

# Preparation of human immortalized B-cells secreting antibodies against food allergens

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An immortalized B-cell library consisting of cells secreting antibodies to food allergens has been prepared. Peripheral B-cells from seven healthy donors were transformed with Epstein-Barr virus and an immortalized human B-cell library with 2,202 ampoules of multi-clone B-lymphoblastoid cells was obtained. The B-cell library contained various types of B-lymphoblastoid cells secreting antibodies to rice, soybeans, milk, and eggs, and can be used to analyze food allergens.

## Introduction

Allergies that are mediated by IgE antibodies are designated as type I hypersensitivities. When an antigen (allergen) binds IgE molecules on the mast-cell surface and cross-links two high-affinity IgE receptors, the mast-cell releases chemical mediators such as histamine and serotonin. Histamine causes smooth-muscle contraction, increased vascular permeability, and itching, and serotonin causes vasospasm and smooth muscle contraction.

Mouse monoclonal antibodies and sera from patients allergic to certain food have been used to analyze antigenic determinants of food allergens (Gonzalez *et al.*, 1992; Kaminogawa *et al.*, 1985; Burks *et al.*, 1988; Plebani *et al.*, 1997). However, mouse monoclonal antibodies and sera from patients may recognize different antigenic determinants. Furthermore, it seems very difficult to collect a sufficient amount of sera from patients of food allergies because most patients were children. For these reasons, it seemed to be useful to establish human cell lines secreting antibodies specific to food allergens.

Epstein-Barr virus (EBV) is a herpes virus that infects human B-cells to cause infectious mononucleosis. It is also known that EBV transforms and immortalizes human B-cells *in vitro* (Kozbor and Roder, 1981). Those immortalized cells (B-lymphoblastoid cells, BLC) secrete antibodies. In this paper, we report preparation of a human BLC library and detection of BLC secreting antibodies to food allergens.

## Materials and methods

### Allergens

Ovalbumin, ovomucoid,  $\alpha$ s1-casein, and  $\beta$ -lactoglobulin were purchased from Sigma. Rice crude allergens were prepared according to the method of Matsuda *et al.*, (1991) by extraction with 0.5 M NaCl. Soybean crude allergens were extracted from soybean powder by an extraction with 1 M NaCl.

### Preparation of peripheral blood lymphocytes and transformation of the lymphocytes with Epstein-Barr virus

Peripheral blood lymphocytes (PBL) were prepared from peripheral blood from seven healthy donors by discontinuous density centrifugation on lymphocyte separation medium (Organon Teknika Co., USA). PBL were washed twice with ERDF medium (2:1:1 mixture of RPMI1640, Dulbecco's modified Eagle medium, and Ham's F12 medium, Gibco). PBL were then suspended into ERDF medium ( $10^6$  cells/ml) supplemented with 10% fetal calf serum, cyclosporin A (2  $\mu$ g/ml) and 1/10 volume of culture supernatants of marmoset B95–8 cells (kindly gifted from Dr. Sakuraba, Tokyo Metropolitan Institute of Medical Science) containing Epstein-Barr virus (EBV). The cell mixture was then plated into 96-well microculture plates ( $10^5$  cells/well). The medium (ERDF medium supplemented with 10% FCS) was changed every 2 or 3 days. After 2 to 3 weeks of the culture, transformed cells were transferred to 24-well plates and cultured for 5 to 7 days. Finally, the cells were transferred to 6 cm dishes and cultured for 3 to 5 days. Transformed cells (B-

lymphoblastoid cells, BLC) were harvested by centrifugation and the cells and the culture supernatants were frozen and stored.

