

Development of hybridoma cells producing monoclonal antibody against human epsilon (ϵ) chain

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Abstract. Epsilon (ϵ) chain is one of the five classes of immunoglobulins that plays an important role in allergic diseases. Production of monoclonal antibodies by a single clonotype against different epitopes of ϵ chain have high priority in development of diagnostic kits. In this study, an attempt was made to produce monoclonal antibodies against human ϵ chain. Balb/c mice were immunized with semipurified ϵ chain and spleen cells were fused with Sp2/0 mouse myeloma cell line in the presence of poly ethylene glycol. Supernatant of hybridoma cells were screened for detection of antibody by Enzyme-linked Immuno Sorbent Assay (ELISA) method. Cloning of selective high absorbance wells were done with limiting dilution method. The suitable clones (monoclones) were selected by ELISA and confirmed by immunoblot. The subclasses of the chosen monoclonal antibodies were determined and the clones freezed and kept in liquid nitrogen.

During this study three successful fusions were carried out, which resulted in over than 100 clones with high production of anti- ϵ chain. Twelve clones with the highest titers were selected for cloning. After limiting dilution more than 50 monoclonal antibodies were produced and the unsuitable one (C1F2) displayed higher absorbance in reaction with purified IgE, relatively high cross-reactivity with IgM, and the highest cross-reactivity with IgG. In Immunoblotting, presence of relatively high-density band in reaction with IgE was confirmed. The unsuitable monoclonal antibody was shown to be IgG1 subclass with kappa light chain. It seems that, this monoclonal antibody could not be successfully useful in diagnostic kits.

Keywords: Monoclonal antibody, human ϵ chain

1. Introduction

Development of hybridoma cells capable of permanent production of specific antibody directed against a predefined immunogen was first reported in 1975 by Kohler and Milstein [7,10]. The method was based on fusion between myeloma cells and spleen cells from suitably immunized animals. Spleen cells die in a short time under ordinary tissue culture conditions, while myeloma cells have been adapted to grow permanently in the culture [7]. Therefore, fusing of both cells results in a new hybrid cell producing specific antibody against immunogen.

The potential use of monoclonal antibodies (mabs) as reagents was widely adapted, leading to rapid growth in the field [10]. The utilization of mab avoids many of the problems inherent in the use of polyclonal antisera, e.g; specificity, cross-reactivity and longevity [1,18]. At the present, the production of mab has become a common technique in many laboratories and in all fields of research, diagnosis and treatment, mabs have an important role [1,8,10].

As reported by Fagan et al. (1982) and Chandler et al. (1983), many different mabs have been made against human ϵ chain [19]. Mabs have also been produced against ϵ chain of other species, including mice, rats, cattle and dogs. In all of these cases purified or semi purified ϵ chain has been used as immunogen and the

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subclass of produced mab has been IgG1 [2,4,10,19, 20].

In this study, spleen cells from well-immunized mouse were fused with myeloma cells in the presence of Poly Ethylene Glycol (PEG). Then, positive clones were detected and cloning was performed by Limiting Dilution (L.D) method. Finally, the secreted mabs were screened and partially characterized.

2. Materials and methods

Immunization of mice: Female Balb/c mice, 4–6 weeks old, were obtained from Razi research and vaccine production institute and kept in a relatively optimum condition. Each mouse was first immunized intraperitoneally with 50 microgram of semi purified human ϵ chain emulsified in complete Freund's adjuvant (CFA, Sigma). Three weeks later, the second injection was made with the same amount of antigen emulsified in Incomplete Freund's adjuvant (IFA). One week after the second injection, titration of serum antibody was determined and well-immunized mouse was selected for the final injection. A final intravenous-intraperitoneal injection of the same amount of ϵ chain in 50 μ l Phosphate Buffer Saline (PBS) was given 3 days before fusion [19,20].

