulated with IL-2 and IL-4**

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Abstract

In vitro immunization (IVI) techniques have a great potential in the production of human monoclonal antibodies (MAbs) against various antigens. An IVI method of human peripheral blood lymphocytes (PBL) has been developed with a human lung adenocarcinoma cell line in our laboratory. Although several cancer specific human MAbs were successfully generated by using this IVI method, it was not available for soluble antigens, which prompted us to improve the method for generation of human MAbs against soluble antigens. IVI with soluble antigens was effectively caused by the addition of muramyl dipeptides, interleukin-2 and interleukin-4. It was found that the difference of sensitivity of lymphocytes depending upon donors could be overcome by finding the optimal concentrations of IL-2 and IL-4. IVI of human PBL was performed with cholera toxin B subunit (CTB) and the immunized B cells were transformed by Epstein-Barr virus. Anti-CTB antibody was detected using an indirect ELISA. B cells producing anti-CTB antibodies were directly cloned by a soft agar cloning method.

Introduction

Human monoclonal antibodies (MAbs) are useful for diagnosis and treatment of various diseases. A number of murine MAbs have been applied for medical uses such as *in vitro* diagnosis. However, murine MAbs have some serious disadvantages, i.e. the rapid plasma disappearance and development of human anti-murine antibody response (Schroeder et al., 1990), for administration to human body. Because human MAbs do not basically have the antigenicity in human bodies, they can be directly injected in the body without harmful effects. Thus, for administration of MAbs into the human body, human MAbs are believed most suitable for clinical use.

Although human MAbs are anticipated to prove promising in the future in clinical use, passive immunization, such as injection of dangerous antigens

in human bodies, should not be permitted for ethical and moral reasons. One of the methods to solve this problem is establishment of efficient *in vitro* immunization methods in which primary B cells are activated to produce antigen-specific B lymphocytes (Koda and Glassy, 1990). Recently, a number of human MAbs have been produced against a variety of antigen using *in vitro* immunized lymphocytes (Power et al., 1990; Hoffmann et al., 1990; Gupta et al., 1992; Alexander et al., 1994). However, poor re-productibility was an problem in conventional *in vitro* immunization methods.

Peripheral blood generally contains few antigen-reactive specific B cells (James et al., 1987; Glassy, 1993) and circulating B cells are normally arrested in a phase in which additional stimuli are required to induce antigen-specific clonal activation (Borrebaeck, 1989). In a previous paper, we reported the establish-

ment of a new *in vitro* immunization method against cultured lung adenocarcinoma cell line (Kawahara et al., 1992) and succeeded in generating several cancer specific human MABs (Shoji et al., 1994a; Shoji et al., 1994b). However, this method could not be available for immunization against soluble antigens. The purpose of this paper is to improve this *in vitro* immunization method for immunization against soluble antigens and to generate human MABs against cholera toxin B subunit as an example of soluble antigens by the new *in vitro* immunization method. We attempted to alter the culture condition, especially combination and concentration of cytokines added to the culture of lymphocytes.

Materials and methods

Antigens and reagents

Keyhole limpet hemocyanin (KLH), ovalbumin and cholera toxin B were obtained from Calbiochem-Novabiochem Corporation (La Jolla, CA, U.S.A.), Chemicon international Inc. (Temecula, CA, U.S.A.) and List Biological Laboratories, Inc. (Campbell, CA, U.S.A.), respectively. Recombinant human IL-2, IL-6 and IL-10 were purchased from Genzyme corporation (Cambridge, MA, U.S.A.). Recombinant human IL-4 and recombinant IL-5 were obtained from Pepro Tech EC LTD. (London, England) and R&D systems (Minneapolis, MN, U.S.A.), respectively. Muramyl dipeptide (MDP) was purchased from Chemicon international Inc. (Temecula, CA, U.S.A.). L-Leucyl-L-leucin methyl ester was obtained by Boehringer GmbH (Mannheim, Germany).