#### Enzyme-linked immunosorbent assay (ELISA)

Each allergen was diluted with 0.05 M NaHCO<sub>3</sub> (1 to 5 µg/ml), 50 µl of the solution was plated into ELISA plates, and the plates were kept at 4°C for 16 h. The allergen solution was discarded and the plates were blocked with a commercial blocking reagent (Block Ace, Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) for 1 h. For αs1-casein and β-lactoglobulin, 2% (w/v) bovine serum albumin solution was used to block the plates. Plates were then washed three times with phosphate buffered saline containing 0.05% Tween 20. The culture supernatants of BLC (50 µl) were pipetted into the plates and incubated for 1 h at room temperature. The plates were then washed three times, and 50 µl antibodies (anti-human IgA, IgG, IgM, and IgE) conjugated with horseradish peroxidase (Bio-source International, USA) were added to the plates. After 1 h of incubation at room temperature, the plates were washed six times and peroxidase activity immobilized on the plates was measured by adding 100 µl of substrate solution (0.3 mg/ml 2,2'-azino-di-(3-ethyl benzthiazolin sulfonic acid), 0.03% H<sub>2</sub>O<sub>2</sub> in 0.1 M citrate buffer pH 4.0).

#### SDS-Polyacrylamide-gel electrophoresis (SDS-PAGE) and the western blot analysis of rice allergens

Rice crude allergens were boiled for 5 min with SDS-sample buffer containing 2.5% 2-mercaptoethanol. The crude allergens were separated by an SDS-PAGE (Laemmli, 1970) using a 10 to 20% (w/v) gradient gel (Daichi-Kagaku, Japan). The proteins on the gel were transferred to a PolyScreen transfer membrane (NEN Life Science Products, USA), and the membrane was blocked with Block Ace solution. The membrane was placed on a multi-slot reacting apparatus (ATTO Co., Japan), and the culture supernatants of BLC were reacted with the membrane. Anti-human IgM antibodies conjugated with horseradish peroxidase were then reacted. Finally, the membrane was developed with a Konica immunostaining kit (Konica, Japan). Prestained molecular marker proteins (New England Bio Labs, USA) were used as standard marker proteins.

#### Results and discussion

Peripheral blood lymphocytes (PBL) were prepared from seven healthy donors. PBL infected with Epstein-Barr virus (EBV) were seeded into 96 well micro-culture plates. After 2 to 3 weeks of culturing, wells with transformed lymphocytes were transferred to 24 well plates and cultured. Cells

**Table 1** Detection of Antibodies to Food Allergens of BLC Supernatants.

| Allergens       | Positive ampoules <sup>a</sup> |
|-----------------|--------------------------------|
| Non-specific    | 9                              |
| Rice            | 151                            |
| Soybean         | 176                            |
| Ovalbumin       | 183                            |
| Ovomucoid       | 29                             |
| αs1-Casein      | 31                             |
| β-Lactoglobulin | 32                             |

<sup>a</sup> Antibodies reacted with the food allergens were detected by ELISA with a mixture of antibodies to human IgA, IgG, IgM, and IgE conjugated with horseradish peroxidase.

**Table 2** Detection of Ig Class of BLC Supernatants.

| Allergens       | positive ampoules     |     |     |     |     |
|-----------------|-----------------------|-----|-----|-----|-----|
|                 | Analyzed <sup>a</sup> | IgA | IgG | IgM | IgE |
| Non-specific    | 2                     | 0   | 0   | 2   | 0   |
| Rice            | 20                    | 0   | 0   | 20  | 0   |
| Soybean         | 24                    | 2   | 4   | 23  | 0   |
| Ovalbumin       | 25                    | 0   | 0   | 25  | 0   |
| Ovomucoid       | 3                     | 0   | 0   | 3   | 0   |
| αs1-Casein      | 2                     | 0   | 1   | 1   | 0   |
| β-Lactoglobulin | 5                     | 0   | 0   | 5   | 0   |

<sup>a</sup> BLC supernatants with high reactivity to the food allergens were chosen and the Ig class was determined by ELISA with antibodies to human IgA, IgG, IgM, and IgE conjugated with horseradish peroxidase.

were further cultured in 6 cm dishes, and resulting B-lymphoblastoid cells (BLC) were stored in liquid nitrogen. We obtained a total of 2,202 multi-clone stocks of BLC for the cell library. Because we estimated that approximately 100 cells out of 100,000 PBL were transformed to BLC (in one ampoule frozen), our immortalized B-cell library might contain more than 200,000 clones of BLC.