Fusion: Spleen cells from well-immunized mouse were fused with myeloma cell Sp2/0 at a ratio of 3:1, using 50% (w/v) PEG 1450 (Merck) [12,16, 17]. The fused cells were resuspended in 50 ml of RPMI 1640 (Sigma) containing 20% Fetal Calf Serum (FCS, Hyclone, superplasma, USA origin), Hypoxanthine, Aminopterin and Thymidine (HAT medium). The cells were aliquoted into five 96-well microtiter plates (Falcon) at $2 - 5 \times 10^5$ per well (100 μ l/well) [4]. The media of the wells were changed at 3rd, 5th, 7th and 9th days after fusion. Positive clones were observed in the bed of dead cells (due to the presence of HAT medium) at 10 days after fusion.

Antibody Screening: Following 2 weeks of culture, supernatants from wells were first screened by ELISA to detect specific binding to IgE [3,12,17]. Polystyrene 96-well microtitre plates were coated with 100 μ l of purified IgE solution (5 μ g/ml) in 0.15 M PBS buffer pH 7.4 for 1 h at 37°C. Non-specific binding sites on the plates were blocked with 1% Bovine Serum Albumin (BSA) in PBS for 1 h at 37°C and the plates were then washed three times with PBS containing 0.05 (v/v) Tween-20 (PBS-T).

100 μ l of supernatants of hybridomas were added to each well and incubated for 1 hrs at 37°C. Then, the plates were washed five times with PBS-T. 100 μ l of peroxidase conjugated rabbit anti-mouse immunoglobulin (diluted 1/4000 in PBS-T, Sigma) was added to each well and incubated for 45 min at 37°C. After five further washes, 100 μ l of the substrate solution; OrthoPhenylene Diamine (OPD, DAKO, 0.4 mg/ml) in 0.1 M Phosphate-citrate buffer pH 5.1 containing 0.04% H₂O₂ was added to each well. After 15–20 min of incubation at 25°C in dark, the enzyme reaction was stopped by the addition of 50 μ l of 5% H₂SO₄ to each well and absorbance determined at 492 nm.

Cloning of hybridoma cells: After primary screening with ELISA, clones with high secretion of anti- ϵ chain antibody were selected for cloning by limiting dilution procedure [7,8]. Supernatants from wells containing a single clone were screened for anti- ϵ chain antibody. Then an index of the suitable and relevant clones of the first, second and third fusions (monoclones, B1FL1, G10F7, and C1F2) with high titer anti- ϵ chain antibody were used for recloning to obtain clones with stable production. Hybridoma cells were considered stable when the supernatants of 80–100% of wells were positive by ELISA.

Partial Characterization of mabs: A sample of mabs secreted by the clones related to the first, second and third fusions were partially characterized by the following procedures:

2.1. Cross-reactivity

For screening of supernatants for anti-human (IgG, IgA and IgM) cross-reactivity, microtitre plates were coated with each purified immunoglobulin (5 μ g/ml in coating buffer, 100 μ l/well) by incubation for 1 h at 37°C. Plates were blocked with 1% BSA as described above. Following a soak and wash cycle, supernatants were added and incubated as described above. The rest of the assay was proceeded as described for the ELISA procedure.

2.2. Isotyping

The class and subclass of the mabs were determined by an enzyme immunoassay employing a mouse – hybridoma sub typing kit (Boehringer, Germany, 1183117) containing peroxidase labeled antimouse – Ig class – and subclass – specific antibody.