Isolation of human lymphocytes

Human peripheral blood lymphocytes were separated by density-gradient centrifugation from several normal donors. In brief, 25 ml of peripheral blood was layered on 20 ml of lymphocyte separation medium (LSM; Organon Teknika, Durham, NC, U.S.A.) and was centrifuged at $400 \times g$ for 30 min. The stratum of lymphocytes were harvested and washed three times with ERDF medium. Lymphocytes from peripheral blood was treated with 0.25 mM Leu-Leu-OMe to remove the cytotoxic T cells, CD8⁺ suppressor T cells, and natural killer cells before use.

In vitro immunization

In vitro immunization of human PBL was performed in 24 well flat bottom culture plates (Becton Dickinson). The Leu-Leu-OMe treated PBL were cultured for 7 days in ERDF medium containing 10% heat inactivated fetal bovine serum (FBS), MDP (10 $\mu\text{g/ml}$), IL-2 (10 units/ml), IL-4 (10 ng/ml), 2-mercaptoethanol (20 μM) and antigens.

Enzyme-linked immunosorbent assay

Ninety-six well microtiter plates were coated with CTB or BSA in 0.1 M Na-carbonate buffer (pH 9.6) at concentration of 0.2 $\mu\text{g/well}$ and incubated at 48 °C overnight. The plate were washed three times with PBS containing 0.05% Tween 20 (PBS-T), the supernatants of EBV transformed B cells were diluted 1:2 in PBS-T, added in 50 μl aliquots per well, and incubated at 48 °C overnight. The well were washed three times with PBS-T and incubated for 2 h at 37 °C with 100 $\mu\text{l/well}$ horseradish peroxidase-conjugated goat antibodies against human IgM. After washing three times with PBS-T, 100 $\mu\text{l/well}$ of substrate solution (0.1 M citrate buffer (pH 4.0) containing 0.003% H₂O₂ and 0.3 mg/ml p-2,2'-azino-di (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) were added, and after 20 min, the absorbance at 405 nm was measured by using an ELISA reader.

EBV transformation

After *in vitro* immunization, human B cells were Epstein-Barr virus (EBV) infected using supernatants from the B95-8 marmoset cell line (ATCC number: CRL-1612) cultured in ERDF medium supplemented with 10% FBS. EBV-transformed cells were washed with ERDF medium containing 20% FBS and seeded into 96 well culture plates at 1×10^5 cells/well.

Cloning

EBV-B cells producing antibodies reactive to CTB were cloned by limiting dilution at density of 1 cell/well in 96 well culture plates or by soft agarose colony forming method. The supernatants from the positive wells were screened by ELISA.

Table 1. Effects of recombinant human cytokines on *in vitro* immunization with soluble antigens

Cytokines ^a	Increase of Ig production ^b		Cell cluster formation ^c	Reactivity to KLH ^d
	IgG	IgM		
IL-2	3.2	18.3	++	+
IL-2, IL-4	3.2	34.7	+++	+++
IL-2, IL-5	2.9	9.9	+	–
IL-2, IL-6	3.3	15.6	+	–
IL-2, IL-10	3.5	3.5	–	–
IL-2, IL-4, IL-6	5.0	33.8	+++	++
IL-2, IL-5, IL-6	4.0	14.6	+	+
IL-2, IL-6, IL-10	4.0	3.9	–	–
IL-2, IL-4, IL-5, IL-6, IL-10	3.9	4.6	+	+
none	4.4	10.2	++	+

^a Recombinant human cytokines were added to the culture medium at following final concentrations: IL-2 (10 units/ml), IL-4 (10 ng/ml), IL-5 (5 ng/ml), IL-6 (10 units/ml), IL-10 (10 ng/ml).

^b Values of increase of Ig production were defined by the following formula: Ig conc. of day 7/Ig conc. of day 4.

^c Degree of cell cluster formation were expressed as follows: negative (–), weakly positive (+), positive (++), and strongly positive (+++).

^d Strength of reactivities of culture supernatants were expressed as follows: the absorbance at 405 nm was below 0.2 (–), 0.2–0.4 (+), 0.4–0.6 (++), and over 0.6 (+++).

