

Effective induction and acquisition of human monoclonal IgE antibodies reactive with house-dust mite extracts

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Received 11 February 1999; received in revised form 27 July 1999; accepted 13 September 1999

Abstract

IgE plays a critical role in acute hypersensitivity such as anaphylaxis, asthma, and atopic dermatitis. IgE antibody is, therefore, an essential reagent for studying the mechanisms of these diseases. However, it is difficult to obtain IgE antibody in amounts sufficient for research use because IgE-producing lymphocytes are very rare. To overcome this problem, we investigated the requirements for generating IgE-secreting human hybridomas using in vitro immunization of peripheral blood lymphocytes. First, culture conditions were optimized for IgE production by a combination of the immunomodulatory mediators interleukin-2, interleukin-4, interleukin-6, and muramyl dipeptide. Second, the addition of mite antigen to the cultures resulted in an increased production of antigen-specific IgE as well as antigen-specific IgG and IgM. When activated lymphocytes in these cultures were fused with Burkitt lymphoma cells, ICLU-B, antigen-specific IgE-secreting hybridomas were obtained with high efficiency. These results demonstrate that our culture and in vitro immunization system for human peripheral blood lymphocytes is useful for obtaining antigen-specific IgE. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Human IgE; In vitro immunization; Human hybridoma; Human monoclonal IgE antibody

1. Introduction

It is well known that IgE plays an important role in acute hypersensitivity such as anaphylaxis, asthma, and atopic dermatitis. IgE reacts with the allergen and associates with FcεRI receptors on the surface of mast cells. These mast cells then release inflam-

matory factors such as histamine and leukotrienes. Thus, the IgE antibody triggers allergic symptoms. Antigen-specific IgE antibody is a powerful tool for studying type I allergy. Generally, the concentration of IgE in serum is lower than that of IgM or IgG because IgE-producing lymphocytes are much rarer than IgM- or IgG-producing lymphocytes. Consequently, it is difficult to obtain monoclonal IgE antibodies, even if intact human lymphocytes from peripheral blood are used in cell fusion. To solve this problem, we sought to achieve an effective induction of antigen-specific IgE from normal human PBL.

Recently, the role of various immunoactivators such as the interleukins, adjuvant peptides, and mito-

Abbreviations: FBS, fetal bovine serum; MDP, muramyl dipeptide; Mite-Df, extract from house-dust mite, *Dermatophagoides farinae*; Leu-Leu-O-Me, leucil-leucine-methyl ester

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gens in the expression and enhancement of lymphocyte function has been investigated. IL-4 causes isotype switching of PBL so that IgE production is induced (Snapper et al., 1988) and IL-7 or IL-13 enhance IL-4-induced IgE production (Punnonen et al., 1997; Pascale et al., 1998). The regulation of IgE production mediated by CD40 or CD58 markers has also been reported (Bergman et al., 1996; Kimata et al., 1996), but there are few reports that these activators enhance antigen specific IgE production.

Many investigators have attempted to obtain human monoclonal antibodies reactive with various antigens, for example, bacterial, viral, and tumor antigen (James and Bell, 1987; Mukerjee et al., 1998). Generally, these human monoclonal antibodies have been produced using PBL or lymph node lymphocytes derived from various patients. However, since the number of lymphocytes that can be obtained from patients is limited, it is difficult to obtain monoclonal antibodies reactive with a desired antigen. It is also suspected that such lymphocytes are damaged by various medical treatments. Therefore, in vitro immunization of normal human lymphocytes would be an advantageous method because fresh peripheral blood lymphocytes can be supplied repeatedly.

We attempted to induce antigen-specific IgE antibodies by immunizing normal human lymphocytes in vitro and monoclonal IgE antibodies were obtained by producing human–human hybridomas.

2. Materials and methods

2.1. Cell and cell culture

Lymphocyte separation medium (LSM) was purchased from Organon Teknika (Durham, NC). ERDF basal medium was obtained by Kyokuto Seiyaku (Tokyo, Japan) (Murakami, 1989). Human blood samples were obtained from four healthy donors, and peripheral blood lymphocytes (PBL) were separated from each blood sample with LSM. ICLU-B, a 6-thioguanine-resistant human Burkitt lymphoma, was used as a human fusion partner. The fusion partner, which was recently established in our laboratory, did not produce or secrete any antibodies in the culture supernatant or cytoplasm. ICLU-B was maintained in

ERDF containing 10% fetal bovine serum (FBS; Life Technologies, Rockville, MD). PBL were cultured in 5% FBS–10% human plasma-ERDF medium. Human plasma was separated from the heparinized blood of each original donor. All cells were incubated under humidified 5% CO₂/95% air atmosphere at 37°C.