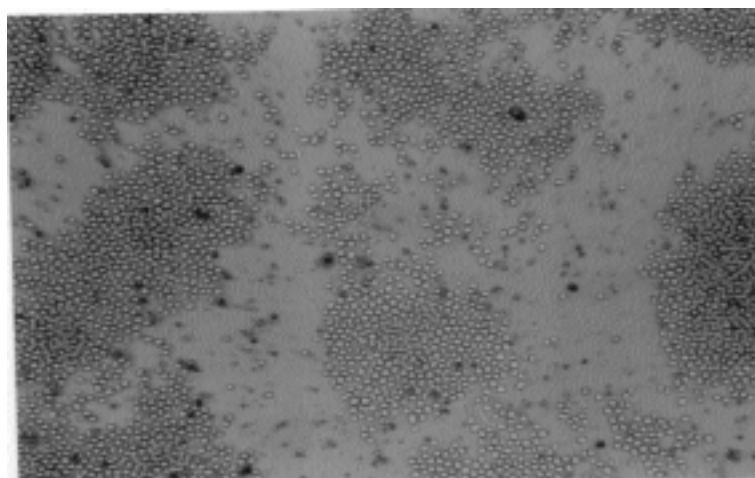


Fig. 1. A sample of positive clones that is seen as shining clusters in the bed of dead cells due to the presence of HAT medium (magnifications: 4 $\times$).

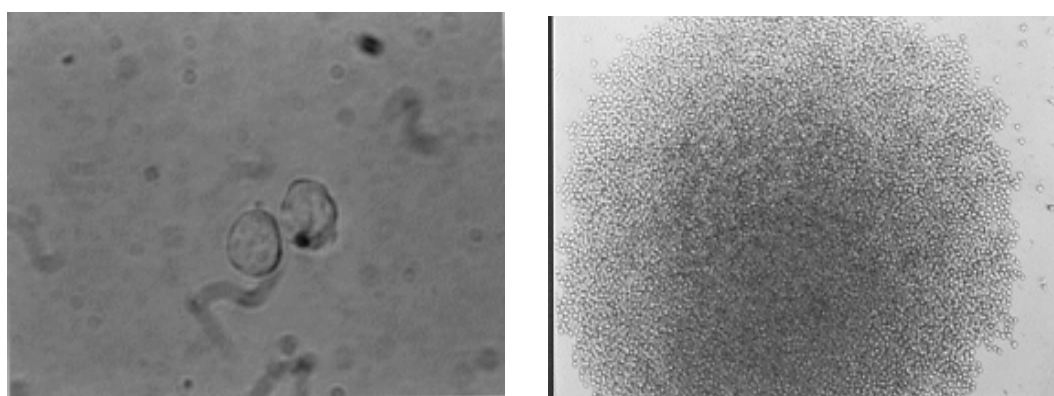


Fig. 2. A sample of mabs (monoclones) resulted from cloning by limiting dilution method that are seen as 2 and more than 5 cells (magnifications: 40 $\times$, 4 $\times$, respectively).

2.3. Immunoblotting

This technique was performed according to tank method, using transblot buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) [14,20]. Nitrocellulose membrane and several thicknesses of whatman chromatography paper were soaked for 20 min in transfer buffer. Wet nitrocellulose membrane was overlayed on wet whatman papers, by taking precaution to avoid bubbles. Then, the electrophoresed semipurified human IgE in a non-reducing condition using 10% polyacrylamide in the presence of SDS overlayed on wet nitrocellulose membrane [9]. Then, several wet whatman papers placed on the top of the gel, followed by eight thicknesses of dry whatman paper. Transfer of proteins from the gel to the nitrocellulose membrane was completed by a 3 hrs incubation with 100V con-

stant voltage. Free binding sites on the membrane were blocked by 2 hrs incubation with 2% BSA dissolved in PBS-T. After washing 3 times with PBS-T, the membrane was cut into strips and incubated for 2 hrs at 37°C with supernatant of selective monoclones related to the first, second and third fusions. After washing 5 times with PBS-T, each strip was then incubated for 2 hrs at 37°C with peroxidase conjugated rabbit antimouse immunoglobulin (1.4000, sigma). The strips were washed and the color reaction was developed with 1 mg/ml Di Amino Benzidine (DAB, sigma) dissolved in substrate buffer (10 mM Tris-HCl, pH 7.4) containing 0.01% H₂O₂ [15]. Color developed within 5–10 min at RT. Finally, in order to stop the reaction, the strips were washed twice in distilled deionized water for 5 min for each wash, and then air-dried [20].

