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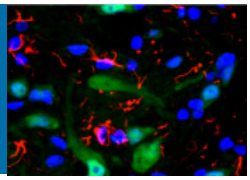

Original article

Methods of cell fusion in hybridoma technology

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Abstract: Background: Cell fusion, the process by which two or more cells merge to form a single cell. In recent years, two different cells in isolated culture can also be fused by artificial methods to form a somatic cell hybrid and generate a new hybrid cell. Nowadays, cell fusion techniques have become an important tool for studying cytogenetics, cellular immunity, viruses and tumors. **Methods:** This experiment was performed using the limited dilution method for cell clonogenization culture. Cells were collected and counted in positive culture, and the cell density was adjusted to 5-10 cells/ml. 0.1 ml was added to each well of a 96-well plate, and the culture was continued by half volume change after one week of incubation in the incubator. The supernatant of each well was tested at the right time, and the cells in the positive culture wells were selected for continued limited dilution until the cells in the wells were sure to be monoclonal, and the culture was expanded. The 0.2 ml of 43% PEG solution was added to a centrifuge tube, gently agitate the cell mass and leave it at room temperature for 2 minutes. The 10 ml of complete culture medium was added and centrifuged at 800 rpm for 5 min, then the cells were suspended in 2× HAT culture medium (adjust the concentration to 6×105/ml using spleen cells as the base), take a 24-well plate and add 0.5 ml to each well; add 0.1 ml to each well of another 96-well plate. place in a 37°C 5% CO2 incubator and incubate for one week before the first change of culture medium. After 7-10 days of fusion, the culture medium was changed by half amount of HAT culture medium, and then by half amount every 2-3 days. After two weeks, the culture medium could be changed to HT culture medium for half volume exchange or still HAT culture medium for half volume exchange. **Results**: Hybrid cell colonies appeared after 2-3 weeks of cell fusion, and the cells were large, round and transparent. **Conclusions:** This paper provides a reference for further research on the application of cell fusion in hybridoma technology.

Key words: Cell fusion; hybridoma technology; bone marrow cells; splenocytes

Introduction

Cell fusion, a process in which two or more cells merge to form a single cell. In recent years, two different cells in isolated culture can also be fused by artificial methods to form somatic cell hybrids(Li YF, et al., 2017), to produce a new hybrid cell. Nowadays, cell fusion technology has become an important tool for studying cytogenetics, cellular immunity, viruses, and tumors.

The cell fusion process starts with the fusion of cytoplasm, and then the nuclei merge together through mitotic cell division to form a new hybrid cell (Hou Y, et al., 2017). Under normal circumstances, fusion does not occur when two cells come in contact because of the presence of an intact cell membrane in each. However, in the presence of specific inducers, the cell membrane can be changed in such a way that two or more cells can fuse and form giant cells. The commonly used inducers are Sendai virus and polyethylene glycol (PEG)(Hou Y et al., 2017), which are round particles consisting of a central RNA and an outer lipoprotein (the substance that induces cell fusion). Sendai virus induces cell fusion in four stages: (1) two cells are cultured together, virus is added, and the virus attaches to the cell membrane at 4°C and causes the cells to coalesce with each other; (2) at 37°C, the virus reacts with the cells and the cell membrane is damaged, at which time Ca2+ and Mg2+ are required, and

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the optimal pH is 8.0-8.2; (3) the cell membrane junction is penetrated and the peripheral junction is repaired, at which time Ca2+ and ATP are required; (4) fusion into giant cells still requires ATP. PEG has the same fusion effect as Sendai virus (Huang WW, et al., 2018). The advantages are that it is easy to obtain, simple to use, stable fusion effect, and has replaced Sendai virus. PEG structure is HOH2C(CH20CH2) nCH2OH, molecular weight greater than 200 and less than 6000 can be used as cell fusion agent (Piliszek A, et al., 2007). PEG concentration is in M/W, e.g. 10 g of PEG is mixed with 10 ml of Eagle's solution (assuming 1 ml of culture solution is 1 g weight) to make 50% PEG solution. peg) is autoclaved and mixed with warmed Eagle's solution. PEG with higher molecular weight often solidifies after adding Eagle's solution, and needs to be placed at 70°C to make it a solution, and later stored at room temperature, it will no longer solidify. Usually, PEG with a molecular weight of 1000 is preferred as a fusion agent, and 50% PEG solution can produce the most hybridized cells; if a smaller molecular weight PEG is used, a concentration of 55% is preferred (Piliszek A et al., 2007). The PEG solution has the highest cell fusion rate at pH 6.0, and the cell density at fusion should not be too high to produce more hybrid cells when fused before confluence into a monolayer.