2.2. Estimation of applicable concentration of various additives

The additives used in this study were recombinant human interleukin-2 (IL-2), IL-4, and IL-6 purchased from Genzyme (Cambridge, MA). Muramyl dipeptide (MDP) was obtained from Chemicon International (Temecula, CA). In brief, 1×10^6 cells/ml of PBL were cultured in 5% FBS–10% human plasma-ERDF medium containing each of the additives at a dose from 1 U/ml to 200 U/ml. After 10 days, the concentration of total Ig (IgE + IgM + IgG) in the supernatant was measured by ELISA. The dose that produced the highest concentration of the antibodies was defined as the optimal concentration of each additive. The concentration of MDP to be added to the medium was determined in the same way.

2.3. Activation of PBL for production of antigen specific IgE

An extract of house-dust mite, *Dermatophagoides farinae* (Mite-Df), was used as an antigen and was purchased from LSL, (Tokyo, Japan). Leucileucine-methyl ester (Leu-Leu-O-Me) was obtained from Bachem (Bubendorf, Switzerland). 1×10^6 PBL cells/ml were suspended with 5% FBS–10% human plasma-ERDF medium containing various combinations of MDP with interleukins and 10 µg/ml Mite-Df. The cell suspension was inoculated into a 24-well culture plate and gently placed in an incubator. After 10 days in culture, the concentration and reactivity of IgE, IgM, and IgG antibodies were measured with ELISA. To examine the effects of Leu-Leu-O-Me treatment on IgE production, PBL were suspended with the medium containing 2.5 mM Leu-Leu-O-Me (Borrebaeck et al., 1987, 1988). After 40 min incubation, the treated PBL were cultured in 5% FBS–10% human plasma-ERDF medium containing various combinations of MDP with inter-

leukins and 10 $\mu\text{g/ml}$ Mite-Df. The concentration of the antibodies in the supernatant was again measured by ELISA.

2.4. Production of human–human hybridomas derived from activated PBL

Polyethylene glycol (MW. 4000)(PEG) was purchased from Merck (Darmstadt, Germany). Amethopterin was obtained from Sigma (St. Louis, MO). Hypoxanthine and thymidine were purchased from Wako (Osaka, Japan). 1×10^7 ICLU-B cells were mixed with 2×10^7 activated PBL. The mixture was centrifuged, and the supernatant was removed. 1 ml of PEG with ERDF medium (1:1 v/v) was added to the remaining cell pellets. A further 9 ml of ERDF medium were then added to the mixture so that the total volume was adjusted to 10 ml. The mixture was recentrifuged and the resulting cell pellets were suspended in 50 ml of 15% FBS-ERDF medium. The suspension was inoculated into a 96-well culture plate so that each well was charged with 100 μl of the cell suspension. After a day of fusion, 100 μl of HAT medium were added to each well. Half of the culture medium was replaced with fresh HAT medium every 2 or 3 days. After 2 weeks or more, growing cells were observed in the culture plates.

2.5. Detection of reactivities of antibodies from the activated PBL and monoclonal IgE antibodies from the hybridomas obtained

Peroxidase-conjugated anti-human IgE, IgM and IgG antibodies were purchased from Biosource International (Camarillo, CA). Human serum IgE was obtained from Chemicon International (Temecula, CA), and human serum IgM and IgG were purchased from BioPur (Bubendorf, Switzerland). The reactivities of the antibodies were measured by ELISA. A 96-well multiplate was coated with 10 $\mu\text{g/ml}$ of Mite-Df diluted with PBS. These antigen-coated wells were blocked with 1.0% BSA-PBS to prevent non-specific reaction. Then, culture supernatants of the PBL or hybridomas were added to the wells and allowed to react for 1 h at 37°C. After the reaction, all wells were washed three times with PBS containing 0.05% Tween 20 (TPBS). Peroxidase-conjugated anti-human IgE, IgM, or IgG antibodies as the sec-

ond antibodies were added to these wells separately. A substrate solution (0.1 M citrate buffer [pH 4.0] containing 0.003% H_2O_2 and 0.3 mg/ml *p*-2,2'-azino-di(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) was added and allowed to react for 15 min. Absorbance at 415 nm was measured in each well with an ELISA reader. The specificity of the monoclonal IgE antibody was also investigated in the same way.