Table 1
Comparing of the mean absorbance of supernatant of a sample of mabs related to the first, second and third fusions at 492 nm

N0	Controls and monoclones	Mean absorbance in reaction with IgE	Mean absorbance in reaction with IgM	Mean absorbance in reaction with IgG	Mean absorbance in reaction with IgA
1	Sp2/0 (N.C)*	0.080	0.065	0.093	0.051
2	Normal Mouse serum (N.C)	0.162	0.071	0.375	0.095
3	Immune mouse serum (P.C)**	0.798	0.365	0.971	0.082
4	B1FL1 (1)	0.469	0.830	0.348	0.064
5	G10F7 (2)	0.900	0.118	0.333	0.076
6	C1F2 (3)	1.024	0.400	1.546	0.115

*Negative Control.

**Positive Control.

Table 2
Results of subclass isotyping of mouse mabs related to the first, second and third fusions at 405 nm

No.	Monoclonal name	Anti-mouse IgA-POD	Anti-mouse IgG-POD	Anti-mouse IgG1-POD	Anti-mouse IgG2a-POD	Anti-mouse IgG2b-POD	Anti-mouse IgG3-POD	Anti-mouse IgM-POD	Anti-mouse Lambda-POD	Anti-mouse Kappa-POD
1	B1FL1 (1)	0.112	2.096	0.388	1.871	0.134	0.092	0.102	0.176	0.968
2	G10F7 (2)	0.075	0.926	3.270	0.091	0.085	0.068	0.063	0.074	0.943
3	C1F2 (3)	0.085	1.313	3.069	0.190	0.133	0.074	0.086	0.094	1.395
4	Sp2/0 (N.C)	0.007	0.100	0.100	0.085	0.073	0.005	0.006	0.007	0.008

3. Results

Development of monoclones: The final results of three successful fusions were about 100 wells containing positive clones (Fig. 1) with high absorbance in reaction with IgE. Twelve wells with the highest absorbance were selected for cloning. The final results of at least 30 cloning with limiting dilution method were more than 50 wells containing monoclones (Fig. 2), out of which the wells containing the suitable monoclones with high concentration of mab were selected.

Cross-reactivity of mabs: cross-reactivity of mabs were studied by ELISA. The results indicated that the mab secreted by the G10F7 clone had lower cross-reactivity with IgG, IgM and IgA and mab secreted by C1F2 (monoclonal related to the third fusion) clone had higher reactivity with IgE (Table 1).

Isotyping of mabs: The class and subclass of mabs were analyzed by ELISA procedure. The obtained data indicated that mab secreted by C1F2 clone belonged to IgG1 subclass with Kappa light chain (Table 2).

Immunoblot analysis: Supernatant of different well-developed clones were overlaid onto the SDS-PAGE analyzed IgE. The obtained result showed a sharp band with high density at 190 KD position for G10F7 clone. However, B1FL1 clone showed a band with low density at 190 KD position and C1F2 clone showed a sharp band with high density at 190 KD position and a relatively sharp band with high-density at 150 KD position (Fig. 3).

4. Discussion

Monoclonal antibodies are widely used in diagnostic and therapeutic aims [10]. Development of mab has resolved many problems derived from polyclonal antisera, including low specificity and wide cross-reactivity [18]. Mab shows great benefit in preparing ELISA kit for measuring IgE level in atopic patients and parasitic disorders. All these reasons directed us towards this study. The immunized mouse with high titer of antibody was selected for fusion. The spleen of this mouse had large number of B cells with high secretion of antibody and could result in hybrids with high secretion of mab [5,6]. The last immunization on three days before fusion had important role in localization of immunized B cells (immunocytes) in spleen of mice. During the first fusion about 10% of wells showed positive clones. Isolation of clones with high secretion of specific antibody resulted in several monoclones with high specificity and low cross-reactivity. Clones with high absorbance in reaction with purified IgE were selected for cloning by limiting dilution method and about 30% of wells indicated single clone (monoclonal) that is a relevant and significant percentage. The range of 30% adaptation is fit with mathematical equation $a = e^{-b}$. This equation is regarded as poisson's distribution and if $b = 1$, thus $a = 37$. Therefore if in each well only one cell is dropped, this probability shows that at least 37% of wells are empty from any cell. In this case, growth of clones in the wells are clear reasons for monoclonality [6,11].