Results

Establishment of protocol for in vitro immunization with soluble antigens

We examined the effect of various recombinant human cytokines which are concerned with the proliferation and differentiation of B lymphocytes. Peripheral blood lymphocytes (PBL) derived from healthy donors were incubated with various combinations of IL-2, IL-4, IL-5, IL-6 and IL-10 in the presence of KLH, as an antigen and MDP for 7 days. Table 1 summarizes the effects of various cytokines on *in vitro* immunization against the soluble antigen. It was found that IgM production and cell proliferation were greatly increased in the combination of IL-2 and IL-4. Similarly, we could observe high production of IgM reactive to KLH. These results indicated that the protocol using the combination of IL-2 and IL-4 was the most effective for *in vitro* immunization against soluble antigens.

We established the new protocol of *in vitro* immunization for soluble antigens. Figure 1 shows a scheme of the new protocol. Major difference of the protocol for soluble antigens from that for cancer cell antigens was the use of IL-4 instead of IL-6 in the culture medium for *in vitro* immunization of lymphocytes.

In vitro immunization with cholera toxin B subunit

To demonstrate the effectiveness of the new protocol, we performed *in vitro* immunization of human PBL with cholera toxin B subunit (CTB) as an example of soluble antigens. Figure 2 shows reactivity of antibodies produced after *in vitro* immunization of PBL with CTB. We examined the effects of several combinations of IL-2, IL-4 and IL-6 on *in vitro* immunization with CTB. Although there were not much differences on immunoglobulin secretions among every combination, the combination of IL-2 and IL-4 was most effective on the level of CTB-specific antibodies. Exogenous addition of IL-6, which was effective for *in vitro* immunization with cultured cell antigen, had no effect on immunization with soluble antigens.

Individual differences of human lymphocytes on sensitivity on in vitro immunization

All examinations mentioned above were carried out using PBL derived from single donor. We examined whether this protocol was also effective for lymphocytes derived from different donors or not. Figure 3 shows results of *in vitro* immunization of lymphocytes derived from three different donors. We could detect

Figure 1. Protocol of *in vitro* immunization for soluble antigens.

Figure 2. Induction of specific antibodies by *in vitro* immunization of human peripheral blood lymphocytes with cholera toxin B subunit (CTB). The Leu-Leu-OMe treated human peripheral blood lymphocytes were inoculated at 1×10^6 cells/ml of viable cells into 24 well flat bottom culture plates with 10% FBS-ERDF medium supplemented with muramyl dipeptide (MDP), several combinations of cytokines and CTB ($10 \mu\text{g/ml}$), and incubated at 37°C in an atmosphere of 5% CO_2 .

Table 2. Optimum concentrations of IL-2 and IL-4

Donor	Antigen ^a	Optimum conc.	
		IL-2	IL-4
A	KLH, CTB, OVA	10 (units/ml)	10 (ng/ml)
B	KLH, CTB, OVA	50	1
C	CTB	N.D. ^b	N.D.
D	KLH, OVA	10	0

^a KLH = keyhole limpet hemocyanin, CTB; cholera toxin B subunit, OVA; ovalbumin.

^b N.D. = not determined.

the increase of specific antigen-antibody reaction on lymphocytes derived from donor A, but not on donor B and C. This result suggested a possibility that each lymphocytes derived from different donors demanded their own optimum concentrations of IL-2 and IL-4.

Then we tried to optimize the concentrations of IL-2 and IL-4 on *in vitro* immunization of lymphocytes derived from different donors. Table 2 shows the optimum concentrations of IL-2 and IL-4 on *in vitro* immunization of lymphocytes derived from different donors. It was found that each lymphocytes from different donors demanded their own optimum concentrations of IL-2 and IL-4. For this reason, if

we can find optimum concentrations of IL-2 and IL-4 for each lymphocytes beforehand, the effective immunization will be caused by this protocol using the combination of IL-2 and IL-4.