3. Results

3.1. Optimization of culture conditions for efficient antibody production

We determined the optimal concentrations of the immunomodulatory mediators, i.e., IL-2, IL-4, IL-6, and MDP in our cultures of human PBL to produce immunoglobulins (the sum of IgE, IgM, and IgG as measured by ELISA). Both IL-2 and IL-4 enhanced antibody production in a concentration-dependent manner (Table 1). However, we found IL-6 had no effect on antibody production under our conditions,

Table 1
Effects of IL-2, IL-4, IL-6, and MDP on Ig production by human PBL

Additive	Concentration of additive ^a (U/ml or $\mu\text{g/ml}$)	Antibody concentration ^b ($\mu\text{g/ml}$)
None	–	0.79 ± 0.07
IL-2	1	0.82 ± 0.06
	10	0.94 ± 0.07
	100	1.16 ± 0.09
	200	1.11 ± 0.07
IL-4	1	0.76 ± 0.04
	10	0.79 ± 0.09
	100	0.85 ± 0.08
	200	0.83 ± 0.09
IL-6	1	0.75 ± 0.08
	10	0.79 ± 0.04
	100	0.76 ± 0.06
	200	0.74 ± 0.05
MDP	1	0.87 ± 0.08
	10	1.31 ± 0.12
	100	1.17 ± 0.09
	200	1.13 ± 0.11

^aConcentrations of interleukins or MDP is shown as U/ml and $\mu\text{g/ml}$, respectively.

^bData are means $\pm$ SEM of concentration of Ig from triplicate cultures.

Table 2

Effects of the combined use of additives and Leu-Leu-O-Me treatment on antibody production by human PBL^d

Treatment ^a	Additive ^b				Antibody concentration ($\mu\text{g}/\text{ml}$) ^c		
	MDP	IL-2	IL-4	IL-6	IgM	IgG	IgE
Leu-Leu-O-Me							
–	–	–	–	–	0.54 ± 0.03	0.42 ± 0.05	ND
–	+	+	+	–	0.78 ± 0.09	0.66 ± 0.06	ND
–	+	+	–	+	0.72 ± 0.02	0.88 ± 0.02	ND
–	+	–	+	+	0.59 ± 0.03	0.46 ± 0.04	ND
–	–	+	+	+	0.53 ± 0.04	0.55 ± 0.02	0.03 ± 0.02
–	+	+	+	+	0.81 ± 0.09	1.02 ± 0.13	0.09 ± 0.03
+	–	–	–	–	0.43 ± 0.08	0.61 ± 0.03	ND
+	+	+	+	+	1.58 ± 0.15	1.69 ± 0.14	0.01 ± 0.01

^a + : With treatment; – : without treatment.^b + : Added; – : not added.^c ND: not detected.^d Data are shown as means $\pm$ SEM in triplicate experiments.

while MDP at $1 \mu\text{g}/\text{ml}$ increased antibody production significantly, and this effect plateaued at $10 \mu\text{g}/\text{ml}$. Therefore, we chose the following concentrations of these reagents for the subsequent experi-