Hybridoma technology consists of three main components: cell fusion, antibody screening and clonogenic culture. Hybridoma technology is a technological revolution in the field of medical biology and has now been rapidly extended to agricultural production for the extraction and purification of rare bioactive substances and for the treatment of cancer. It is an important component of bioengineering, and once a hybridoma cell line is established, it will live forever and the products obtained are unlimited, with a wide range of applications that are gaining increasing attention.

Preparation of hybrid cells

Culture of myeloma cells

Myeloma cell lines used for fusion have been turned into gene-deficient cells (hypoxanthine-guanine phosphoribosyl transferase (HGPRT-) or thymidine kinase (TK)) that are resistant to 8-hybridized purine or 6-mercaptopurine (hypoxanthine analogs) or 5-bromodeoxyuridine (BUdR, thymidine nucleoside analogs), a marker of their biological properties. They die when cultured with the HAT system culture medium. Myeloma cells grow in suspension or in a slightly adherent form. Cells are usually maintained in the exponential growth phase (15-20 hours of cell culture) and 0.2-1 ml of cells are removed every 3-5 days into 10 ml of new culture medium, whose maximum cell density should not exceed 5×105-106/ml. The exact passaging change culture depends on the growth characteristics of different cell lines. To prevent excessive cell growth, DMEM (Dulbecco's modified eagle medium) containing high sugar and supplemented with pH stabilizer 4-hydroxyethylpiperazine ethanesulfonic acid (HEPES) is generally used. The composition of the culture medium is as follows: 1000 ml DMEM, 3.0 g L-glutamine (200 mM), 0.11 g sodium pyruvate (100 mM), 0.25 ml 2-mercaptoethanol (0.1 M), 5.0 g HEPES, 110 ml calf serum, and 100 IU/ml each of dual antibodies (streptomycin and penicillin) (note: sodium pyruvate and 2-mercaptoethanol may not be added). (Note: sodium pyruvate and 2-mercaptoethanol may not be added).

Preparation of immune mice

Only some of the hybridoma cells produced are capable of secreting the required specific antibodies. The rate of appearance of positive hybridomas depends not only on the number of specific B cells in splenocytes, but also on the maturation stage of these cells, so the method of immunizing animals is one of the most important factors determining the outcome of fusion. The method of high-dose antigen injection 3-4 days before fusion is now generally used. The immunization procedure is to take 6–8-week-old BALB/c female rats that are white variant laboratory rats, originating from Mus musculus, and immunize them for 2 weeks with a basal immunization and a further intravenous booster, which is used for fusion 3-5 days later. The use of adjuvants and prior treatment of antigens during immunization depends on the strength of the antigen.

In general, soluble antigens are better with complete adjuvant. The amount of antigen is also related to the strength of the antigen, for example, 100 μg for the first time and 50 μg for the second time for IgG. The immunization route can be administered via intraperitoneal injection for the first time. For cellular antigens, no adjuvant is used and 1×106-107 is injected intraperitoneally each time.

Preparation of spleen cells

After the mice were executed by cervical dislocation, the spleen was disinfected with alcohol, removed under aseptic conditions, the connective tissue and fat were removed, and rinsed once with 5 ml of serum-free culture solution. The spleen was placed in a sterilized 90-100 mesh stainless steel or nylon gauze mesh, a small incision was made in the middle of the spleen, the syringe core was gently squeezed from one end, and the spleen was rinsed once with serum-free culture medium. The cells were allowed to pass through the mesh and collected into a flat dish. Alternatively, 3 ml of serum-free culture medium was injected into the spleen with a syringe, and cells were obtained by repeated aspiration several times. The collected cells were placed in a centrifuge tube to make a cell suspension and centrifuged at 1000 rpm

for 5 minutes. After removing the supernatant, add a small amount of culture medium to make a cell suspension, count, and set aside.