Acquisition of EBV-B cell lines secreting CTB-specific human MAbs

To immortalize the B lymphocytes after *in vitro* immunization with CTB, we adopted the Epstein-Barr virus (EBV) transformation. The results of screening of EBV-B lymphocytes supernatants in ELISA were shown in Figure 4. Several EBV-B cells secreted antibodies strongly reacting with CTB. But most supernatants also reacted to BSA as a control. Lymphocytes cultured in the well named no. 13C among all wells had the highest specificity to CTB. Positive EBV-B cells in the 13C well were amplified and cloned by the soft agar cloning method. We could obtain 4 clones secreting IgM antibodies reactive to CTB among 280 clones picked up. However, only one clone, 13C/C7, had a significant specificity to CTB (Figure 5).

Figure 3. Individual differences of lymphocytes in *in vitro* immunization. The Leu-Leu-OMe treated human PBL from three different donors (A, B and C) were immunized with CTB in 10% FBS-ERDF medium supplemented with MDP (10 μ g/ml), IL-2 (10 units/ml) and IL-4 (10 ng/ml). After 7 days culture, supernatants were subjected to ELISA for measurement of reactivities against CTB.

Discussion

As an alternative method of *in vivo* immunization methods, *in vitro* immunization methods have been developed. *In vitro* immunization is based on antigen-specific activation of B lymphocytes in primary culture, and these cells are subsequently transformed with EBV for the immortalization of the antigen-specific B cells. Human monoclonal antibodies specific for several different peptides, proteins and several tumor cells have been produced by the *in vitro* immunization technique. However, poor re-productibility was an major obstacle in conventional *in vitro* immunization methods. We developed an *in vitro* immunization method against cultured tumor cells before, however, this method could not be available for immunization against soluble antigens. The goal of *in vitro* immunization is to induce the proliferation and differentiation of antigen-specific B cells in culture. In this study, we attempted to improve the *in vitro* immunization method by finding the best combination of cytokines permitting *in vitro* stimulation and clonal expansion of activated cells for immunization against soluble

antigens, e.g. keyhole limpet hemocyanin (KLH) or cholera toxin B subunit (CTB).

Antibody production to T cell dependent antigens requires a collaborative interaction between B cells, helper T cells and antigen presenting cells (APCs). B cells which secrete a specific antibody are highly efficient APCs. Optimal antigen presentation and antigen-specific B cell activation require highly enriched B and helper T cell population. We eliminated the cytotoxic T cells, CD8⁺ suppressor T cells, and natural killer cells by treatment of PBL with Leu-Leu-OMe. Production of antigen-specific antibodies was hardly detected when we carried out *in vitro* immunizations without this treatment (data not shown).

We examined various combinations and concentrations of human recombinant cytokines concerned with the proliferation and differentiation of B cells. The combination of IL-2 and IL-4 was the most effective for immunization with KLH among all combinations examined. It appeared that IL-4 was essential to the antigen specific B cell induction. Several investigators have reported its ability to both proliferate and differentiate activated B cells (Defrance et al., 1987;

Figure 4. Antigen-specific reactivities of immunoglobulin secreted by EBV-transformed B lymphocytes after *in vitro* immunization with CTB. The culture supernatant of eleven established EBV-transformed B cell lines were examined on the reactivity against CTB and BSA by ELISA.

Defrance et al., 1988; Paul et al., 1987; Yokota et al., 1988). IL-4, formerly B cell growth factor (BCGF), which acts only activated B cells at an early stage of the response (Sahasrabudde et al., 1988), may prepare B cells to acquire the responsiveness to IL-2 and possibly to other interleukins (Boyd et al., 1985). IL-2 is a T cell growth factor, which may be an essential factor for the expansion of the pool of helper T cell responding to the antigen. Jelinek and Lipsky have previously reported a markedly enhanced and sustained proliferation of activated B cells when cultures contained a combination of IL-2 and BCGF (Jelinek and Lipsky, 1987). Moreover, Laroche-Traineau et al. reported the protocol using BCGF, IL-2 and mixed lymphocyte culture supernatant (MLCS), was proved to be the best among the one-step *in vitro* immunization procedures (Laroche-Traineau et al., 1994). In their protocol, the effect of the combination of IL-2 and IL-4 (BCGF) was not able to be evaluated correctly since MLCS contained many unknown factors. Our results agree with these conclusions and indic-

ate that IL-2 and IL-4 synergistically induce antigen-specific B cells in the *in vitro* immunization of PBL with soluble antigens.

