Short communication

Different individual immune responses elicited by *in vitro* immunization

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Received 29 July 2002; accepted in revised form 27 September 2002

Key words: human antibody, interleukin-2, interleukin-4, *in vitro* immunization

Abstract

We have previously demonstrated that the addition of muramyl dipeptide (MDP), interleukin-2 (IL-2) and IL-4 effectively raises antibody production from L-Leucyl-L-leucine methyl ester (LLME)-treated human peripheral blood lymphocytes (PBLs) against specific soluble antigen when immunized *in vitro*. However, PBLs from individual donors were separate optimal conditions regarding concentrations for IL-2 and IL-4, which in turn required us to optimize each individual PBLs to effectively produce antigen specific human antibody by *in vitro* immunization. These individual differences in the requirement for IL-2 and IL-4 reflects the differences in individual immune responses against a specific soluble antigen, which can be elicited by *in vitro* immunization. In the present study, we investigated these individual differences in the requirement for IL-2 and IL-4 to induce antibody production *in vitro* in the PBLs of 12 volunteers (9 healthy donors and 3 allergenic patients). IL-2 requirements for antibody production varied dependent upon each donor, while higher amounts of IL-4 inhibited IgM and IgG production in all of the healthy donors. However, some of the characteristic features for PBLs donated from allergenic included lowered IgM production compared to PBLs derived from healthy donors, and very high IgE production in the absence of cytokines and allergen. These results demonstrate that the sensitivity of PBLs against antigen sensitization differs between healthy donors and atopic patients, which suggests that the frequency of antigen sensitization might be reflected in differing activation states and/or differing subpopulations of lymphocytes *in vivo*.

Abbreviations: APC, antigen presenting cell; DC, dendritic cell; IL-2, interleukin-2; IL-4, interleukin-4; LLME, L-Leucyl-L-leucine methyl ester; MDP, muramyl dipeptide; PBLs, peripheral blood lymphocytes.

Introduction

Antibodies have great potential for use in the treatment of cancer, autoimmune disorders and viral or bacterial infections. A number of murine or humanized monoclonal antibodies (mAbs) have been tested for clinical use, and have been met with limited success. MAbs from mouse origin are relatively easy to produce, however, the therapeutic value is restricted by the immunogenicity in humans when the human anti-mouse antibody (HAMA) response occurs (Ho-

zumi et al., 1993). Therefore, mAbs from human origin are thought to be best suited for clinical use, but techniques to produce human mAb have not yet been well established. One method to produce human mAb is the *in vitro* immunization technique, in which antigen-specific B lymphocytes taken from peripheral blood are activated by a specific antigen, and are induced to produce antigen specific mAb (Bird et al., 1981; James et al., 1987). We have previously improved this technique and demonstrated that muramyl dipeptide (MDP), interleukin-2 (IL-2) and IL-4 were

Table 1. Healthy donors and allergenic patients

No.	Age	Sex	Antibody level in sera			
			IgM (mg ml ⁻¹)	IgG (mg ml ⁻¹)	IgE (mg ml ⁻¹)	Allergic symptom to rice
1	23	Male	ND ^a	ND	ND	No
2	23	Male	0.55	4.61	0.24	No
3	24	Female	ND	ND	ND	No
4	31	Male	0.59	6.19	0.52	No
5	23	Female	1.34	3.28	3.66	No
6	24	Female	ND	ND	ND	No
7	28	Female	1.44	4.50	0.26	No
8	26	Male	0.68	4.07	0.36	No
9	34	Male	0.52	4.35	0.22	No
10	23	Male	ND	ND	ND	No
11	31	Male	0.09	2.13	69.66	No
12	24	Male	0.18	2.58	14.42	No

^a ND = Not determined.

effective as additives for inducing the production of antigen-specific antibody from human PBLs *in vitro* (Ichikawa et al., 1999). In the course of our study, we found that the ratios and amounts of IL-2 and IL-4 are important factors for inducing antibody production from B cells. Especially, the amounts of IL-2 and IL-4 required to induce antibody production were found to be varied depending upon individuals, suggesting that this *in vitro* technique can be applied to analyze the individual differences in the immune responses. Here we evaluated the possibility of utilizing the *in vitro* immunization technique to analyze the sensitivity of lymphocytes against antigen sensitization and/or to anticipate the activation state or population of lymphocytes *in vivo*.