Suspension cell fusion

Preparation of 30%-50% PEG solution

PEG with a molecular weight of 1000 was taken, autoclaved (6.8 kg, 15 min), placed in a 56°C water bath and dissolved, and then warmed in a 37°C water bath. Take the serum-free culture solution in a medium warm bath at 37°C (separately). When dispensing 40% concentration, aspirate 0.4 ml PEG and 0.6 ml serum-free culture solution mixed, and set aside in a warm bath at 37°C. It should be noted that the PEG solution is more acidic, and the pH of the serum-free culture solution can be adjusted to 8.0 in advance, or a small amount of 1 mol NaOH or NaHCO3 solution can be added to raise its pH when preparing it.

Cell fusion

The two different parental cells were mixed at 5×106 each, centrifuged to precipitate, and the supernatant was aspirated. The cells were fused by adding 1 ml of prepared 50% PEG solution and blowing with a pipette to bring them into contact with the cells for 1 minute. It was added 9ml Eagle's culture solution, centrifuged and precipitated, and the supernatant was aspirated. 5ml of growth culture solution was added and inoculated in five 60mm diameter dishes, each dish was added with growth culture solution to 5ml and incubated in a 5% CO2 incubator at 37℃. After 6-24 hours of incubation, the culture was changed to selection medium to screen the hybridized cells.

Hybridoma cell fusion

Cell preparation

Myeloma cells were collected, cells were washed 3 times with serum-free culture medium (37℃) and cell viability (greater than 90%) was counted; mouse spleen cells were collected, cells were washed 3 times with serum-free culture medium (37℃) and cell viability (greater than 90%) was counted. Then splenocytes and myeloma cells were mixed at 1:5 or 1:10, followed by centrifugation, removal of supernatant, and aspiration of excess supernatant with sterile filter paper.

Hybridoma cell fusion

The cell mass was gently stirred by adding 0.2 ml of 43% PEG solution in a centrifuge tube and placed at room temperature for 2 minutes. The cells were suspended in 2 times HAT culture medium (with spleen cells as the base, the concentration was adjusted to 6×105/ml), and 0.5ml was added to each well of a 24-well plate; 0.1ml was added to each well of another 96-well plate and placed in a 37℃ 5% CO2 incubator for the first time after one week of incubation.

Post-fusion cell culture

After 7-10 days of fusion, HAT culture medium was used for half volume exchange, and then every 2-3 days for half volume exchange. After 2-3 weeks, hybridized cell colonies appeared with large, round and transparent cells. When the colonies proliferate and grow to 1/3 of the wells, antibody detection is performed.

Clonogenic culture

Clonification culture is also a crucial aspect for hybridoma cell lines that secrete a single antibody. In most cases, the hybridoma cell colonies that produce specific antibodies are not derived from a single cell and therefore may be mixed with non-antibody secreting clones. It often proliferates faster than antibody-secreting clones, and timely antibody detection and clonogenic culture is important to avoid crowding out of antibody cells by the transitional proliferation of non-antibody-secreting cells in the same culture. In addition antibody-secreting hybridoma cell lines that are isolated may mutate and lose their ability to secrete antibodies during the culture process. In this case repeated selection is required to clone the antibody-secreting specific hybridoma line.

In this experiment, the cell cloning culture was performed using the limited dilution method. The cells were collected and counted, and the cell density was adjusted to 5-10 cells/ml. 0.1 ml was added to each well of a 96-well plate, and the culture was continued by half volume change after one week of incubation in the incubator. The supernatant of each well was tested at the right time, and the cells in the positive culture wells were selected and continued to be diluted in a limited way until the cells in the wells were monoclonal, and the culture was expanded.

Detection of antibodies

At 10-15 days of cell fusion, a well-by-well examination is required. Once exuberant growth of hybrid cell colonies is found, preliminary screening for antibody activity should be done. On the one hand, since the growth of negative clones will cause the loss of positive clones, they should be found and screened as soon as possible. On the other hand, when the positive clone is small and a screening is negative, a second and third screening should still be done to check out the holes for antibody activity, and it is important to perform the clone culture as early as possible. Due to the small amount of culture supernatant to be tested, the large number of samples and the low antibody content, thus the detection method must be very sensitive, rapid, reliable and able to complete the determination of a large number of samples in a short

time. The most commonly used methods are immunofluorescence (IF) test, radioimmunoassay (RIA), enzyme linked immunosorbent assay (ELISA), etc. ELISA is generally used.

Bulk preparation of monoclonal antibodies

The supernatant of in vitro culture of hybridoma cells contains a certain amount of antibodies. In order to obtain a large amount of antibodies, one needs to increase the culture vessel and collect a large amount of supernatant; the second needs to concentrate the antibodies, the procedure is to first inoculate a certain density of cells, culture for 24 hours, replace the new culture medium once, and then continue to culture for 1-2 weeks, during which the cells will continue to die, while the antibody content can gradually increase.