Exogenous addition of IL-6, which was effective for *in vitro* immunization with such an antigen as cultured cells as described by Kawahara et al., had no effect on immunization with soluble antigens. Sufficient IL-6 production from lymphocytes was observed in the culture supernatant after *in vitro* immunization without IL-6 (data not shown). This result suggested that Th2 type helper T cells were effectively activated by soluble antigens during *in vitro* immunization supplemented with IL-2 and IL-4. It was also suggested that IL-6 was necessary for the terminal differentiation of B cells, however, exogenous addition of IL-6 to *in vitro* immunization was unnecessary.

Anti-CTB antibodies generated by this *in vitro* immunization method were all IgM isotype. IgG type is favorable for the clinical purpose, however, we have not succeeded to generate the antigen-specific IgG with the *in vitro* immunization method. However, we

Figure 5. CTB-specific reactivity of monoclonal antibody from 13C/C7 clone established after soft agar cloning. An CTB-specific B cell clone, 13C/C7 was established by cloning by soft agar cloning method from the anti-CTB antibodies producing EBV-transformed B cell line, 13C. CTB-specific reactivity of monoclonal antibody from 13C/C7 clone was examined by ELISA.

observed IgE production was significantly increased in long term culture (data not shown). IL-4 plays an important role in the isotype switching to IgE, on the other hand interferon- γ (IFN- γ) is concerned with the isotype switching to IgG. Therefore the addition of IFN- γ may be effective for acquisition of antigen specific IgG.

To expand antigen-specific B cells immunized *in vitro*, we adopted an EBV immortalization method because of low fusion efficiency of human fusion partner cells. EBV transformation was effective to expand small number of B cells immunized with antigen *in vitro*. However, EBV transformed B (EBV-B) cells were genetically unstable and low-antibody secretors stopped to secrete antibodies rapidly with time. We succeeded in cloning several anti-CTB antibody secreting B cell clones by a conventional soft agar cloning method. However, their proliferation and antibody production rates were strikingly decreased because it took almost two months to establish EBV-B cell lines secreting CTB-specific IgM antibodies. Alfonso et al. reported that positive selection of antigen specific antibody producing cells using antigen-coated magnetic beads was effective to rescue unstable clones (Alfonso et al., 1995). Rapid cloning of EBV-B cells secreting specific antibodies after EBV transformation is desir-

able. Culture of cloned EBV-B cells with cytokines, such as IL-6, may help maintain antibody production and prevent the instability. Although the virus production of the EBV-B cell is usually suppressed, EBV genes are retained and virus production may be induced under certain conditions. Therefore, we must keep this in mind when the human antibodies produced by this method.

Isolation of cDNA from the EBV-B cells is one of the available means for securing the purposive immunoglobulin genes. However, cDNA could not be isolated from 13C/C7 clone because sufficient numbers of cells could not be provided.

At present, equine antiserum is used for the treatment of various bacterial infectious diseases. However, there is considerable danger in serotherapy using equine antiserum because xenoantiserum bring about anaphylactic shock or serum sickness. We are expecting that the development of human MAbs against various bacterial endotoxins by this *in vitro* immunization method will cause substitution the human MAbs for equine antiserum in the serotherapy against bacterial infectious diseases.

Conclusions

We have presented that the *in vitro* immunization of human peripheral blood lymphocytes with soluble antigens was effectively caused by the addition of MDP, IL-2 and IL-4. Although the effects of IL-2 and IL-4 were different depending upon donors, the difference could be overcome by finding optimal concentrations of IL-2 and IL-4 for lymphocytes from each donor. The EBV transformed B cell clones secreting CTB-specific monoclonal antibody could be established by the soft agar cloning method. We are quite confident that the *in vitro* immunization method will be a powerful tool for the production of soluble antigen specific human monoclonal antibodies.

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