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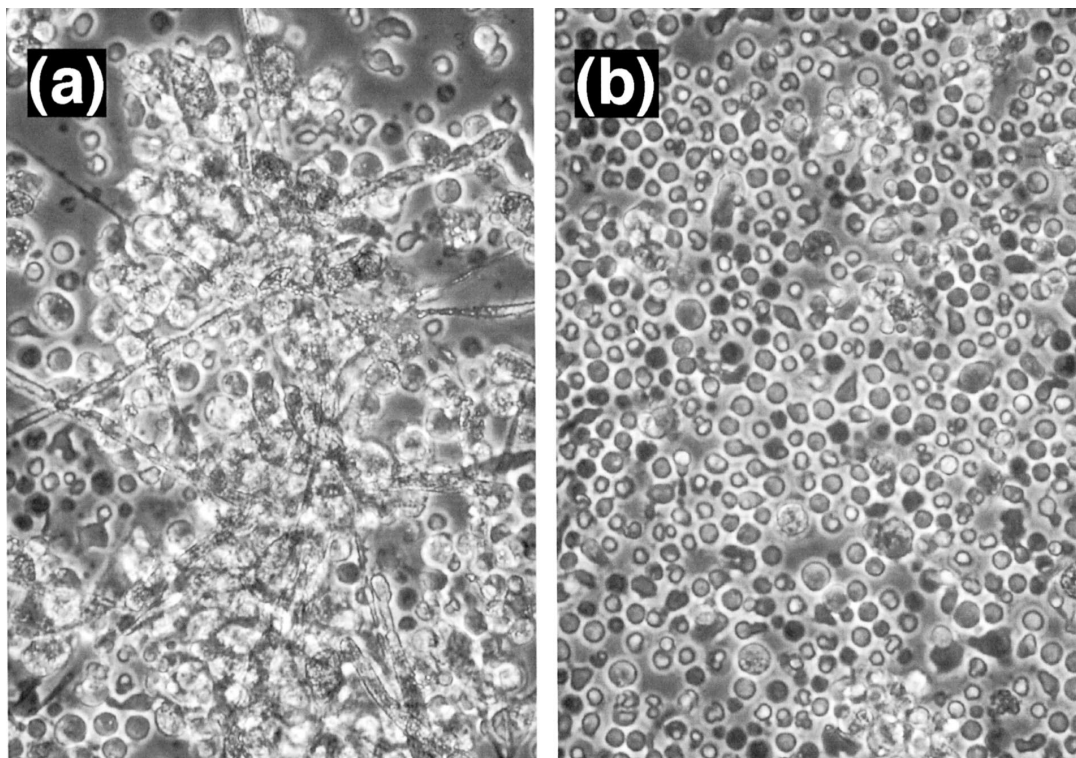


Fig. 1. Morphological changes in activated PBL. PBL were cultured in 5% FBS–10% human plasma-ERDF medium containing MDP, IL-2, IL-4, IL-6, and Mite-Df. After 10 days, these cells were observed by microscopy. PBL were activated with these additives (a), and without the additives (b) (Magnification $\times 400$).

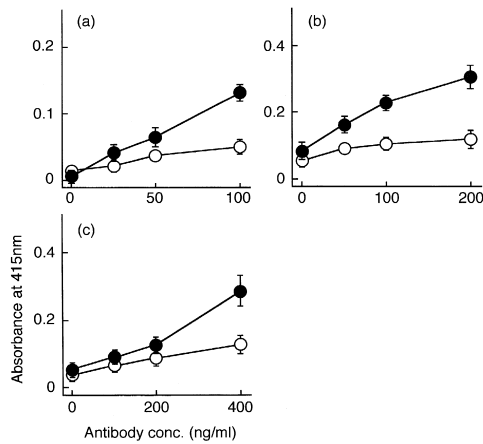


Fig. 2. Reactivity of antibodies secreted from PBL activated with MDP, IL-2, IL-4, and IL-6 against Mite-Df. The PBL were cultured in 5% FBS–10% human plasma-ERDF medium containing MDP, IL-2, IL-4, and IL-6 for 10 days. The PBL culture supernatant was reacted with Mite-Df. To compare the reactivity of each IgE (a), IgM (b), and/or IgG (c) in the supernatant, peroxidase conjugated anti-human IgE, IgM, and IgG as second antibodies were used in ELISA, respectively. The closed circles show the reactivity of the antibody in the supernatant, and the round circles show the reactivity of conventional antibody purified from human serum as a control.

ments: IL-2, 100 U/ml; IL-4, 100 U/ml; IL-6 10 U/ml; MDP, 10 µg/ml.

Next, the effects of various combinations of these reagents were examined on the production of IgE, IgM, and IgG by cultured human PBL, as separately measured by ELISA. Table 2 summarizes the results. Production of IgG and IgM was enhanced by most of

these combinations whereas IgE production was detected only with the combination of IL-2, IL-4, and IL-6. IgE production was further enhanced three times by adding MDP to the combination.

It is well known that *in vitro* immunization can be enhanced by Leu-Leu-O-Me because it removes cytotoxic and suppressor T cells. Therefore, we examined the effects of this reagent on antibody production under our culture conditions. As expected, the production of IgG and IgM was strongly enhanced by the presence of Leu-Leu-O-Me. However, IgE production induced by the presence of the above interleukins was suppressed by this reagent. Consequently, this reagent was omitted in the subsequent experiments.

3.2. Morphological changes in IgE-secreting PBL

Fig. 1(a) shows the morphology of PBL cultured for 10 days in the presence of IL-2, IL-4, IL-6, and MDP. Some PBL under these conditions adhered to the substrata with needle-like protrusions. These protrusions appeared to be connected to cells with similar morphology. In contrast, the PBL cultures with no detectable IgE production contained no such cells (Fig. 1b).