Another method of collecting antibodies is by in vivo transplantation. Female mice of the same strain at 6-8 weeks of age are injected intraperitoneally with 0.5 ml of phytolacca or paraffin oil and inoculated intraperitoneally with 107 hybridoma cells one week later. The hybridoma cells will proliferate and produce ascites in the peritoneal cavity, and the level of antibodies in the ascites can reach mg levels. The ascites can then be re-infused into the peritoneal cavity of another few mice to produce more ascites. The cell culture supernatant has low levels of antibodies mixed with large amounts of bovine serum proteins, and the ascites, although high in antibodies, also contains the mouse's own proteins, and thus must also be purified and characterized.

Selection of fusion cells

Artificial methods

For example, cell fusion induced by Sendai virus can form binucleated and multinucleated cells. In the process of cell fusion, not only fusion occurs between two different types of cells, but also the two types of cell parental cells can fuse with each other. Cells containing two different parental nuclei are called heteronucleosomes or heteronucleated cells. Cells formed by the fusion of the same parental cells are called homonucleosomes or homonucleated cells. Most multinucleated cells die within a week, but only a few heteronucleated cells, especially binucleated heteronucleated cells, survive and, through mitosis, merge the chromosomes of two different parental cells into a single nucleus to form a syncytium or syncytiotrophoblast, or hybrid cell.

HAT system method

Cells are grown for several generations in culture medium containing 3H thymidine nucleosides, and these cells are used to fuse with another parental cell that is not labeled to show one labeled nucleus with another unlabeled cell by radioautography, i.e., heteronuclear cells, or the HAT system method, a special medium for isolating hybrid cells designed according to the purine and pyrimidine biosynthetic pathway, i.e., aminopterin can primary break the nucleotide The main pathway of IMP synthesis, so the cells can only synthesize nucleotides through the HGPRT can make hypoxanthine acidification to form hypoxanthine, and then converted to AMP and GMP in turn, through the emergency pathway dependent on exogenous sources. When HGPRT-/TK+ cells were crossed with HGPRT+/TK- cells as parental cells, both unfused parental cells and fused homologous cells died in the HAT culture medium. This is due to the blockage of the main pathway of nucleotide synthesis in these cells and the inability to utilize exogenous nucleotide material. Only the fused heteronuclear cells became HGPRT+/TK+ cells due to enzymatic compensation and were able to grow in the HAT culture medium and thus were screened for preservation.

Preparation of HAT culture solution (100×)

Molecular weight of hypoxanthine is 136.1, 1.0×10-4M =13.6 μg/ml, weigh hypoxanthine 1.361mg, put it in 100ml double distilled water and heat it to 45-50℃ to dissolve it, or add 1M HCl 4ml to dissolve it first.

Aminopterin molecular weight is 440.4, 4.0×10-7M=0.176 μg/ml, weigh 1.76mg of aminopterin, add 90ml of double distilled water, add 0.5ml of 1 M NaOH to dissolve it, then add double distilled water to 100ml, finally add 0.5ml of 1M HCl to neutralize it.

The molecular weight of thymine nucleoside is 242.2, 1.60×10-5M=3.87 μg/ml, and 38.7 mg of thymine nucleoside can be dissolved in 100 ml of double distilled water. For convenience, hypoxanthine and thymine nucleoside were placed together in 100 ml of double distilled water and heated to 45-50°C to dissolve them, i.e. HT and A two 100× stock solution. Filter and de-bacterize separately and store in -20℃ refrigerator. Aminopterin needs to be stored away from light. Any culture can be mixed with HAT culture, but glycine should be added, because the tetrahydrofolate reductase that converts serine to glycine will also be inhibited by aminopterin.

The molecular weight of glycine is 75.07, 3.0×10-6M=0.225 μg/ml, just weigh 2.25mg of glycine and dissolve it in 100ml of double distilled water.

Discussion

Scientists have discovered that the phenomenon of fusion of animal cells into multinucleated cells after being infected by viruses exists in nature. Scientists used this natural phenomenon to successfully induce animal cell fusion in vitro and to culture hybrid cells into viable ones (Chen J, 2020). This provides technical support for the fusion and culture of hybridoma cells. In addition to virus induction, chemical

reagents and electrofusion under microgravity conditions in space have been improved and innovated, and the success rate of hybridoma cell fusion and culture has been rising.