Materials and methods

Antigen and reagents

Rice allergen was prepared according to the method described by Dr. T. Matsuda et al. (Matsuda et al., 1988, 1991). Recombinant human IL-2 was purchased from Genzyme (Cambridge, MA). Recombinant human IL-4 was purchased from Pepro Tech (London, U.K.). MDP was purchased from Chemicon (Temecula, CA). LLME was obtained from BACHEM (Torrance, CA).

Isolation of human lymphocytes

Human PBLs were separated by density-gradient centrifugation using lymphocyte separation medium (LSM; Organon Teknika, Durham, NC). In brief, 25 ml of peripheral blood was layered onto 15 ml of LSM and centrifuged at 400 × g for 30 min at room temperature. Lymphocytes were harvested and washed three times with ERDF medium (KYOKUTO Pharmaceutical, Tokyo, Japan). Lymphocytes were preincubated in ERDF medium supplemented with 0.25 mM LLME for 20 min at room temperature. These cells were washed three times in culture medium and were utilized in *in vitro* immunization.

Experiment throughout this study was carried out in accordance with the principles of the Declaration of Helsinki.

In vitro immunization

In vitro immunization of human PBLs was performed in 24 well culture plates (Becton Dickinson Labware, Franklin Lake, NJ). The LLME-treated PBLs were cultured for 8 days in ERDF medium supplemented with 10% heat inactivated fetal bovine serum, IL-2 (0, 1, 10 or 50 units ml⁻¹), IL-4 (0, 1, 10 or 50 ng ml⁻¹), MDP (10 µg ml⁻¹), 2-mercaptoethanol (50 µM) and rice allergen (10 µg ml⁻¹).

Table 2. Culture conditions for *in vitro* immunization

Sample No.	IL-2 (U ml ⁻¹)	IL-4 (ng ml ⁻¹)	MDP (mg ml ⁻¹)	2-ME (mM)	Allergen (mg ml ⁻¹)
1	0	0	10	50	10
2		1			
3		10			
4		50			
5	1	0	10	50	10
6		1			
7		10			
8		50			
9	10	0	10	50	10
10		10			
11		10			
12		50			
13	50	0	10	50	10
14		1			
15		10			
16		50			
17	0	0	0	0	0

Enzyme-linked immunosorbent assay (ELISA)

Ninety-six well microtiter plates (Nunc, Naperville, IL) were coated with 100 μ l of 1000-fold diluted anti-human IgM (TAGO, Burlingame, CA), IgG (TAGO) or IgE antibodies (TAGO) in 0.1 M sodium carbonate buffer (pH 9.6) and incubated at 4 °C overnight. After the plates were washed three times with 2.24×10^{-2} M phosphate buffer containing 1.37×10^{-1} M NaCl and 0.05% Tween 20 (TPBS), 50 μ l aliquots of serially diluted supernatants of *in vitro* immunized PBLs were added and incubated at 4 °C overnight. The wells were washed three times with TPBS and 100 μ l of 2000-fold diluted horseradish peroxidase-conjugated goat antibodies against human IgM (TAGO), IgG (TAGO) or IgE (TAGO) were added, and subsequently incubated for 2 h at 37 °C. After washing three times with TPBS, 100 μ l of substrate solution (0.1 M citrate buffer (pH 4.0) containing 0.003% H₂O₂ and 0.3 mg ml⁻¹ 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; WAKO, Osaka, Japan) was added, and incubated for 20 min.

Absorbance at 405 nm was measured using an ELISA reader.

When examining RA-specific antibody production, microtiter plates coated with 1 μ g ml⁻¹ RA were incubated with serially diluted supernatants of *in vitro* immunized PBLs, and subsequently with appropriately diluted horseradish peroxidase-conjugated goat antibodies against human IgM, IgG or IgE. Color development reaction was carried out as above.