3.3. Reactivity of antibody produced by activated PBL in the presence of Mite-Df

PBL were cultured in the presence of the cocktail of IL-2, IL-4, IL-6, and MDP together with 10

Table 3

Acquisition efficiencies of IgE-producing hybridomas derived from activated PBL

Donor of PBL	Activation ^a	Fusion efficiency ^b (clones/10 ⁵ partner cells)	IgE productivity ^c (%)	No. of specific antibodies ^d
H.K.	+	1.10	19.2	1
	–	1.04	0	0
M.S.	+	0.90	25.6	0
	–	0.86	0	0
M.Y.	+	0.96	20.5	2
	–	0.78	0	0
Y.K.	+	0.98	15.0	0
	–	1.20	0	0

^a + : PBL were activated with the mixture of MDP, IL-2, IL-4, and IL-6; – : PBL were not activated by any additive.

^b Fusion efficiency is expressed as the number of acquired hybridomas per 10⁵ fusion partner cells.

^c IgE productivity indicates the ratio of the number of IgE-producing hybridomas relative to all hybridomas obtained.

^d Number of specific antibodies in each fusion.

$\mu\text{g/ml}$ Mite-Df, house-dust mite extract. Antibodies of IgE, IgG, and IgM classes all reacted with Mite-Df antigen (Fig. 2). Of note is the fact that the reactivity of antigen-specific IgE with mite antigen was two times higher than that of conventional IgE. This suggested that our culture system was suitable for the induction of IgE hybridomas specific for mite antigen.

3.4. Establishment of human–human hybridomas producing mite antigen-specific IgE

PBL activated in the presence of mite antigen were fused with the human fusion partner, ICLU-B. Table 3 shows the fusion efficiency, IgE production, and the number of Mite-Df-specific IgE hybridomas obtained in fusion experiments. Although the fusion efficiency was not affected by the activation of the cells with IL-2, IL-4, IL-6, and MDP, IgE-producing hybridomas were obtained only by using PBL cultured in the presence of these immunoactivators. Approximately 20% of the hybridomas obtained produced IgE antibodies. Three of these hybridomas were found to be specific for Mite-Df. Monoclonal IgE produced by clone 3E7 exhibited the highest

reactivity against Mite-Df. Fig. 3. shows that 3E7-derived IgE reacted specifically with Mite-Df, but the reactivity of this IgE with other known allergens such as cedar pollen, house dust, or ovalbumin was very weak or undetectable.

4. Discussion

Antigen-specific human monoclonal IgE antibodies are rarely described. There are two reasons why it is difficult to obtain monoclonal IgE antibodies. First, IgE-producing lymphocytes are rare in the PBL used for a cell fusion; second, the productivity of antibodies originally generated by fusion partners affects the productivity of monoclonal antibodies from the resultant hybridomas.

It was reported that pokeweed mitogen induced IgE synthesis in the presence of a blocking antibody to the IFN- γ receptor (Jujo et al., 1993). IgE production was induced by PBL activated with anti-CD40 antibody and IL-4 (Zurcher et al., 1995). IL-4 was commonly used in these studies. PBL produced IgE in the presence of IL-4 in our study. However, IgE production by PBL activated with only IL-4 was temporarily induced and was unstable in long-term culture. In addition, few antibodies were detected in the culture supernatant, even if IgE was recognized on the cell surface. When hybridomas were produced using these PBL, the production of monoclonal IgE antibodies was very unstable. However, the combination of IL-4 with MDP, IL-2, or IL-6 stimulated IgE production by PBL so highly that the amount of IgE in the culture supernatant was increased 10 times. As a result, IgE-producing hybridomas were frequently obtained using PBL activated with these immunoactivators. These results suggest that MDP, IL-2, and IL-6 not only enhance IgE production induced by IL-4 but also stabilize the IgE production.