When two cells are in close contact, their cell membranes may fuse together, and the fused cells contain two different nuclei, called heterokaryons (heterokaryons). Under the right conditions, they can fuse together to produce a single nucleated cell with the genetic information of the original two cells, called a hybrid cell. Over the years, during the process of performing somatic cell fusion, it has often been found that hybrid cells lose their chromosomes and retain only one characteristic of the parental cells.

In 1975, Köhler and Milstein applied the fusion of mouse myeloma cells with mouse spleen cells sensitized by sheep cells and obtained a fraction of fused hybrid cells that both continued to grow and secreted anti-sheep erythrocyte antibodies, calling this hybrid cell system a hybridoma(Köhler G, et al., 1975), which can be applied to prepare a single antigenic determinant cluster of monoclonal antibodies to a single antigenic determinant cluster. In fact, before that, in 1970, Sinkovics et al. had reported that virus-specific antibody-producing lymphocytes and virus-induced tumor cells could naturally form hybridomas in vivo to secrete specific antibodies (Sinkovics JG, et al., 1970). The first successful murine-human hybridoma was reported by Schwaber and Coken in 1973(Schwaber J, et al., 1973). In 1974 Bloom and Nakamura first applied human B cells fused with human myeloma cells to produce lymphokines (Bloom AD, et al., 1974). In 1980 Luben and Molle demonstrated that mouse thymocytes cultured in vitro for 10 days were capable of producing lymphokines and could replace the immune T cells needed to produce the primary immunization (also called primary immunity) response in vitro (Luben RA, et al., 1980). They applied this thymocyte culture (called conditioned culture) to mouse spleen cell cultures and added an antigen (lymphokine-osteoclast activating factor) to stimulate the splenocytes to produce an immune response, and subsequently used these cells to produce monoclonal antibodies to osteoclast activating factor by hybridization with murine myeloma cells, establishing an initial immune response in vitro and shortening the time to immunization in vivo. 1978 Miller and Lipman took the hybridoma monoclonal antibody technology a step further by applying EBV trans-formed human B lymphocytes to produce monoclonal antibodies(Raab-Traub N, et al., 1978).

It is expected that in the near future, such specific antibodies against malignant tumor cells may become a special delivery vehicle to combine cancer-killing toxins and therapeutic agents such as chemotherapeutic drugs, and specifically transport them around malignant tumor cells to exert their proper efficacy, which will open up a broad prospect for human cancer treatment.

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Conflict Interests

No conflict of interest

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细胞融合在杂交瘤技术中的应用方法

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[摘要]：**背景：**细胞融合，即两个或两个以上细胞合并形成一个细胞的过程。近年来，用人工方法也可以使两个离体培养的不同细胞发生融合，形成体细胞杂交，产生一个新的杂种细胞。现在，细胞融合技术已经成为研究细胞遗传、细胞免疫、病毒和肿瘤等的重要手段。**方法：**本实验使用有限稀释法进行细胞克隆化培养。收集细胞并计数阳性培养细胞，调整细胞密度为 5-10 个 /ml。在 96 孔板中每孔加 0.1ml，培养箱培养一周后半量换液，继续培养。适时检测每孔培养的上清，挑选呈阳性培养孔的细胞继续进行有限稀释，直至确信孔中细胞为单克隆为止，进行扩大培养。加 0.2ml 43% 的 PEG 溶液于离心管中，轻轻搅动细胞团，置于室温中 2 分钟。加入 10ml 完全培养液，以 800 转 / 分钟离心 5 分钟，用 2 倍 HAT 培养液悬浮细胞（以脾细胞为基数，调节浓度为 6×105/ml），取 24 孔板，每孔中加 0.5ml；另 96 孔板中每孔加 0.1ml。置于 37℃ 5% CO2 培养箱中，培养一周后进行第一次更换培养液。融合后 7-10 天用 HAT 培养液半量换液，以后每 2-3 天半量换液一次。两周后可改用 HT 培养液半量换液，或仍用 HAT 培养液进行半量换液。**结果：**细胞融合 2-3 周后出现杂交细胞集落，细胞体积大，圆且透明。**结论：**本文为进一步研究细胞融合在杂交瘤技术中的应用提供参考。

[关键词]：细胞融合；杂交瘤技术；骨髓细胞；脾细胞

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