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Hybridoma Production by Electroporation

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Introduction

In 1975, Kohler and Milstein described a technique for the generation of antigen-specific monoclonal antibodies.[1] Myeloma cells were fused with spleen cells from immunized animals to produce hybridomas which have the capacity for continuous growth and the ability to secrete monoclonal antibodies to the immunizing antigen. Since the development of this technique, monoclonal antibodies have become essential tools for both basic and clinical research.

Recently, modifications of the original technique have been made to increase the yield of hybridoma clones producing antibodies of desired specificity and affinity. One such technique, avidin-mediated electrofusion, exploits the high affinity of the interaction between biotin and streptavidin.[2, 3, 4] Antigen-specific B lymphocytes are incubated with biotinylated antigen and brought into close physical proximity with biotinylated myeloma cells through a streptavidin bridge. Fusion of these cells can then be accomplished by electrical pulses.

The Gene Pulser® apparatus has been well documented as an electroporation apparatus on a number of cell types including primary human lymphocytes,[5] T-cell and macrophage cell lines,[6] gram-positive bacteria,[7] and gram-negative bacteria,[8] but only recently has its use been reported for electrofusions.[9] This bulletin describes the use of the Gene Pulser as a fusion apparatus for the production of hybridomas to a selected model immunogen, keyhole limpet hemocyanin (KLH).

Methods

Immunizations

Female BALB/c mice (6–8 weeks old) were initially immunized by intraperitoneal injections of 50 µg KLH (Sigma Chemical Co., St. Louis, MO) emulsified in complete Freund's adjuvant. Subsequent immunizations were administered in incomplete Freund's adjuvant. A total of four immunizations were administered at weekly intervals. Sera from immunized mice were monitored by enzyme-linked immunosorbant assay (ELISA) for anti-KLH antibodies three weeks after the initial immunization. Spleens from mice having high-titer anti-KLH sera were used for this study.

Biotinylation of KLH

KLH was biotinylated as previously described.[3] KLH was extensively dialyzed against 0.1 M NaCl, 70 mM $NaHCO_3$, 30 mM Na_2CO_3, pH 9.0. Following dialysis, the KLH concentration was adjusted to 2 mg/ml. KLH (2 mg) was mixed and incubated for 1 hour at 23 °C with 1 mg NHS-Biotin (Bio-Rad Laboratories, Hercules, CA). The product was dialyzed extensively against PBS, filter-sterilized, and stored at 4 °C.

Spleen and Myeloma Cell Preparation

Spleen and myeloma cells were prepared as previously described.[3] Briefly, single cell suspensions of spleen cells from immunized mice were prepared in RPMI by carefully mincing spleens between the frosted edges of sterilized glass slides. Cells were washed twice at 4 °C in RPMI 1640 media containing DNAse I (Sigma Chemical Co., St. Louis, MO) at 50 µg/ml (RPMI/DNAse) and resuspended at 5×10^7 cells/ml. Cells were then incubated with biotinylated KLH (100 µg/ml) for 3 hours at 4 °C, and resuspended at 1×10^8 cells/ml. Spleen cells bearing biotinylated KLH were incubated with streptavidin (50 µg/ml; Bio-Rad Laboratories, Hercules, CA) for 30 minutes at 4 °C. Spleen cells were then washed three times with RPMI/DNAse and resuspended at 1×10^8 cells/ml prior to incubation with biotinylated myeloma cells.

P3X63-Ag.8.653 murine myeloma cells were obtained from cultures in exponential growth, washed three times, and resuspended at 2×10^7 cells/ml. Myeloma cells were incubated with 50 µg/ml of NHS-biotin for 1 hour at 4 °C, washed twice with RPMI/DNAse, and resuspended in RPMI/DNAse at 1×10^8 cells/ml.

Electrofusions

For the electrofusions, spleen cells which had been incubated with biotinylated KLH and with streptavidin (5×10^7 cells) were mixed with biotinylated myeloma cells (5×10^7 cells), centrifuged at 200 x g for 5 minutes and incubated for 1 hour at 37 °C. Cells were then washed and resuspended at 1×10^8 cells/ml in low ionic strength buffer (1.5 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 272 mM sucrose, pH 7.2). A portion of the cell suspension was washed separately, resuspended in RPMI and fused using polyethylene glycol (PEG) as previously described.[4] Electrofusions were carried out as previously described[9] by exposing 0.5 ml of the remaining cell suspension to the following Gene Pulser conditions: (a) 200 V, 25 µF,

2 pulses; (b) 200 V, 25 µF, 3 pulses; or (c) 400 V, 1 µF, 2 pulses. The electrical pulses were applied in standard Bio-Rad 0.4 cm electrode gap cuvettes. Immediately following electrofusion, the cells were diluted with HAT/RPMI medium and plated in the central wells of 96-well microtiter plates at 5×10^5 cells/well.