Generally, IgE was undetectable in culture supernatants when normal PBL were incubated with a culture medium. Secreted IgE was not detected in cultures containing surface IgE positive lymphocytes (data not shown). Therefore, the optimal concentration of each immunoactivator was the concentration at which the largest amount of IgE, IgM, and IgG was produced by PBL. IgE production was induced

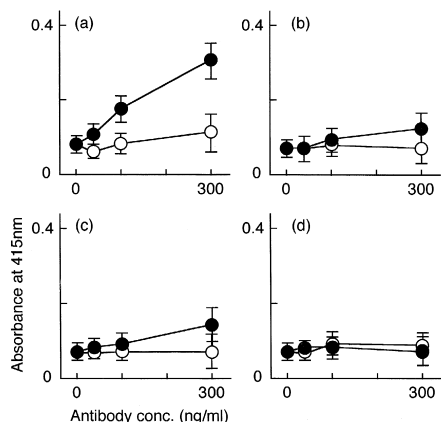


Fig. 3. Reactivity of monoclonal IgE antibody 3E7 against various allergens. A hybridoma clone which produced monoclonal IgE antibody, 3E7, was cultured in 10% FBS-ERDF medium for 5 days. The concentration of 3E7 antibody in the culture supernatant was measured and adjusted to 300 ng/ml. This antibody was reacted with Mite-Df (a), cedar pollen (b), house dust (c), and egg white (d). The closed circles show the reactivity of the 3E7 antibody, and open circles show the reactivity of conventional IgE antibody purified from human serum as a control.

with the combination of IL-2, IL-4, and IL-6 at the determined concentration, which was further enhanced by adding MDP. These results suggested that IgE production was remarkably enhanced by the synergistic use of these additives, although each additive alone could not induce IgE production.

A portion of PBL activated with MDP, IL-2, IL-4, and IL-6 adhered to and expanded on the bottom of the culture vessel. Notably, these cells were always observed when antigen-specific IgE was detected in the culture supernatant. We assumed that these cells with needle-like protrusions might be $\gamma\delta$ T lymphocytes as a result of analysing of the lymphocyte surface antigens and observations of their morphology (Salerno and Dieli, 1998). Recently, it was reported that $\gamma\delta$ T lymphocytes expressed CD40L receptors and then the cells induced isotype switching of B cells in the presence of IL-4 (Horner et al., 1995). It may be that the differentiation of T lymphocytes to $\gamma\delta$ T lymphocytes plays an important role in IgE induction in our system.

Some reports have indicated that specific antibody production is enhanced by treating PBL with Leu-Leu-O-Me because it removes cytotoxic T and CD8 positive lymphocytes (Borrebaeck et al., 1987, 1988). However, IgE production was not induced by this treatment in our study. On the other hand, when IFN- γ was added to the culture medium, the population of lymphocytes treated with Leu-Leu-O-Me produced IgE at the same level as those not receiving the treatment (data not shown). These results suggested that IFN- γ was related to the enhancement of IgE production in vitro, although it was thought that IgE production was inhibited by IFN- γ . We are examining the mechanism of enhancement of IgE production by IFN- γ .

Human fusion partners for the production of human monoclonal antibodies have been reported from many laboratories. The classes of monoclonal antibodies produced by hybridomas are strongly dependent on the original antibody class of the fusion partner used in the cell fusion. For example, 90% of all hybridomas derived from HK-128 partner, which was originally an IgG producer, generated monoclonal IgG antibodies (Kawahara et al., 1990). Similarly, 90% of all hybridomas derived from HO-323, which was originally an IgM producer, generated monoclonal IgM antibodies (Ohashi et al., 1986).

Some hybridomas obtained from these cells produced both IgG and IgM antibodies. However, all of them deleted the production of IgG or IgM within one month of culture. These results suggested that it was necessary to establish a new human fusion partner that did not originally produce antibodies. Therefore, a human fusion partner, ICLU-B, was established in our laboratory, which was derived from human Burkitt lymphoma. Hybridomas derived from ICLU-B reliably produce monoclonal antibodies that were dependent on the antibody productivity of the original lymphocytes. The acquisition of IgE antibody only became possible when the fusion partner, ICLU-B was established.

Mite-Df is a crude antigen extracted from house-dust mites. However, it was found that antigen-specific IgE antibody production could be induced using ovalbumin or cedar pollen instead of Mite-Df as the antigen (data not shown). Since ovalbumin is a highly purified protein, this method may be suitable for the generation of monoclonal human IgE antibody specific for purified as well as non-purified antigens.

Acknowledgements

We thank Dr. Toshiaki Kawakami (La Jolla Institute for Allergy and Immunology) for his kind advice. We would also like to thank Dr. H. Oda, Miss M. Suzuki, N. Matsuda, and Mr. N. Tahara for their technical assistance. This work was supported by the grants from Program for Promotion of Basic Research Activities for Innovative Biosciences.

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