Culture supernatants of BLC were assayed for antibodies to several food allergens. Crude extracts from rice and soybean, two hen egg allergens (ovalbumin and ovomucoid), and two cow milk allergens (αs1-casein and β-lactoglobulin) were used as allergens. As shown in Table 1, we detected many cells secreting antibodies to food allergens. This result suggested that we obtained numerous BLC secreting specific antibodies to many antigens.

The Ig class of antibodies was determined by ELISA. As shown in Table 2, antibodies of IgM class were dominant. EBV is known to bind CR2 molecule (CD21, a 145 kDa complement receptor) on B-cells of peripheral blood that express IgM class antibodies on the cell surface (Brown and Miller, 1982). That would be the reason for dominant

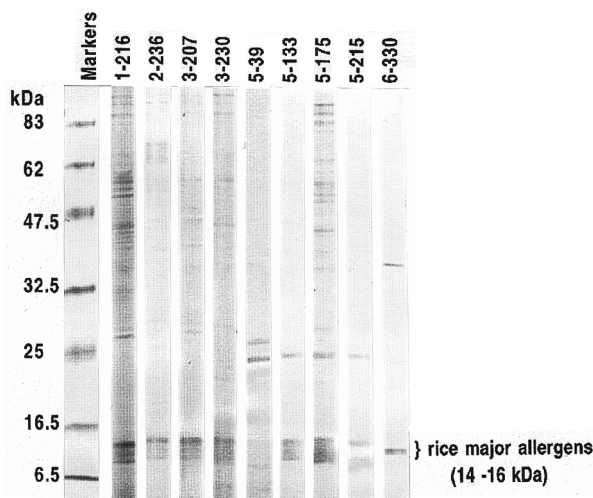


**Figure 1** Western Blot Analysis of Rice Allergens with the Supernatants of BLC. Rice allergens were separated by an SDS-PAGE and transferred to a membrane. Rice protein bands reacted with nine supernatants of BLC were detected with anti-human IgM antibodies conjugated with horseradish peroxidase.

secretion of IgM by BLC from peripheral lymphocytes (Shinmoto *et al.*, 1992).

We chose nine BLC secreting antibodies to rice allergens to detect proteins recognized with the antibodies. As shown in Figure 1, the culture supernatants of BLC reacted with several kinds of rice protein including rice major allergens having the molecular weights of 14 to 16 kDa (Matsuda *et al.*, 1991). Proteins with higher molecular weights of 24 kDa (5-39, 5-133, 5-175, and 5-215), 26 kDa (1-216), 35 kDa (6-330) and 45 kDa (1-216 and 5-175) were also detected. It is not clear whether the proteins with different molecular weights had the same antigenic determinants, or the supernatants were the mixture of several antibodies reactive to rice proteins with different molecular weights.

To clarify the reactivities of the antibodies, BLC should be clones.

An immortalized B-cell library consisting of more than 2,000 stocks of BLC could be an excellent source of antibodies to a wide spectrum of allergens (Nagatsuka *et al.*, 1994). It seemed to contain antibodies to various food allergens. The disadvantages of this method lie in the instability of the BLC clones secreting antibodies of interest and, thus, in the difficulty in establishing single clones. We are trying to eliminate the disadvantage by cell fusion of BLC with mouse myeloma cells to establish stable human-mouse hybridomas. In the preliminary results, we obtained five stable hybridomas secreting human IgM class antibodies to rice allergens.

The presence of BLC secreting antibodies to rice allergens from normal donors suggested that the BLC library contained antibodies to most food allergens. We consider that our new technology will also enable us to evaluate the allergenicity of novel proteins derived from less commonly allergenic food or non-food sources.

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