As a comparison, one set of fusions was performed without streptavidin mediation. Spleen and myeloma cells were mixed and fused as described above using either PEG or the Gene Pulser apparatus.

Supernatants were assayed for anti-KLH antibody production using an ELISA as previously described.[3]

The effects of electrofusion power conditions on myeloma cell viability were tested prior to fusions. Myeloma cells were pulsed in low ionic strength buffer and cell viabilities were determined by trypan blue dye exclusion as previously described.[4]

Results

Prior to electrofusions, the Gene Pulser conditions were tested for their effects on P3X63-Ag.8.653 myeloma cell viability. Viabilities of pulsed myeloma cells ranged from 46 to 58% (Table 1). A recent report suggests that optimization of pulse conditions should be performed prior to electrofusions. Successful electrofusions occurred with pulse conditons that resulted in myeloma cell viabilites of 40–60%.[4, 9] Electrofusions were subsequently performed using these three pulse conditions.

Table 1. Effect of Gene Pulser Conditions on Myeloma Cell Viability

Pulse Conditions	Myeloma Cell Viability (%)
200 V, 25 µF, 2 pulses	58
200 V, 25 µF, 3 pulses	51
400 V, 25 µF, 2 pulses	46

Streptavidin-mediated electrofusions were performed and hybridoma supernatants were assayed at various times post-fusion (pf). At 9 days pf, high percentages of positive clones were detected with all fusion conditions (Table 2). After 3 weeks, the percentage of positive clones from the PEG fusion dropped slightly from 88% to 65%. A relatively high percentage of clones produced with the highest voltage (400 V, 1 µF, 2 pulses) continued to secrete antigen-specific antibodies over 22 days pf, whereas the percentage of positive clones from the lower voltage (200 V, 25 µF, 3 pulses) dropped significantly.

Table 2. Anti-KLH Antibody Production by Hybridomas Generated from Streptavidin-mediated Electrofusions.

Fusion Conditions	% Hybridomas Positive for Antigen* 9 Days	22 Days
200 V, 25 µF, 2 pulses	82	ND†
200 V, 25 µF, 3 pulses	80	43
400 V, 1 µF, 2 pulses	83	74
PEG	88	65

* As assessed by ELISA performed 9 and 22 days post-fusion.
† ND = not done

The relative fusogenic capabilities of PEG and the Gene Pulser were also compared in electrofusions performed without streptavidin mediation (Table 3). At 8 days pf, a lower percentage of positive clones was seen with PEG than with the Gene Pulser condition tested. Dramatic differences between PEG and electrofusions were noted in percentages of positive clones at 21 days pf. All three pulse conditions yielded higher percentages of positive clones than PEG. Similar results were obtained with supernatants assayed 33 days pf. Overall, electrofusions produced higher frequencies of antigen-specific clones than did PEG.

Table 3. Anti-KLH Antibody Production by Hybridomas Generated in the Absence of Biotin/Streptavidin.

Fusion Conditions	% Hybridomas Positive for Antigen* 8 Days	21 Days	33 Days
200 V, 25 µF, 2 pulses	81	36	ND†
200 V, 25 µF, 3 pulses	ND	51	49
400 V, 1 µF, 2 pulses	ND	79	63
PEG	50	21	24

* As assessed by ELISA performed 8, 21, and 33 days post-fusion.
† ND = not done

Discussion

The Gene Pulser apparatus was found to be as effective as PEG at generating clones of interest using the streptavidin-mediated fusion system. Both PEG and the highest voltage pulse condition resulted in the maintenance of a high number of positive clones through 22 days of culture. Interestingly, the Gene Pulser apparatus was more effective than PEG at generating positive clones in fusions performed without streptavidin mediation. Positive clones were maintained more effectively with the Gene Pulser unit than with PEG. All pulse conditions were better than PEG, with the highest voltage pulse condition being the most effective. Unlike the PEG fusions, Gene Pulser electrofusions performed without streptavidin mediation resulted in high frequencies of antigen-specific hybridomas. These findings indicate that it may not be necessary to use lengthy procedures such as streptavidin-mediation to increase the yield of antigen-specific clones. The Gene Pulser apparatus may be very useful for fusion applications, especially fusions performed without streptavidin mediation.

It has been reported that electrofusions are more effective between cells of similar size.[10] Non-activated spleen lymphocytes are generally smaller than myeloma cells, whereas plasma cells (activated B-cells) are approximately the same size as myeloma cells. Spleens from immunized mice contain increased numbers of plasma cells, but the majority of lymphocytes are non-activated T- and B-cells. Because of their similar sizes, plasma cells and myeloma cells may fuse more readily when pulsed, thereby decreasing the number of undesired fusions (such as non-activated lymphocytes with myeloma cells). Thus, use of the Gene Pulser apparatus may cause fusions between cells of similar size, resulting in a higher number of antigen-specific clones.

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