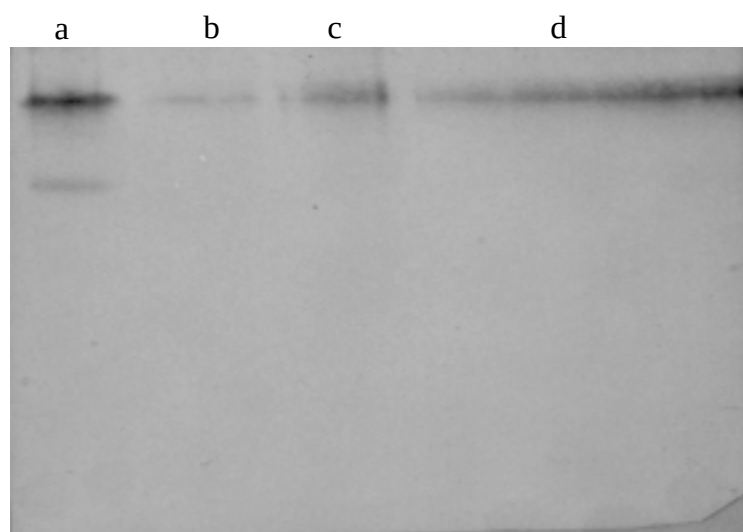


Fig. 3. Immunoblotting of supernatant of a sample of mabs.

Clones with high cross-reactivity with IgG and IgM were considered as undesired clones. Since high cross-reactivity with IgG and IgM was seen in clones obtained from the first fusion (B1FL1 clone), in order to find out a desirable clone, the second and third fusions were done with intact and ϵ chain of IgE 'respectively' for comparison with each other and with the results of the first fusion which resulted in multiple clones and monoclones.

As shown in Table 1, a monoclonal (G10F7) was obtained from the second fusion with high reactivity with IgE and lower cross reactivity with IgG and IgM. This monoclonal was chosen as reliable clone for the preparation of anti-IgE mab.

It is necessary to mention that the overall amino acid composition of IgE is similar to that of IgM and IgG. The percentage of homology, expressed in term of the number of the identical amino acids in the sequence, is 27% for ϵ and γ and 37% for ϵ and μ [8]. High cross-reactivity of the most mabs with IgM and IgG is due to these identical amino acids. Thus monoclonal (G10F7) obtained in the second fusion seemed to be a relevant one which has been prepared for the first time in Iran. The results of immunoblotting confirmed the results of ELISA, because supernatant of the suitable monoclonal (G10F7) in reaction with IgE (Fig. 3, c, d) indicated only one band with high density at 190kD position.

Using immunoblotting analysis, a sample from mab related to the B1FL1 clone (first fusion) and a sample from mab secreted by C1F2 clone (third fusion) were compared. As shown in Fig. 3, mab of B1FL1 clone showed a band with low density at 190kD (Fig. 3, b),

but mab of C1F2 showed two different bands; a band with high density in 190kD and a band with relatively high density at 150 kD (Fig. 3,a). High cross-reactivity of C1F2 with IgG is probably due to: Denaturation of ϵ chain during reduction with DDT and purification process with gel filtration and or the presence of intact IgE and IgG in the bed of semipurified ϵ chain so that seems mabs have been produced against IgE and ϵ chain at the same time or against IgG impurity in semipurified IgE.

The comparison of the immunoblotting patterns of clones related to the 1–3 fusion indicates that the later clone (C1F2) is unsuitable clone with high cross-reactivity and could not be used successfully in a diagnostic kit.

In conclusion, the results of this study showed that the mab secreted by a clone from the third fusion (C1F2) with IgG1 subclass and considerable cross-reactivity with IgM and IgG was an unreliable mab for use in diagnostic kits.

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