Results and discussion

Differences in individual immune response evaluated by in vitro immunization

Human PBLs isolated from 9 healthy donors (Table 1, Nos. 1 to 9) were separately immunized *in vitro*. In *in vitro* immunization, the antibody productions were induced only in the presence of antigen (data not shown) and the number of CD19⁺ CD27⁻ naïve B cells decreased by stimulation with antigen (data not shown). These results indicated that antibodies were produced via not only activation of memory B cells

Figure 1. Antibody production from *in vitro* immunized PBLs. PBLs isolated from healthy donors and allergic patients were immunized *in vitro* using rice allergen (RA) in the presence of IL-2 (1, 10, 50 U ml⁻¹), IL-4 (1, 10, 50 ng ml⁻¹), MDP (10 µg ml⁻¹) and 2-ME (50 µM). After 8 days of culture, the amount of IgM, IgG and IgE and Ra-specific IgM, IgG and IgE secreted into the culture supernatants was measured by ELISA. The x-axis show the culture condition for the *in vitro* immunization (refer to Table 2). PBLs derived from donors were grouped into five groups of similar expression patterns (A to E). Figure 1 shows a representative example from each group.

but also immunization of naïve B cells in *in vitro* immunization, and suggest that this technique may be applicable for analysis of the immune responses *in vitro*. Then we tried to evaluate the effect of varying concentrations of IL-2 and IL-4 using this technique. The amounts of IL-2 and IL-4 added to the LLME-treated PBLs are shown in Table 2. After 8 days of culture, amounts of IgM, IgG and IgE secreted into the culture supernatants were measured by sandwich ELISA. Production profiles for each immunoglobulins were roughly divided into 3 groups (Figures 1A–C). First, we tested for the requirements of IL-2 on the

induction of antibody production by *in vitro* immunization. The PBLs donated from the individuals in group A showed no dependency to IL-2 in regards to antibody production (Figure 1A). PBLs donated from the remaining healthy individuals (group B and C) demonstrated varied responses to IL-2. PBLs from the 4 healthy donors in group B showed an IL-2 dependent antibody production increase (Figure 1B), but cells from the other two healthy donors in group C demonstrated an IL-2 dependent antibody production decrease (Figure 1C). These results suggest that these different antibody production patterns in response to

IL-2 after *in vitro* immunization might reflect a different lymphocyte activation state *in vivo* for each individual donor.

Next we evaluated the IL-4 requirements for inducing antibody production. IL-4 was shown to inhibit IgM and IgG production, but to promote the IgE production in a dose-dependent manner in all cells derived from healthy donors (Figures 1A–C). This strongly supports reports suggesting that higher concentrations of IL-4 is able to induce switching to the IgE isotype in PBLs when immunized *in vitro* (Vercelli et al., 1989). Furthermore, differences in the ratio of IgM and IgG antibodies produced by *in vitro* immunization may be explained by the varying ratio of lymphocyte subset within the total PBLs population of an individual donor such as naïve B cells and memory B cells, or by the differing abilities of B cells in the PBLs of individual donors to be sensitized with antigen or to react to adjuvant and cytokines.

Evaluation of immune responses between healthy donors and allergenic patients by in vitro immunization

After being *in vitro* immunized, the response from the PBLs donated by 3 allergenic patients (Table 1, Nos. 10 to 12) were evaluated. Results show that the production of IgM is significantly lower than healthy donors (Figures 1D and E). This taken together with data demonstrating that the amount of IgM in the sera is significantly lower than the amounts for IgG and IgE in allergenic patients (Table 1), indicates that the number of naïve B cells which are to be subjected to antigen sensitization are low, and further that memory B cells existing in PBLs predominantly proliferate to produce IgG and IgE antibodies in response to adjuvant and cytokines. We could not elucidate the reason why the number of memory B cells producing IgG and IgE is higher in PBLs isolated from allergenic patients, and why naïve B cells in allergenic patients are difficult to sensitized with antigen by *in vitro* immunization, these issues need to be clarified in a future study.

Another possibility is that when naïve B cells in the PBLs from allergenic patients are sensitized with antigen by *in vitro* immunization, they can be rapidly induced to switch subclasses from IgM to IgG or IgE in response to stimulation with adjuvant and cytokines. The data which shows a high IgE content in the sera of allergenic patients can be explained by the ability of B cells to induce class switching upon antigen sens-

itization. The predisposition of these B cells to switch subclasses might explain the pathophysiological state of allergenic patients.

In the present study, we conclude that *in vitro* immunization can be applied to analyze different individual immune responses. This technique may lead to the ability to easily measure the sensitivity of B cells to antigen sensitization which may result in varying activation states or differentiated subpopulations of B cells *in vivo*.

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